ISSN: 1949-4998 Volume 2, Number 5, May 2010

Scientific Research

Health

ISSN: 1949-4998

www.scirp.org/journal/health

Journal Editorial Board

ISSN 1949-4998 (Print) ISSN 1949-5005 (Online)

http://www.scirp.org/journal/health

TABLE OF CONTENTS
Volume 2, Number 5, May 2010

HEALTH

Journal Information

SUBSCRIPTIONS

The *HEALTH* (Online at Scientific Research Publishing, www.SciRP.org) is published monthly by Scientific Research Publishing, Inc., USA.

Subscription rates:

Print: $50 per issue.
To subscribe, please contact Journals Subscriptions Department, E-mail: sub@scirp.org

SERVICES

Advertisements
Advertisement Sales Department, E-mail: service@scirp.org

Reprints (minimum quantity 100 copies)
Reprints Co-ordinator, Scientific Research Publishing, Inc., USA.
E-mail: sub@scirp.org

COPYRIGHT

PRODUCTION INFORMATION

For manuscripts that have been accepted for publication, please contact:
E-mail: health@scirp.org

Vol.2, No.5, 391-394 (2010)
doi:10.4236/health.2010.25058

Concurrent pulmonary *Mycobacterium avium* complex (MAC) infection and active Hürthle cell thyroid carcinoma: is there a connection?

Kara M. Meinke Baehr, Whitney S. Goldner*

University of Nebraska Medical Center, Department of Internal Medicine–Diabetes, Endocrinology, and Metabolism, Omaha, USA
*Corresponding Author: wgoldner@unmc.edu

Received 5 October 2009; revised 5 January 2010; accepted 8 January 2010.

ABSTRACT

We present two cases of pulmonary MAC infection in women with Hürthle cell thyroid carcinoma. Both cases were asymptomatic octogenarian women with active Hürthle cell thyroid carcinoma and prolonged periods of hypothyroidism prior to diagnosis of pulmonary MAC. Mycobacterium avium complex has never been reported in association with any type of thyroid cancer, specifically Hürthle cell carcinoma. A review of the literature and possible associations between the two are discussed in this article.

Keywords: Hürthle Cell Thyroid Cancer; Hypothyroidism; Mycobacterium Avium Complex; Cellular Immunity

1. INTRODUCTION

Hürthle cell thyroid carcinomas are a type of well-differentiated thyroid carcinoma and are either classified as a subset of follicular carcinoma or a separate category of well-differentiated thyroid cancer [1]. Hürthle cell carcinomas usually produce thyroglobulin, but are often less iodine avid, and are more aggressive than the other types of well-differentiated thyroid carcinoma such as papillary or follicular [2,3]. There is a female predominance with peak ages of incidence in the seventh and eighth decades [2]. Specific treatment is controversial, but generally total thyroidectomy and remnant ablation is recommended [1]. Follow-up for Hürthle cell thyroid cancer is similar to papillary and follicular thyroid cancers, which entails anatomic imaging of the neck and chest. This is usually done with neck ultrasound and computed tomography (CT) scans of the chest and occasionally neck; and when indicated, TSH-stimulated I-131 whole body scan is performed. Laboratory follow- up includes tumor marker evaluation with serum thyroglobulin (Tg) and antibodies. Thyroid hormone suppression is also a mainstay of therapy. Despite its more aggressive course, there are no known associations between Hürthle cell thyroid cancer and immunocompromised states; specifically, there are no published reports of Hürthle cell thyroid carcinoma and pulmonary MAC infection.

MAC refers to mycobacterium infections caused by free-living nontuberculous organisms that are inhaled or ingested from the environment [4]. Among nontuberculous species, MAC is the most common cause of pulmonary disease in the United States. Diagnosis of pulmonary MAC requires both imaging consistent with pulmonary disease and microbiologic confirmation in a symptomatic patient to necessitate treatment [4]. Increased rates of MAC in immunocompromised patients with AIDS were reported in the 1990s; however, with improved anti-retroviral therapies, MAC infections in HIV infected patients have been declining [5]. The increased susceptibility of patients with AIDS to disseminated nontuberculous mycobacteria infections helps provide insight into the pathogenesis of these infections in non-immunocompromised hosts. Disseminated MAC infections usually occur when the CD4+ T-lymphocyte count is < 50 µl, suggesting that some intrinsic activity of the T-lymphocytes is important for immunity to mycobacteria [6]. We present two cases of pulmonary MAC infection in octogenarian women with active Hürthle cell thyroid carcinoma.

2. CASE 1

Case 1 is a 79 year-old female initially diagnosed with stage 2 Hürthle cell carcinoma (pathologic stage T2N0M0) in February 1999. She underwent total thyroidectomy and remnant ablation with 100 mCi of I-131. Posttreatment scan showed uptake in the central neck consistent with thyroid remnant without evidence of local or distant

metastases. She had no follow-up until 2006 when she presented to our institution for evaluation of multiple pulmonary nodules. Excisional biopsy of one of the lesions was consistent with metastatic Hürthle cell carcinoma. Thyroid ultrasound showed no evidence of thyroid cancer in the neck. Thyroglobulin (Tg) was 136 ng/mL (goal < 0.1 ng/ml in remission) with negative antithyroglobulin antibodies, and thyroid stimulating hormone (TSH) of 11.5 mcIU/mL. I-123 whole body scan showed uptake in the thyroid bed and superior mediastinum and stimulated Tg was 155 ng/mL. She underwent repeat radioactive iodine ablation with 206 mCi of I-131 using dosimetry to calculate her dose. Her post-treatment scan showed heterogeneous uptake throughout the lungs, most intense in the left lower lobe, and uptake in the thyroid bed.

Despite successful uptake on radioactive iodine scans, six months after treatment, Tg had risen to 341 ng/mL. Repeat chest CT was unchanged. PET scan showed uptake in the right middle lobe pulmonary nodule and bilateral lower lobe lesions that were calcified with lower SUVs suggesting more benign disease. Since most of the pulmonary lesions were not PET avid, indicating they were likely radioiodine avid, she received another 249.6 mCi of I-131 with dosimetry in March 2008. Post-treatment scan showed faint uptake throughout the lungs bilaterally, but no uptake to correspond with the largest pulmonary nodules, suggesting the pulmonary metastases were no longer radioiodine avid. Following treatment, Tg continued to rise and was 3976 ng/mL in July 2008 on thyroid hormone suppression. Follow-up chest CT showed numerous pulmonary nodules, two lesions increased in size in the right upper lobe, along with scattered tree-in-bud opacities, bronchial wall thickening, and bronchiectasis suggesting mycobacterium avium complex (MAC). Induced sputum was culture positive for MAC. The patient was referred to infectious disease and no treatment was recommended since she was asymptomatic.

A PET scan showed disease progression within the mediastinum and right hilum with a maximum SUV of 12.4 in a subcarinal mass and multiple PET avid pulmonary nodules. The hilar mass was felt to be non-resectable, so she was treated with external beam radiation therapy for her mediastinal mass and sensitizing chemotherapy with doxorubicin in November 2008. After starting chemotherapy, she developed worsening productive cough, chills, and fatigue and significant oxygen desaturation. Since MAC was still present in her sputum, she was started on triple antibiotic therapy (azithromycin 500 mg daily, rifampin 300 mg daily, and ethambutol 800 mg daily) for her symptomatic MAC in January 2009. She has symptomatically improved and no longer has a productive cough; however, continues to require supplemental oxygen. Her most recent Tg was 1582 ng/ml (down from maximum 3976 ng/ml) with negative antibodies and a TSH of 0.45 mcIU/ml. Chest CT shows interval decrease in bilateral consolidations, unchanged bilateral bronchiectasis and 2 right lower lobe nodules, and a decrease in the subcarinal lymph node size.

3. CASE 2

Case 2 is an 82 year-old female diagnosed with stage 2 Hürthle cell carcinoma (T3N0M0) in July 2004 and underwent total thyroidectomy and remnant ablation with 45.8 mCi of I-131. Post-treatment scan showed only uptake in the thyroid bed. In September 2005, she underwent a TSH-stimulated (with thyrogen) whole body scan that showed 1.6% minimal uptake in the area of the thyroid bed. Tg stimulated to 0.2 ng/ml with a TSH of 315 mcIU/mL. She refused additional radioactive iodine therapy due to severe sialoadenitis with the first treatment. Thyroid ultrasound revealed residual thyroid tissue in the right bed measuring 1.2 × 0.7 × 1.1 cm and no evidence of focal nodules or lymphadenopathy.

Repeat thyroid ultrasounds in September 2006 and August 2007 showed no evidence of recurrence. Tg level ranged 0.3-0.8 ng/ml, antibodies remained negative, and TSH ranged from undetectable to 3.6 mcIU/ml during this time. Tg increased to 2.9 ng/mL in March 2008 with an elevated TSH of 31.5 mcIU/ml; however, decreased to 1.7 ng/mL in August 2008 (TSH 6.9 mcIU/ml) and further to 1.5 ng/mL with a TSH of 0.14 mcIU/ml one month later. Thyroid ultrasound in August 2008 showed minimal right-sided residual thyroid tissue measuring 0.4 × 0.6 × 0.7 cm. Non-contrast chest CT showed a right upper lobe semi-solid nodule measuring 1cm that was suspicious for cancer. CT also revealed bronchiectasis changes in anterior portions suspicious for MAC; and her induced sputum was MAC culture positive. PET scan was normal. After infectious disease consultation, she was not treated for her MAC because she was asymptomatic.

Follow-up chest CT in February 2009 showed stable mixed solid and ground glass nodules in the right upper lobe, bilateral patchy consolidations, and an enlarging precarinal lymph node. This lymph node was biopsied and pathology was negative for malignancy. Tg has continued to increase and is currently 8.4 ng/ml in August 2009, and TSH is 0.087 mcIU/ml. She is still asymptomatic.

4. DISCUSSION

These cases are the first to report pulmonary MAC infection in persons with active Hürthle cell thyroid carcinoma. Interestingly, both these patients were asymptomatic, octogenarian women and diagnosis was initially suggested by radiographic findings and later confirmed

with culture positive induced sputum. Typical radiologic findings consistent with pulmonary MAC infection on chest radiographs or computed tomography (CT) include nodular infiltrates, multifocal bronchiectasis, cavitation, and multiple small nodules [7]. The nodules and bronchiectasis are usually present within the same lobe and occur most frequently in the right middle lobe and lingula [8,9]. The keys to our patient's diagnoses were the presence of new, non-dependent bronchiectasis that was different from the other pulmonary disease previously seen on past CT scans.

Four different clinical syndromes of pulmonary MAC infection have been described. These include classic cavitary disease seen commonly in middle-aged smoking males [6]; nonclassic forms such as bronchiectasis and multiple nodules found in elderly women [6,8], MAC lung disease in cystic fibrosis patients [10], and a hypersensitivity pneumonitis associated with hot tub use [11]. The clinical syndrome that is most likely in our two cases includes the group who presents with mid-lung nodular bronchiectasis with multiple nodules and tree-in-bud opacities due to MAC with no underlying lung disease or immunosuppression. This bronchiectatic form of the disease is frequently termed the "Lady Windermere syndrome" and presents in slender, middle-aged to elderly Caucasian women and involves the right middle lobe and/or lingula [6,12]. The first report of this form of MAC cites 21 such patients, most non-smoking women with a mean age of 66 years, who presented with symptoms of persistent cough and purulent sputum [13]. This group is homogeneous, consisting of older, Caucasian, otherwise healthy women, raising the possibility of a host defense immune defect to explain disease susceptibility [14]. Whereas mutations in the interferon-gamma and interleukin-12 production and response pathway have been proposed, no consistent immune phenotype in pulmonary nontuberculous mycobacteria infection has been clearly established [15]. Both of our patients were Caucasian women in their late 70's and early 80's with Hürthle cell thyroid carcinoma and no obvious form of immunosuppression; however, they were both asymptomatic which is in contrast to how the Lady Windermere syndrome group usually present. There are no studies linking thyroid cancer or its treatment with immunosuppression. However, Hürthle cell thyroid carcinoma often has a more aggressive course than papillary or follicular thyroid carcinoma [12] and tends to occur more frequently in older women [2]. Case 1 had pulmonary disease from her metastatic Hürthle cell carcinoma, and case 2 had a pulmonary nodule, not definitively metastatic thyroid cancer, so neither of the patients was completely devoid of lung disease as described in the Lady Windermere form of MAC pulmonary infection.

Another common feature for both cases was a prolonged period of hypothyroidism evidenced by an increased TSH prior to diagnosis of MAC. Case 1 was iatrogenically hypothyroid prior to both treatments with radioactive iodine ablation using dosimetry in April 2007 and March 2008. Case 2 was hypothyroid due to medication non-adherence from March through August 2008 which immediately preceded the diagnosis of pulmonary MAC infection on chest CT. Animal studies have shown a decrease in lymphocyte function during hypothyroidism, with a return of normal lymphocyte function during euthyroid states [16]. The number of peripheral white blood cells, mainly lymphocytes, in hypothyroid (thyroidectomized) animals is reduced to half of normal levels [17]. Other studies have shown dysregulation of CD4+ T lymphocyte responsiveness in hypothyroid dogs suggesting a relationship between hypothyroidism and cellular immunity [18]. Unfortunately specific studies regarding cellular immunity in hypothyroid humans are lacking. Radioactive iodine treatment can cause bone marrow suppression after treatment; however, this happens in all types of thyroid cancer, not exclusively Hürthle cell carcinoma, making it a less likely etiology [19]. Given both women experienced prolonged hypothyroidism immediately prior to their diagnosis of MAC, we propose that the prolonged hypothyroidism may have predisposed to suppression of cell-mediated immunity in an already vulnerable host (older, Caucasian women with potential lung pathology). This may have resulted in both patients having an increased susceptibility to acquire pulmonary MAC infection. There are no reports of altered immune function associated with any thyroid carcinomas, and specifically none that associate Hürthle cell thyroid carcinoma with altered immune function; however, this is also a potential mechanism, especially given Hürthle cell thyroid carcinoma often has a more aggressive course, and should be considered and evaluated further. Also, radiologic detection of MAC may be more common in patients with more aggressive thyroid cancer due to close monitoring of disease done by performing more frequent radiographic scans.

5. CONCLUSIONS

To our knowledge, pulmonary MAC has not previously been reported in association with any type of thyroid cancer. We present two interesting cases of octogenarian women with active Hürthle cell thyroid carcinoma who developed pulmonary MAC infection after prolonged periods of hypothyroidism. Further observations and studies are necessary to understand the potential relationship between the two diseases.

REFERENCES

[1] Watson, R., Brennan, M., Goellner, J., *et al.* (1984) Invasive Hürthle cell carcinoma of the thyroid: Natural his-

tory and management. *Mayo Clinic Proceedings*, **59(12)**, 851-855.

[2] Grossman, R. and Clark, O. (1977) Hürthle cell carcinoma. *Cancer Control*, **4**, 13-17.

[3] Yutan, E. and Clark, O. (2001) Hürthle cell carcinoma. *Current Treatment Options in Oncology*, **2(4)**, 331-335.

[4] Good, R. and Snider, D. (1982) Isolation of nontuberculous mycobacteria in the United States. *Journal of Infectious Diseases*, **146(6)**, 829-833.

[5] Pallella, F. Jr., Delaney, K., Moorman, A., *et al.* (1998) Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV outpatient study investigators. *New England Journal of Medicine*, **338(13)**, 853-860.

[6] Parrish, S., Myers, J. and Lazarus, A. (2008) Nontuberculous mycobacterial pulmonary infections in non-HIV patients. *Postgraduate Medicine*, **120(4)**, 78-86.

[7] Erasmus, J., McAdams, H., Farrell, M. and Patz, E. Jr. (1999) Pulmonary nontuberculous mycobacterial infection: Radiologic manifestations. *Radiographics*, **19(6)**, 1487-1503.

[8] Levin, D. (2002) Radiology of pulmonary *Mycobacterium avium-intracellulare* complex. *Clinics in Chest Medicine*, **23(3)**, 603-612.

[9] Koh, W., Lee, K., Kwon, O., Jeong, Y., *et al.* (2005) Bilateral bronchiectasis and bronchiolitis at thin-section CT: Diagnostic implications in nontuberculous mycobacterial pulmonary infection. *Radiology*, **235(1)**, 282-288.

[10] Olivier, K., Weber, D., Lee, J., *et al.* (2003) Nontuberculous mycobacteria I: Multicenter prevalence study in cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*, **167(6)**, 828-834.

[11] Hartman, T., Jensen, E., Tazelaar, H., Hanak, V. and Ryu, J. (2007) CT findings of granulomatous pneumonitis secondary to *Mycobacterium avium-intracellulare* inhalation: "Hot tub lung." *American Journal of Roentgenology*, **188(4)**, 1050-1053.

[12] Reich, J. and Johnson, R. (1992) *Mycobacterium avium* complex pulmonary disease presenting as an isolated lingular or middle lobe pattern: The Lady Windermere syndrome. *Chest*, **101(6)**, 1605-1609.

[13] Prince, D., Peterson, D., Steiner, R., *et al.* (1989) Infection with *Mycobacterium avium* complex in patients without predisposing conditions. *New England Journal of Medicine*, **321(13)**, 863-868.

[14] Huang, J., Kao, P., Adi, V. and Ruoss, S. (1999) *Mycobacterium avium-intracellulare* pulmonary infection in HIV-negative patients without preexisting lung disease. *Chest*, **115(4)**, 1033-1040.

[15] Kim, R., Greenberg, D., Ehrmantraut, M., *et al.* (2008) Pulmonary nontuberculous mycobacterial disease. *American Journal of Respiratory and Critical Care Medicine*, **178(10)**, 1066-1074.

[16] Schoenfeld, P., Myers, J., Myers, L. and LaRocque, J. (1995) Suppression of cell-mediated immunity in hypothyroidism. *Southern Medical Journal*, **88(3)**, 347-349.

[17] Fabris, N. (1973) Immunodepression in thyroid-deprived animals. *Clinical & Experimental Immunology*, **15(4)**, 601-611.

[18] Tani, H., Nabetani, T., Sasai, K. and Baba, E. (2005) Proliferative responses to canine thyroglobulin of peripheral blood mononuclear cells from hypothyroid dogs. *Journal of Veterinary Medical Science*, **67(4)**, 363-368.

[19] Mazzaferri, E. and Kloos, R. (2001) Current approaches to primary therapy for papillary and follicular cancer. *Journal of Clinical Endocrinology & Metabolism*, **86(4)**, 1447-1463.

Vol.2, No.5, 395-399 (2010)
doi:10.4236/health.2010.25059

The effect of detergent as polluting agent on the photosynthetic activity and chlorophyll content in bean leaves

Branislav R. Jovanić[1]*, Srdjan Bojović[2], Bratimir Panić[1], Božidar Radenković[3], Marijana Despotović[3]

[1]Institute of Physics, Belgrade University, Zemun, Serbia; *Corresponding Author: branislav.jovanic@ipb.ac.rs
[2]Institute for Biological Research "Siniša Stanković", Belgrade, Serbia
[3]Faculty of Organization Science, Belgrade, Serbia

Received 28 October 2009; revised 10 January 2010; accepted 12 January 2010.

ABSTRACT

The paper investigates effects of detergent for domestic use on the photosynthetic activity and chlorophyll content in intact bean leaves. The plants were watered for 21 days with a solution of domestic washing powder of 0.60 g r/l. It was established that the activity of photosynthetic apparatus in the plant leaf $PhAC_{Norm}$ [%] decreases exponentially with the length of plant treatment/watering. At the end of the treatment (21st day) the activity of photosynthetic apparatus in the dosed plant leaf was no more than 45% of that in control plant (those which were not watered with detergent solution). With increased plant treatment duration the changed chlorophyll concentration ΔChl_{Norm} [%] rose non-linearly in plant leaves. The highest change ΔChl_{Norm} [%] was observed on the 21st day and amounted to 12%.

Keywords: Chlorophyll; Detergent; Plant; Photosynthesis; Pollution; Water

1. INTRODUCTION

High technologies and technological processes are always accompanied with products which pollute the environment to varying extents. Very few of these products are not pollutants. This is the reason why environmental study is becoming increasingly important for the survival of plant and animal world and ultimately of humankind itself. It should be noted that a culprit for environmental pollution should not be sought only in outdated or new technologies. Sources of pollution can be, which is often ignored, some domestic processes in urban environment, such as food preparation or personal hygiene. The subject of this study is an investigation into water pollution resulting from everyday domestic hygienic procedures. There are hardly any households without a washing machine connected with pipes to sewage for discharge of used water with detergent. Used water is discharged into the nearest river or a lake and together with it detergent. Undoubtedly, with time detergent concentration in the river/lake goes up and the direct consequence of this is a dramatic change in the biosphere. Significant pollution in ground water was observed in Tehran [1]. There are other numerous examples of polluted rivers and lakes with industrial detergents. For example it was found out that the Caspian Sea waters and Volga Terek, and Sulak rivers were extremely polluted with a high detergent concentration [2]. The Asa River in Nigeria is dramatically polluted with industrial detergents [3]. Likewise the Coastal Zone of the Sea of Okhotsk and Avacha Bay are polluted with detergents [4]. Regardless whether it is river or lake water, it is used in gardening for watering vegetables used for human consumption. Doubtless, this water will cause changes in vegetables which can have an adverse effect on people eating them.

2. MATERIAL AND METHODS

2.1. Methods

Bean (*Phaseolus vulgaris L.*) seeds were grown for 3 weeks. They were placed in a growth chamber adjusted to the identical growing conditions (humidity, lighting, temperature, nutrition of soil). The seedlings were watered daily during all investigated periods with tap water. After this period the plants were divided in two groups: control and stress. Growing conditions were also identical for control and stressed samples and the only difference was the presence or absence of detergent in soil. Concentration of domestic use detergent in the water

used for watering was 0.60 g r/l. The control samples were watered with water without detergent. Therefore, any differences between fluorescent spectra and fluorescence induced curve for stressed versus control plants could only be the result of the presence of detergent.

In all experiments the plant's leaves remain intact (cutting is an additional stress) and we could make several measurements on the same plant at any time. In further text, the subscripts (S) and (C) will denote the stressed or control (nonstressed) conditions, respectively. Photosynthetic activities were determined using well known Kautczhy method. Photosynthesis measurements of light-adapted plants, non-destructive measurements of potential quantum yield (Fv/Fm), were taken using a photodiode connected with 14bit AD card for collecting modulated fluorescence. In front of the photodiode was placed interference filter 690 nm ± 5 nm. Excitation source for fluorescence induction curve was high intensity LED 470 nm/12 mW. The beans were transferred to a darkened laboratory for 5 minutes for adaptation before measuring fluorescence kinetics at 690 nm [5]. Each point in **Figure 1** and **Figure 2** represents mean value of 15 measurements on different leaves which completely satisfies the demand for value measurement and calculation precision [ΔChl(a,b) and $PhAc_{Norm}$] to be higher than 1% [6].

For excitation the leaves and obtained fluorescence spectra the leaf was irradiated by high power LED (470 nm/12 mW). The fibre inlet was placed 15 mm from the leaf surface. The LED beam diameter on the leaves was ~10 mm. The LED light beam was always directed onto the upper surface of the leaves at a 90° angle of the leaf axis, and the optical fibre was set at a 90° angle to the leaves' surface on the same side. Fluorescence emitted radiation from intact leaf was collected and directed through an optical fibre (N.A. of 0.22 and 1000 μm diameter) that was coupled to a portable 2048-element CCD spectrometer (AVANTES 1000 PC). Data collection and spectrum processing were conducted in real time with microcomputer and commercial software OOI Base (AVANTES Inc.). The results for each groups of bean represent an average of the measurement of ten leaves. Fluorescence measurements took 1.5 min for each measured leaf.

Chlorophyll content Chl(a,b) in bean leaf was determined from experimentally obtained fluorescence spectra and well known relation between chlorophyll content and fluorescence intensity ration. It is well known that the ratio of the two chlorophyll fluorescence peaks (F_{730}/F_{690}) in leaves correlates well with amount of chlorophyll content in the bean plant leaves [7]. Therefore chlorophyll content was determined using: a) Fluorescence bean spectra and b) relation between chlorophyll content and the fluorescence intensity ratio FIR defined as the ratio of the fluorescence intensity measured at 730 nm (F_{730}) and 690 nm (F_{690}) FIR = FIR_{690}/FIR_{730}. For the bean linear correlation (r^2 = 0.954) between chlorophyll(a,b) content and FIR is Chl(a,b) = 42.93 – 12 FIR and was obtained from literature data [5]. In order to eliminate errors which can appear due to differences in individual chlorophyll contents in different bean samples we introduced a relative change of the chlorophyll(a,b):

$$\Delta\mathrm{Chl(a,b)} = \mathrm{Chl(a,b)}_{UV} - \mathrm{Chl(a,b)}_{C} = 12\left[\mathrm{FIR}_{UV} - \mathrm{FIR}_{C}\right]$$

FIR_C and FIR_{UV} are the fluorescence intensity ratio for control plant which were not exposed to the UV radiation and the plant which were exposed to the UV radiation. This method was successful in the experiment with a pumpkin exposed to the γ-nuclear radiation [8].

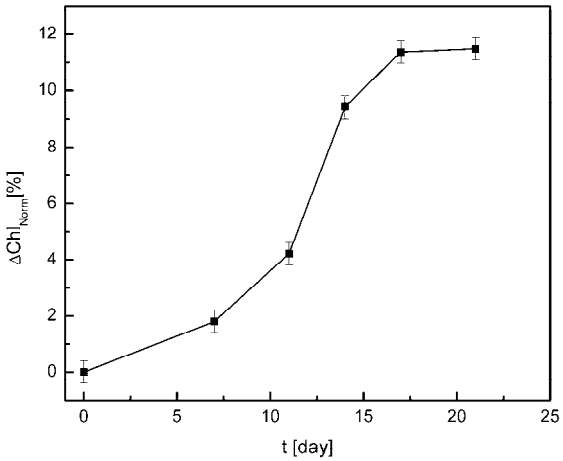


Figure 1. Change in chlorophyll content ΔChl(a,b) in the bean leaves during irrigation with water contain detergent.

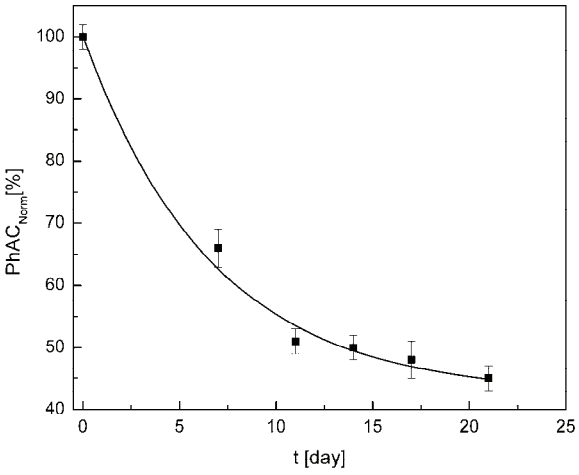


Figure 2. Change of the normalized photosynthesis activity $PhAc_{Norm}$ in the bean leaves as function of time exposition to radiation with detergent.

3. RESULTS

To avoid effects of plant age on chlorophyll concentration the control and dosed plants were of the same age, To avoid determining real chlorophyll concentration, relative chlorophyll concentration reduction was determined: chlorophyll concentration in the dosed plants was marked ΔChl_{Norm} as opposed to the control samples of the same age which were not treated with detergent. The reduction in chlorophyll concentration in the marked values ΔChl_{Norm} over detergent treatment time is shown in **Figure 1**. **Figure 1** shows that with time relative change of chlorophyll concentration increases quickly to reach its peak on the seventeenth day at about 11.5%. Obviously in this period the detergent has adverse effect on chlorophyll by destroying it constantly. After this the relative change of chlorophyll concentration remains steady at the same value. This pattern can be explained with a hypothesis that the plant adapted to the given unfavourable conditions and developed a mechanism to maintain the reduced chlorophyll concentration.

When determining photosynthetic activity PhAc of photosynthetic apparatus in a plant leaf, equally when determining reduction in chlorophyll concentration in control and dosed plants, the plants were of the same age to avoid the effect of plant age. To avoid determining absolute photosynthetic activity, relative reduction of PhAc was determined. The PhAc in detergent treated plant was marked $PhAc_{Norm}$ to distinguish them from control samples of the same age which were not treated with detergent. The obtained changes in marked values $PhAc_{Norm}$ over the detergent treatment time are shown in **Figure 2**. Unlike chlorophyll concentration, photosynthetic activity PhAc constantly, exponentially decreases to reach only 45% of initial activity on the 21st day. This pattern indicates that this water has adverse effects on plants.

4. DISCUSSION

Scientific papers offer data indicating different effects of different detergents on plants. In most cases detergents have adverse effects on plant pigments and morphology and inhibit metabolic processes. It was found the toxic effect of sodium dodecyl sulfate (SDS) and the household synthetic detergents (HSDs) Kristall and Tix (0.1, 1, and 10 mg/l) on the diatom Alga Thalassiosira pseudonana [9]. By the presence in water of detergents for wool domestic washings the native enzyme lost 50% of activity after 20 min of incubation [10]. The effect of detergent on plants varies depending on how the plant is exposed to it. For example, when bean was watered with 0.01% (w/v) solution the nondenaturing, zwitterionic detergent [3-{3-cholamidopropyl}-dimethylammonio]-1-propane-sulfonate) 0.01% (w/v) it induced root hair deformation [11]. Also, in mungbean (Vigna radiata) seeds synthetic detergent induced reduction in dehydrogenase activity [12]. The leaves completely lost their turgor pressure and displayed chlorosis when they are treated with detergents [13]. The biophysical characteristics of the membrane were changed after detergent (Brij 58) treatment [14]. Detergent inhibited growth, metabolic activity took place only for 1 to 5 days, after which metabolic activity also ceased [15]. Also, high concentration of detergent might cause loss of the native configuration of β-carotene [16]. Cell growth and fission inhibition, as well as morphological changes and blocking of chlorophyll a synthesis, were recorded at 10 mg/L concentration of detergent of household synthetic abstergent (HSA) [17]. When β-carotene is treated with a high concentration of detergent [18] this might cause loss of the native configuration [18]. Higher plant thylakoid membranes can be fractionated with various detergents [19].

As can be seen from our data chlorophyll is sensitive to detergent which tallies with other research results. The plant treated with water content detergents showed high inhibitory effect on chlorophyll content in sunflower leaves [20]. The aggregation of chlorophyll is partly inhibited by detergent Triton X-100 [21]. In addition to reducing its concentration, detergents have other effects on chlorophyll. Studies on light harvesting complexes LHCs show that detergent-induced dissociation of LHCs and caused decline in bonding Chl b and Chl a [22]. The results on pigment-protein complexes of Pisum sativum thylakoids treated with detergent Triton X-100 and n-octyl β-D-glucopyranoside show that reversible dissociation of pigment-protein complexes occur [23]. Cell growth and fission inhibition on cryptophytic alga Chroomonas salina (Wils.) Butch. (Cryptophyta), as well as morphological changes and blocking of chlorophyll a synthesis, were recorded at 10 mg/L concentration of household synthetic abstergent (HSA) «Tix» [17] .

In addition to the above discussed effect of detergent on chlorophyll, it was to be expected that similar possibly even identical effect will be observed on photosynthesis. Detergent-induced reversible denaturation of the photosystem reaction [24]. Detergents have strong effect on the fluorescence properties of the light-harvesting complexes of photosystem II [25]. Detergent (Triton X-100) has effect on relaxation dynamics of photosystem II [26]. Some results have shown that low concentration of nonionic detergent Triton X is sufficient for saturation of photosynthesis in terrestrial higher plants [27]. Even a short exposure to detergent effects causes extensive changes in photosynthesis. For example exposing for 10 min in water containing a few drops of liquid detergent induce the increase of photosyntesis [28]. It was concluded that detergent (Triton X-100) causes damage of the donor part of photosystem 2 in

isolated chloroplasts [29]. Detergent treatment of the membranes resulted in loss of PS I activities [30]. Non-ionic detergent n-dodecyl-α, D-maltoside cause disintegration of the potosystem II (PS II) into separated PS II in stacked and unstacked thylakoid membranes from spinach [31]. The addition of the detergent Triton X-100 to the 'chromatophore' facilitated the photooxidative destruction of the antenna BChl [32]. In addition to inhibition of activities PHII detergent can cause morphological changes of these centres. Detergent treatment of stacked thylakoid or BBY membranes usually gives to PS II–LHC II varying size [31].

5. CONCLUSIONS

Detergents in the water for watering plants have adverse effect. In the bean plants which were watered with detergent water, significant changes were observed. Chlorophyll concentration dropped by 12%. The activity of photosynthesis apparatus in leaves decreased by around 45%.

6. ACKNOWLEDGEMENTS

This work was supported by Grants No. 141007 of MNRS. Authors want to thank Mr. P. Hiddinga on kindness in supporting equipments.

REFERENCES

[1] Imandel, K., Razeghi, N. and Samar, P. (1978) Tehran ground water pollution by detergent. *Water, Air, & Soil Pollution*, **9**, 119-122.

[2] Korshenko, A. and GasimGul, A. (2005) Pollution of the Caspian Sea. *Handbook of Environmental Chemistry*, Springer-Verlag Berlin Heidelberg, **5**, Part P, 109-142.

[3] Adekola, B.N. and Eletta, O.A.A. (2007) A study of heavy metal pollution of Asa River, Ilorin. Nigeria; trace metal monitoring and geochemistry. *Environmental Monitoring and Assessment*, **12**, 157-163.

[4] Zhuravel, V.E., Bezverbnaya, I.P. and Buzoleva, S.L. (2004) Microbian indication of pollution of the coastal zone of the sea of Okhotsk and Avacha Bay. *Russian Journal of Marine Biology*, **30(2)**, 121-126.

[5] Burdon, F., Bouchaud, J.P., Tannoudji, A. and Levy, C.C. (2002) *Statistics & Laser Cooling* (*Paperback*), Cambridge University Press, UK.

[6] Lichthenthaler, K.H. and Buschmann, C. (1987) Chlorophyll Fluorescence Spectra of Green Bean Leaves. *Journal of Plant Physiology*, **129(1-2)**, 137-147.

[7] Lichtenthaler, K.H. and Riderle, U. (1988) The role of the chlorophyll fluorescence in the detection of stress conditions in plants. *CRC Critical Reviews in Analytical Chemistry*, **19**, S29-S85.

[8] Jovanić, R.B. and Dramićanin, D.M. (2003) In vivo monitoring of chlorophyll fluorescence response to low-dose γ-irradiation in Pumpkin (Cucurbita pepo). *Luminescence*, **18**, 274-277.

[9] Aizdaicher, N.A. and Reunova, Yu. A. (2002) Effects of detergents on in vitro growth of diatom alga thalassiosira pseudonana. *Russian Journal of Marine Biology*, **28(5)**, 324-328.

[10] Vasconcelos, A., Silva, C.J.S.M., Schroeder, M., Guebitz, G.M. and Cavaco-Paulo, A. (2006) Detergent formulations for wool domestic washings containing immobilized enzymes. *Biotechnology Letters*, **28(10)**, 725-731.

[11] Ca´rdenas, L., Vidali, L., Domı´nguez, J., Pe´rez, H., Sa´nchez, F., Hepler, K.P. and Carmen Quinto, C. (1998) Rearrangement of actin microfilaments in plant root hairs responding to rhizobium etli nodulation signals. *Plant Physiology*, **116(3)**, 871-877.

[12] Nand, L. and Richa, M. (2003) Synthetic detergent induced changes in the seed inhibition pattern and dehydrogenese activity in mungbean (Vigna radiata). *EcoEnvConserv*, **9(3)**, 379-383.

[13] Park, J., Gu, Y., Lee, Y., Yang, Z. and Lee, Y. (2004) Phosphatidic acid induces leaf cell death in arabidopsis by activating the rho-related small G protein GTPase-mediated pathway of reactive oxygen species generation. *Plant Physiology*, **134(1)**, 129-136.

[14] Behzadipou, M., Kluge, M. and Liithjea, S. (2001) Changes in plasma membrane fluidity of corn (Zea mays L.) roots after Brij 58 treatment. *Protoplasma*, **217**, 65-69.

[15] Brandt, K.K., Hesseloy, M.E., Rosloev, E.P., Enriksen, K. and Oyrensen, J.S. (2001) Toxic effects of linear alkylbenzene sulfonate on metabolic activity, growth rate, and microcolony formation of nitrosomonas and nitrosospira strains. *Applied and Environmental Microbiology*, **67(6)**, 2489-2498.

[16] Nanba, O. and Satoh, K. (1987), *Proceedings of the National Academy of Sciences*, USA. **84**, 109-112.

[17] Reunova, Y.A. and Ayzdaycher, N.A. (2003) Effects of detergent on chlorophyll a content and quantity dynamics of microalga Chroomonas salina (Wils.) Butch. (Cryptophyta). *International Journal on Algae*, **5**, 106-110.

[18] Mimuro, M. and Katoh, T. (1991) Carotenoids in photosynthesis: Absorption, transfer and dissipation of light energy. *Pure and Applied Chemistry*, **63(1)**, 123-130.

[19] Green, B.R. (1988) The chlorophyll-protein complexes of higher plant photosynthetic membranes or Just what green band is that? *Photosynthesis Research*, **15(1)**, 30-32.

[20] Gadallah, M.A.A. (2004) Phytotoxic effects of industrial and sewage waste waters on growth, chlorophyll content, transpiration rate and relative water content of potted sunflower plants. *Water, Air, & Soil Pollution*, **89(1-2)**, 33-47.

[21] Szabad, J., Lehoczki, E., Szalay, L. and Csatorday, K. (1984) Lutein-chlorophyll-a energy transfer in detergent micelles, *Biophysics of Structure & Mechanism*, **1(1)**, 65-74.

[22] Eggink, L.L., Park, H. and Hoober, J.K. (2001) The role of chlorophyll b in photosynthesis: Hypothesis, BioMed Central.

[23] Murphy, D.J. and Woodrow, I.E. (1984) The effects of Triton X-100 and n-octyl f-D-glucopyranoside on energy transfer in photosynthetic membranes. *Biochemical Journal*, **224(3)**, 989-993.

[24] Liu, S., Dong, F.Q., Tang, C.Q., Kuang, T.Y., Li, L.B. and Liu, Y. (2006) Photodamage to pigment in the pho-

tosystem reaction center D1/D2/Cytochrome b559 complex. *Journal of Integrated Plant Biology*, **48(7)**, 800-806.

[25] Moya, I., Silvestri, M., Vallon, O., Cinque, G. and Bassi, R. (2001) Time-resolved fluorescence analysis of the photosystem II antenna proteins in detergent micelles and liposomes. American Chemical Society.

[26] Tang, D., Jankowiak, R., Seibert, M. and Small, G.J. (1991) Effects of detergent on the excited state structure and relaxation dynamics of the photosystem II reaction center: A high resolution hole burning study. *Journal of Photosynthesis Research*, **27(1)**, 19-29.

[27] Ivanov, B.N., Ignatova, L.K. and Romanova, A.K. (2007) Diversity in forms and functions of carbonic anhydrase in terrestrial higher plants. *Russian Journal of Plant Physiology*, **54(2)**, 143-162.

[28] Santacruz-Ruvalcaba, F., Gutiérrez-Pulido, H. and Rodríguez-Garay, B. (1999) Efficient in vitro propagation of agave parrasana berger. *Plant Cell, Tissue and Organ Culture*, **56(3)**, 163-167.

[29] Klimov, V.V., Karapetian, N.V. and Krasnovskiĭ, A.A. (1975) The effect of detergent Triton X = 100 on the light induced changes in the fluorescence yield of chloroplasts. *Journal of Molecular Biology* (*Mosk*), **9**, 219-226.

[30] Katoh, S. (2003) Early research on the role of plastocyanin in photosynthesis. *Photosynthesis Research*, **76(1-3)**, 255-261.

[31] Dekker, J.P., Germano, M., Roon, H. and Boekema, E,J. (2002) Photosystem II solubilizes as a monomer by mild detergent treatment of unstacked thylakoid membranes. *Photosynthesis Research*, **72(2)**, 203-210.

[32] Vernon, L.P. (2003) Photosynthesis and the Charles F. Kettering Research Laboratory. *Photosynthesis Research*, **76(1-3)**, 379-388.

Vol.2, No.5, 400-406 (2010)
doi:10.4236/health.2010.25060

Cointegration of event-related potential (ERP) signals in experiments with different electromagnetic field (EMF) conditions

Argiro E. Maganioti[1]*, Hountala D. Chrissanthi[1], Papageorgiou C. Charalabos[2,3], Rabavilas D. Andreas[3], Papadimitriou N. George[2], Capsalis N. Christos[1]

[1]National Technical University of Athens, Department of Electrical Engineering, Division of Information Transmission Systems and Material Technology, Athens, Greece; *Corresponding Author: roumag@mail.ntua.gr
[2]Department of Psychiatry, Eginition Hospital, University of Athens, Athens, Greece
[3]University Mental Health Research Institute (Umhri), Athens, Greece

Received 11 December 2009; revised 20 February 2010; accepted 23 February 2010.

ABSTRACT

Due to their non-stationarity, ERP signals are difficult to study. The concept of cointegration might overcome this problem and allow for the study of the co-variability between whole ERP signals. In this context cointegration factor is defined as the ability of an ERP signal to co-vary with other ERP signals. The aim of the present study was to investigate whether the cointegration factor is dependent on different EMF conditions and gender, as well as the locations of the electrodes on the scalp. The findings revealed that women have a significantly higher cointegration factor than men, while all subjects have increased cointegration factors in the presence of EMF. The cointegration factor is location dependent, creating a distinct cluster of high cointegration capacity at the central and lateral electrodes of the scalp, in contrast to clusters of low cointegration capacity at the anterior and posterior electrodes There seem to be distinct similarities of the present findings with those from standard methodologies of the ERPs. In conclusion cointegration is a promising tool towards the study of functional interactions between different brain locations.

Keywords: EMF; ERP; Stationarity; Cointegration; ACF

1. INTRODUCTION

The electroencephalogram (EEG) is a non-invasive technique, providing a millisecond by millisecond readout of the brain's processing of information, and is relatively inexpensive to implement. Event-related potentials (ERPs) are a reflection of the brain's electrical response to stimulation. Typically, the event-related activity is small and is, thus difficult to view in the single trial. It is usually covered by the ongoing 'spontaneous' EEG. ERPs techniques overcome this initially poor signal to noise ratio by averaging across many trials, typically from about 15 to several hundred [1].

Both EEG and ERP signals are time series. Stationary EEG signals are successfully analyzed in the frequency domain using Fourier transformations [2]. It has been found that electromagnetic fields (EMF), similar to those emitted by mobile phones, have a gender specific effect on the energy of the EEG [3]. However, Fourier transformations cannot be applied on the non-stationary ERP signals. There are a number of alternative approaches that overcome the issue of non-stationarity, such as windowed Fourier [4,5] and wavelet analysis [6,7].

The majority of the studies analysing ERPs focuses on certain components of the ERP signal (P50, N100, P200, N200, P300, N400, P600) [8,9], each of which receives a particular functional interpretation in the physiology of the brain. Very few analyses have been made employing the whole time-series and even less regarding the correlation among the activity of different electrodes. The common methods used for processing ERPs include coherence [10], regression [11], correlation [12] and Granger causality [13]. Most of these methods were first developed in economic sciences.

In the present paper the whole series of the ERP is employed as a unit and its non-stationarity is taken into consideration. The approach, based on a concept introduced by Granger in analysing economic time series, is cointegration [14]. With regards to brain stimuli, cointegration has been used for linear autoregressive EEG modeling [15] and for utilizing the stationarity of the EEG multivariate time series (MTS) [16].

In the present study, cointegration is defined as the ability of two ERP signals to co-vary in time. The aim is to investigate whether this ability is dependent on different EMF conditions and gender, as well as the locations of the electrodes on the scalp.

2. METHODS

2.1. Participants

Two different groups of people, who took part in two separate experiments, were used in this study. The first group consisted of nineteen healthy individuals (9 men and 10 women, mean age = 23.3 ± 2.23 years, mean education = 16.9 ± 1.82 years) and participated in the first experiment. The participants of the second experiment were twenty healthy individuals (10 men and 10 women, mean age = 22.75 ± 2.71 years, mean education = 16.3 ± 1.71 years). In both experiments, the male and female subgroups were homogeneous with regards to age and educational level. All participants were right-handed and had no history of any hearing problem. Informed consent was obtained from all subjects.

2.2. Experimental Setup and Measurement Procedure

The two experiments were in fact the same as far as the evaluation method is concerned. The subjects were evaluated with the digit span Wechsler Auditory test [17]. A warning stimulus of either high (3000 Hz) or low frequency (500 Hz) was presented through earphones to the subjects, who were asked to memorize the numbers that followed. The warning stimulus lasted 100 msec. A one second interval followed the onset of the warning stimulus and then the numbers to be memorized were presented by a male voice. At the end of the number sequence presentation, the same signal tone was repeated. The signals were recorded for a 1500 msec interval, divided into 500 msec before the warning stimulus (EEG) and 1000 msec after that (ERP) [3]. The numbers were recalled by the subject in the same (low frequency tone) or in the opposite order (high frequency tone) than that presented to the participant.

The total task consisted of 52 repetitions for a period of about 45 min. The subjects performed the tasks twice, with and without radiation, with an interval of two weeks between the measurements. The order in which the subject was exposed at the EMF (exposure at the first or second visit) was random and the subjects were unaware of the experimental condition.

The only difference between the two experiments was the frequency of the EMF signal at which the subjects were exposed. The first experiment involved an antenna emitting 900 MHz electromagnetic field, with mean power at 64 mWatt, while in the second one the antenna used emitted 1800 MHz electromagnetic field, with mean power at 128 mWatt. In both experiments the signal was not modulated.

The experimental setup was the same in both cases and included a Faraday room, which screened any electromagnetic interference that could affect the measurements. The subjects sat in an anatomical chair and a certified dipole antenna was fixed near their right ear. Care was taken so that the distance between telephone and ear (about 20 cm) was constant during the whole session. The antenna was driven by a signal generator, which could be switched on or off.

The electrophysiological signals were recorded with Ag/AgCl electrodes. Electrode resistance was kept constantly below 5 kΩ. EEG activity was recorded from 15 scalp electrodes (Fp1, F3, C5, C3, Fp2, F4, C6, C4, O1, O2, P4, P3, Pz, Cz, Fz) based on the International 10-20 system of Electroencephalography [18], referred to both earlobes. An electrode placed on the subject's forehead served as ground. The bandwidth of the amplifiers was set at 0.05 Hz to 35 Hz. During the administration of stimuli, the subjects had their eyes closed in order to minimize eye movements and blinks. Eye movements were recorded through electro-oculogram (EOG) and recordings with EEG higher than 75 μV were rejected which on the average were 2.1 ± 1.4 trials from the total of 52. Warning stimuli, as well as the numbers to recall were presented binaurally via earphones at an intensity of 65dB sound pressure level. The earphones did not have metal components in order to avoid EMF concentration.The evoked biopotential signal was submitted to an analogue-to-digital conversion, at a sampling rate of 1 KHz.

2.3. Data Transformation

For each question 1500 data points, each corresponding to time segments of 1 msec duration for each electrode were saved. In order to maximize the signal to noise ratio for each subject and each channel all values were average referenced on the basis of the grand average across the 52 repetitions of the EEG values. This procedure was done separately for each EMF condition in both experiments. Artifact–contaminated epochs with a signal deviation of > 75 μV in the EEG or 100 μV in the EOG were excluded. The final data for analysis for each subject and condition consists of 1500 amplitude values for each electrode, expressed in μVolts corresponding to the 1500 msec of the time period [3].

A representative chart of the primary recordings of the amplitude values are shown in **Figure 1**.

2.4. Stationarity and Integration

A strict stationary process is a stochastic process whose probability distribution does not vary over time; basic characteristics such as the mean $\left(E\left(x_t\right)\right)$ and the vari-

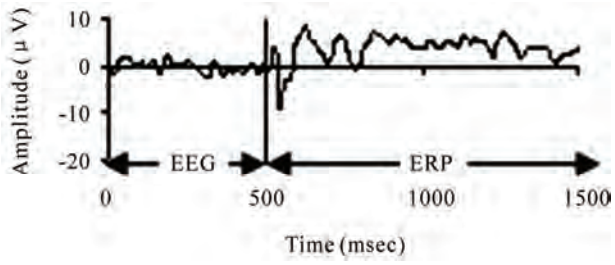


Figure 1. Representative graph of the 1500 amplitude values of the electrode Fz.

ance $\left(\mathrm{var}\left(x_t\right)\right)$ remain constant over time. Stationary time series are easier to analyze and forecast, therefore non-stationary raw data are often transformed, in order to become stationary. Non-stationary processes contain a trend. Trend represents a general systematic linear or non-linear component that changes over time and does not repeat, or at least does not repeat within the time range captured by the data. There are two kinds of trend; deterministic and stochastic. Time series with deterministic trend have constant variance but non-constant mean, whereas the ones with stochastic trend exhibit non-constant variance. Some processes may contain both stochastic and deterministic trends, which means that they combine a random walk $\left(r_t\right)$ and a deterministic trend $\left(\beta t\right)$, plus an error term.

$$x_t = r_t + \beta t + \varepsilon_t$$

A non-stationary process with deterministic trend is transformed into a stationary one by regressing it on time t. The most common method for removing stochastic trends from a non-stationary process is differencing. Differencing of a time series x_t in discrete time t is the transformation of the series x_t to a new time series dif_t where the values dif_t are the differences between consecutive values of x_t. The d_{th} differences of a time series are described by the following expression:

$$dif^{(d)}{}_t = dif^{(d-1)}{}_t - dif^{(d-1)}{}_{t-1},$$

where the top index *d* means the order of the difference.

Many time series need to be differenced more than once in order to achieve stationarity. From this comes the definition of integration: a time series is said to be integrated of order *d*, in short, I(d), if it becomes stationary after differencing *d* times. A series which is I(d) is also said to have *d* unit roots.

2.5. Testing for Stationarity: KPSS Test

The KPSS test [20] is commonly used to test for stationarity in time-series data. Let $\{x_t\}$, $t = 1, 2, \ldots, N$, be the observed series for which we wish to test stationarity. Assume that the series can be decomposed into the sum of a deterministic trend, a random walk, and a stationary error with the following linear regression model

$$x_t = r_t + \beta t + \varepsilon_t$$

where r_t is a random walk, *i.e.*, $r_t = r_{t-1} + u_t$ and u_t is independent identically distributed (iid) $N(0, \sigma_u^2)$, βt is a deterministic trend and ε_t is a stationary error.

To test in this model if x_t is a stationary process, the null hypothesis will be $\sigma_u^2 = 0$, which means that the intercept is a fixed element, against the alternative of a positive σ_u^2. Under the null hypothesis, in the case of stationarity, the residuals $e_t (t = 1, 2, \ldots, N)$ are from the regression of *x* on an intercept and time trend, $e_t = \varepsilon_t$. Let the partial sum process of the e_t be

$$S_t = \sum_{j=1}^{t} e_j ,$$

and σ^2 be the long-run variance of e_t, which is defined as

$$\sigma^2 = \lim N^{-1} E\left[S_N^2\right].$$

The consistent estimator of σ^2 can be constructed from the residuals e_t by the equation below [19]

$$\hat{\sigma}^2(p) = \frac{1}{N}\sum_{t=1}^{N} e_t^2 + \frac{2}{N}\sum_{j=1}^{p} w_j(p) \sum_{t=j+1}^{N} e_t e_{t-j} ,$$

where *p* is the truncation lag, $w_j(p)$ is an optional weighting function that corresponds to the choice of a special window, e.g., Bartlett window (Bartlett, 1950) $w_j(p) = 1 - j/(p+1)$.

Then the KPSS test statistic is given by

$$KPSS = N^{-2}\sum_{t=1}^{N} S_t^2 / \hat{\sigma}^2(p) .$$

Under the null hypothesis of stationarity,

$$KPSS \to \int_0^1 V_2(r)^2 dr ,$$

where $V_2(r)$ is the second level Brownian bridge, given by

$$V_2(r) = B(r) + (2r - 3r^2)B(1) + (-6r + 6r^2)\int_0^1 B(s)ds .$$

The upper tail critical values of the asymptotic distribution of the KPSS statistic are given by Kwiatkowski *et al.* [20]. It has been shown that KPSS test is the most powerful test for the stationarity of time series [20,21], therefore it will used in the present paper.

Further details of the test are given in Zivot and Wang (2002).

2.6. Cointegration

Granger and Newbold [22] have proven that using two I(1) time series, an apparently significant regression and correlation can be obtained, even if the two time series are independent. These regression results were coined as

"spurious regressions".

In order to overcome this limitation, Granger introduced a new method, based on the notion that a linear combination between a pair of non-stationary integrated series can be stationary. This property is known as cointegration [14]. Cointegration is a formulation of the phenomenon that non-stationary integrated series can have linear combinations that have a lower degree of integration than the original series.

In this paper, the tool used for testing the co-variability of different electrodes is cointegration. The method of cointegration involves testing whether the residuals from a cointegrating regression are stationary. Consider two time series y_{1t} and y_{2t} which are both I(d). In general, any linear combination of y_{1t} and y_{2t} will be also I(d). However, if there exists a vector $(1, -\beta)'$, such that the linear combination

$$z_t = y_{1t} - a - \beta \cdot y_{2t}$$

is I(d – b), d ≥ b > 0,then, following Engle and Granger (1987), y_{1t} and y_{2t} are defined as cointegrated of order (d, b) denoted $y_t = (y_{1t}, y_{2t})' \sim CI(d, b)$, with $(1, -\beta)'$ called the cointegrating vector [23].

2.7. Data Processing

In this study, the ERP signals are being analyzed in terms of cointegration. More precisely, the stationarity of the ERPs of each electrode, for all the subjects, is being examined using the KPSS test. If the ERPs are found to be non-stationary (which is the case), they are properly differenced. This procedure is repeated until KPSS test indicates stationarity. In order to achieve stationarity the signals were differenced twice or three times. The null hypothesis of stationarity with upper tail critical value of the asymptotic distribution of the KPSS statistic 0.119 is tested.

Cointegration is tested for those electrodes that are integrated of the same order. The two ERPs are being regressed one against the other and the order of integration or stationarity of the residuals is tested. If the order of integration of the residuals is less than that of the two ERP signals then cointegration exists between the two variables. A 15 × 15 array is created for each subject in each condition and if cointegration exists between electrode i and electrode j—provided that the two electrodes are integrated of the same order—a 1 is placed at the [i, j] cell. Else, if no such relationship exists, a 0 is placed in the cell. For each electrode, the respective column is summed, and the result is a 1 × 15 vector, containing the exact number of cointegrations for each electrode for the specific subject. This number is subsequently normalized by dividing by the maximum number of possible cointegrations (in this case 14). This number is called Cointegration Factor (CF) of the electrodes. According to the above, the values of the electrode CF can range from 0 to 1. In the same way, the mean value of electrode CFs is the Aggregate Cointegration Factor (ACF).

3. STATISTICAL ANALYSIS

The aggregate CF was subjected to two-way ANOVA with gender (male, female) and EMF condition (none, 900 MHz, 1800 MHz) as the independent factors. Likewise the CFs at the fifteen electrodes were subjected to MANOVA with the same independent factors. Finally, in order to examine whether the CF differs among different electrodes (locations), an ANOVA procedure with repeated measures was performed for all the subjects and measurements. The statistical significance was set at 0.05.

4. RESULTS

Univariate analysis of variance with ACF as the dependent variable and EMF condition (off, 900 MHz, 1800 MHz), gender (male, female) and their interaction as the independent factors revealed a significant EMF effect ($F_{2,77}$ = 4, p = 0.022) as well as a significant gender effect ($F_{1,77}$ = 4, p = 0.048), but no interaction effect. **Figure 2** helps to clarify the direction of the differences between EMF conditions and genders. As post-hoc comparisons with Bonferroni corrections show, women have in general a significantly higher ACF than men (p = 0.048). The presence of radiation increases ACF. As a result ACF in the presence of 1800 MHz is significantly higher than in the absence of radiation (p = 0.029).

In order to further qualify the effect of EMF and gender on CF for each electrode individually, the CFs of the 15 electrodes were subjected to MANOVA with EMF condition (off, 900 MHz, 1800 MHz), gender (male, female) and their interaction as independent factors. **Table 1** shows the significance of their effects on the CF for all the electrodes. Significant effects are shown in bold. Once again the variability of the CF of the leads is mostly due to the effect of EMF conditions and to a lesser degree of gender differences, while the EMF x Gender interaction does not have any significant effect. These effects were more obvious at F3, C5, C3, C6, C4, O1, P4, Pz and Cz.

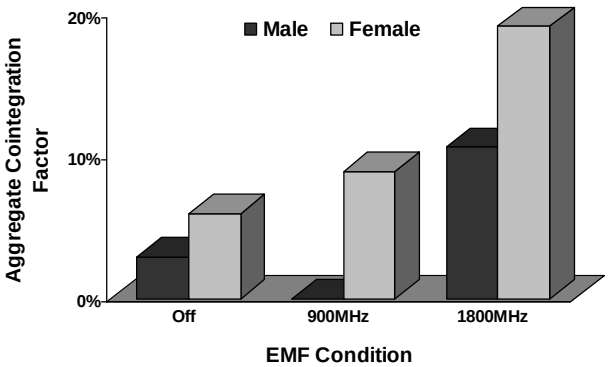


Figure 2. Average of the Aggregate Cointegration Factor for each gender and EMF condition.

Table 1. Significance of the effects of EMF condition and gender on the cointegration factor of the electrodes.

	EMF	Gender	EMF x Gender
Lead	**p-value**	**p-value**	**p-value**
Fp1	0.445	0.302	0.300
F3	**0.036**	**0.044**	0.430
C5	**0.000**	**0.035**	0.218
C3	**0.023**	**0.011**	0.517
Fp2	0.561	0.174	0.709
F4	0.174	0.114	0.346
C6	**0.045**	0.200	0.624
C4	**0.005**	**0.008**	0.676
O1	**0.023**	0.546	0.629
O2	0.457	0.142	0.516
P4	**0.035**	0.505	0.936
P3	0.067	0.130	0.435
Pz	**0.017**	0.295	0.775
Cz	**0.036**	**0.027**	0.503
Fz	0.249	0.102	0.684

The repeated ANOVA procedure with the CFs at the fifteen electrodes as the within subjects factor proved that there exist significant differences between the means of the CFs at different locations ($F_{14,77} = 3.7$, $p < 0.001$).

As **Figure 3** shows high CFs seem to cluster in the central and lateral electrodes, while electrodes with low CFs are grouped mainly in the posterior but also the anterior electrodes.

5. DISCUSSION

As Granger notes, "cointegration signifies co-movements among trending variables which can be exploited to test for the existence of equilibrium relationships within a fully dynamic specification framework" [23]. In the present study, the cointegration factor was defined as the ability of ERP signals to co-vary in time. Results showed that the values of the CFs seem to follow specific patterns forming distinct clusters, which discriminate the central and lateral electrodes, having relatively high CFs, from the anterior and posterior ones. Furthermore, women have a significantly higher CF than men, while all subjects have increased CFs in the presence of EMF.

The above findings seem to be in congruence with other findings regarding typical characteristics of the ERP signal. Specifically, with regards to EMF effects, EEG studies showed an increase of spectral power in the

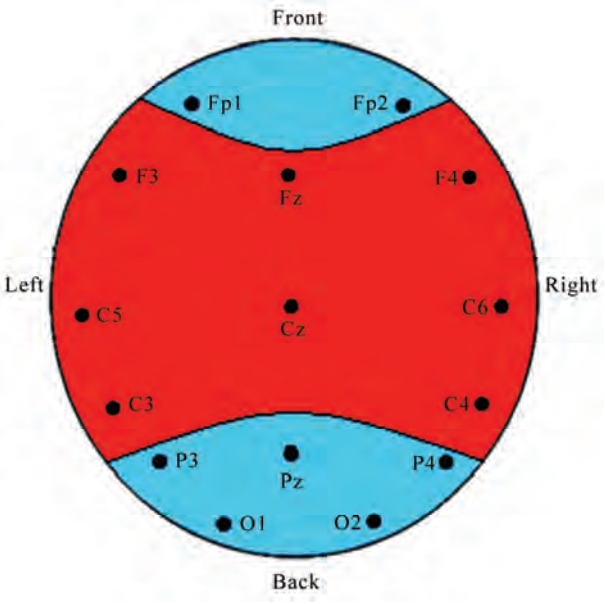


Figure 3. Mean values of the cointegration factors at different locations on the scalp. Red area signifies cointegration factors greater than 7.5%, while the blue areas contain electrodes with cointegration factors less than 7.5%.

alpha band [2,24,25], while ERP studies have demonstrated reduced N100 amplitudes, shortened N100 latencies and prolonged P300 latencies [26]. In this context, it has been shown that EMF modulated the event-related desynchronization/synchronization (ERD/ERS) responses in the approximately 4-8 Hz EEG frequencies [27]. In view of these collected observations the authors concluded that EMF could influence brain activity through thermal and non-thermal mechanisms [28,29].

However, a number of studies failed to find EMF effects upon brain physiology [30,31]. This might be attributed to the fact that these studies focused not on the whole ERP recording, but on certain components of ERP. In contrast, the present study analyzed the total ERP signal, specifically its cointegration capacity, which is likely to represent a more valid picture of the effect of EMF exposure on the ERP.

The gender-related differences of CF may be related to different strategies activated due to sex-related functional brain organization, as indicated from psychophysiological and neurobiological studies [32-35]. There also appears to be consistent evidence that EEG coherence varies systematically with gender [36].

Finally, the auditory nature of the warning stimuli that elicit the ERPs might be the possible reason that affects the ERP activity in the temporo-parietal region [37,38] creating the distinct clusters of CF presently found.

To the best of our knowledge, this is the first attempt to apply the concept of cointegration in the study of co-variability of ERP signals. Cointegration seems to be a promising tool towards the study of functional interact-

tions between different brain locations. Also, the method can be applied on EEG data, obtained from different clinical and technical experimental conditions. Finally, it is our immediate object to demonstrate the feasibility of the cointegration method on additional cogntive tasks that involves activities in the frontal and occipital scalp locations, using data from ongoing experiments.

6. ACKNOWLEDGEMENTS

The authors would like to thank M. Kyprianou, Scientific Investigator, Athens, Greece, for his support on the statistical analysis of the experimental results.

REFERENCES

[1] Fabiani, M., Gratton, G. and Coles, M. (2000) Event-related potentials: Methods, theory and applications. *Handbook of Psychophysiology*, 3rd Edition. Cambridge University Press, New York.

[2] Laufs, H., Kleinschmidt, A., Beyerle, A., Eger, E., Salek-Haddadi, A., Preibisch, C. and Krakow, K. (2003) EEG-correlated fMRI of human alpha activity. *NeuroImage*, **19(4)**, 1463-1476.

[3] Papageorgiou, C.C., Nanou, E.D., Tsiafakis, V.G., Capsalis, C.N. and Rabavilas, A.D. (2004) Gender related differences on the EEG during a simulated mobile Phone signal. *Neuroreport*, **15(16)**, 2557-2560.

[4] Agam, Y. and Sekuler, R. (2007) Interactions between working memory and visual perception: An ERP/EEG study. *NeuroImage*, **36(3)**, 933-942.

[5] Edwards, E., Soltani, M., Deouell, L.Y., Berger, M.S. and Knight, R.T. (2005) High gamma activity in response to deviant auditory stimuli recorded directly from human cortex. *Journal of Neurophysiology*, **94**, 4269-4280.

[6] Yordanova, J., Rosso, O.A. and Kolev, V. (2003) A transient dominance of theta event-related brain potential component characterizes stimulus processing in an auditory oddball task. *Clinical Neurophysiology*, **114(3)**, 529-540.

[7] Quiroga, R.Q., Sakowitz, O.W., Basar, E. and Schürmann, M. (2001) Wavelet Transform in the analysis of the frequency composition of evoked potentials. *Brain Research Protocols*, **8(1)**, 16-24.

[8] Chlubnová, J., Kremláček, J., Kubová, Z. and Kuba, M. (2005) Visual evoked potentials and event related potentials in congenitally deaf subjects. *Physiological Research*, **54**, 577-583.

[9] Sanders, L.D., Newport, E.L. and Neville, H.J. (2002) Segmenting nonsense: An event-related potential index of perceived onsets in continuous speech. *Nature Neuroscience*, **5**, 700-703.

[10] Shibata, T., Shimoyama, I., Ito, T., Abla, D., Iwasa, H., Koseki, K., Yamanouchi, N., Sato, T. and Nakajima, Y. (1998) The synchronization between brain areas under motor inhibition process in humans estimated by event-related EEG coherence. *Neuroscience Research*, **31(4)**, 265-271.

[11] Hauk, O., Davis, M.H., Ford, M., Pulvermüller, F. and Marslen-Wilson, W.D. (2006) The time course of visual word recognition as revealed by linear regression analysis of ERP data. *NeuroImage*, **30(4)**, 1383-1400.

[12] Nikolaev, A.R., Ivanitsky, G.A., Ivanitsky, A.M., Posner, M.I. and Abdullaev, Y.G. (2001) Correlation of brain rhythms between frontal and left temporal (Wernicke's) cortical areas during verbal thinking. *Neuroscience Letters*, **298(2)**, 107-110.

[13] Oya, H., Poon, P.W.F., Brugge, J.F., Reale, R.A., Kawasaki, H., Volkov, I.O. and Howard III, M.A. (2007) Functional connections between auditory cortical fields in humans revealed by Granger causality analysis of intra-cranial evoked potentials to sounds: Comparison of two methods. *Biosystems*, **89**, 198-207.

[14] Engle, R. and Granger, C.W. (1987) Co-integration and error correction representation estimation and testing. *Econometrica*, **55(2)**, 251-276.

[15] Yang, K. and Shahabi, C. (2005) On the stationarity of multivariate time series for correlation-based data analysis. *Proceedings of the Fifth IEEE International Conference on Data Mining*, 805-808.

[16] Brandt, M.E., Ademoglu, A. and Pritchard, W.S. (2000) Non-linear prediction and complexity of alpha EEG activity. *International Journal of Bifurcation and Chaos*, **10(1)**, 123-133.

[17] Wechsler, D. (1955) Manual for the Wechsler adult intelligence scale. Psychological Corporation, New York.

[18] Jasper, H.H. (1958) The ten-twenty electrode system of the international federation. *Electroencephalography and Clinical Neurophysiology*, **10**, 371-375.

[19] Newey, W.K. and West, K.D. (1987) A simple, positive semi-definite, heteroskedasticity and autocorrelation consistent covariance matrix. *Econometrica*, **55(3)**, 703-708.

[20] Kwiatkowski, D., Phillips, P.C.B., Schmidt, P. and Shin, Y. (1991) Testing the null hypothesis of stationarity against the alternative of a unit root. How sure are we that economic time series have a unit root? *Journal of econometrics*, **54**, 158-178.

[21] Hobijn, B., Franses, P.H. and Ooms, M. (2004) Generalizations of the KPSS-test for stationarity. *Statistica Neerlandica*, **58(4)**, 483-502.

[22] Granger, C.W. and Newbold, P. (1974) Spurious regression in econometrics. *Journal of Econometrics*, **2(2)**, 111-120.

[23] Dolado, J.J., Gonzalo, J. and Marmol, F. (2009) A Companion to Theoretical Econometrics, In: Baltagi, B.H., Ed., Blackwell Reference Online, 634-654 http://www.blackwellreference.com/public/book?id=g97814051061764_9781405106764

[24] Croft, R.J., Chandler, J.S., Burgess, A.P., Barry, R.J., Williams, J.D. and Clarke, A.R. (2002) Acute Mobile phone operation affects neural function in humans. *Clinical Neurophysiology*, **113(10)**, 1623-1632.

[25] Curcio, G., Ferrara, M., Moroni, F., D'Inzeo, G., Bertini, M. and De Gennaro, L. (2005) Is the brain influenced by a phone call? An EEG study of resting wakefulness. *Neuroscience Research*, **53(3)**, 265-270.

[26] Hamblin, D.L., Wood, A.W., Croft, R.J. and Stough, C. (2004) Examining the effects of electromagnetic fields emitted by GSM mobile phones on human event-related potentials and performance during an auditory task. *Clinical Neurophysiology*, **115(1)**, 171-178.

[27] Krause, C.M., Björnberg, C.H., Pesonen, M., Hulten, A.,

Liesivuori, T., Koivisto, M., Revonsuo, A., Laine, M. and Hämäläinen, H. (2006) Mobile phone effects on children's event-related oscillatory EEG during an auditory memory task. *International Journal of Radiation Biology*, **82(6)**, 443-450.

[28] Challis, L.J. (2005) Mechanisms for interaction between RF fields and biological tissue. *Bioelectromagnetics*, Supplement 7 Review, **26**, 98-106.

[29] Valentini, E., Curcio, G., Moroni, F., Ferrara, M., De Gennaro, L. and Bertini, M. (2007) Neurophysiological effects of mobile phone electromagnetic fields on humans: A comprehensive review. *Bioelectromagnetics*, **28**, 415-432.

[30] Hamblin, D.L., Croft, R.J., Wood, A.W., Stough, C. and Spong, J. (2006) The sensitivity of human event-related potentials and reaction time to mobile phone emitted electromagnetic fields. *Bioelectromagnetics*, **27**, 265-273.

[31] Yuasa, K., Arai, N., Okabe, S., Tarusawa, Y., Nojima, T., Hanajima, R., Terao, Y. and Ugawa, Y., (2006) Effects of thirty minutes mobile phone use on the human sensory cortex. *Clinical Neurophysiology*, **117(4),** 900-905.

[32] Skrandies, W., Reik, P. and Kunze, C. (1999) Topography of evoked brain activity during mentalarithmetic and language tasks: sex differences. *Neuropsychologia*, **37(4)**, 421-430.

[33] Briere, M.E., Forest, G., Chouinard, S. and Godbout, R. (2003) Evening and morning EEG differences between young men and women adults. *Brain and Cognition*, **53(2)**, 145-148.

[34] Dimpfel, W., Wedekind, W. and Keplinger, I. (2003) Gender difference in electrical brain activity during presentation of various film excerpts with different emotional content. *European Journal of Medical Research*, **8(5)**, 192-198.

[35] Razumnikova, O.M. and Vol'f, N.V. (2007) Gender differences in interhemisphere interactions during distributed and directed attention. *Neuroscience and Behavioral Physiology*, **37(5)**, 429-434.

[36] Barry, R.J., Clarke, A.R., McCarthy, R., Selikowitz, M., Johnstone, S.J. and Rushby, J.A. (2004) Age and effects in EEG coherence: I. Developmental trends in normal children. *Clinical Neurophysiology*, **115(10)**, 2252-2258.

[37] Haarala, C., Aalto, S., Hautzel, H., Julkunen, L., Rinne, J.O., Laine, M., Krause, B. and Hämäläinen, H. (2003) Effects of a 902 MHz mobile phone on cerebral blood flow in humans: a PET study. *Neuroreport*, **14(16)**, 2019-2023.

[38] Cinel, C., Boldini, A., Russo, R. and Fox, E. (2007) Effects of mobile phone electromagnetic fields on an auditory order threshold task. *Bioelectromagnetics*, **28(6)**, 493-496.

Vol.2, No.5, 407-411 (2010)
doi:10.4236/health.2010.25061

Respiratory rehabilitation with abdominal weights: a prospective case study

Stanley John Winser[1], Priya Stanley[1], George Tarion[2]*

[1]Department of Physiotherapy Masterskill University college of Health Science (MUCH), Cheras, Malaysia;
[2]Department of Physical Medicine and Rehabilitation, Christian Medical College, Vellore, India; *Corresponding Author: stanjwpt@gmail.com, stanjw_pt@ yahoo.com, Stanley@masterskill.edu.my

Received 6 January 2010; revised 21 January 2010; accepted 24 January 2010.

ABSTRACT

***Objective*: Abdominal weights was used to strengthen the diaphragm of a C6 ASIA (A) tetraplegic subject with the aim of studying the long term effect of the technique as a part of respiratory rehabilitation. *Setting:* Department of Physical Medicine and Rehabilitation, Christian Medical College, Vellore, Tamil Nadu, India. *Study Design*: Prospective case study. *Material and methods*: The peak EMG amplitude of the diaphragm (DIA), intercostals (INT) and sternoclidomastiod (SCM) were assessed using a surface EMG and inspired lung volume (ILV) was assessed using an adjustable portable spirometer. The measurements were repeated after 3, 6, 9 and 12 months of inspiratory muscle training for a period of 15 minutes daily, 6 days a week for 12 months. *Results and discussion:* Peak amplitudes recorded by the EMG of DIA and SCM muscles showed a progressive increase, INT muscle did not show a consistent change. INV showed a gradual rise from 1772ml to 2760 ml over the study period. These values have the following significance: 1) Use of abdominal weights as a part of respiratory rehabilitation has beneficial long term effects; 2) In patients with tetraplegia, respiratory muscles in particular the diaphragm, are trainable in terms of muscle efficiency; 3) The improvement in the muscle efficiency obtained during the early rehabilitation can be maintained or improved using simple non sophisticated exercises like abdominal weights post discharge. *Conclusions*: Abdominal weights can be used as an effective adjunct to pulmonary rehabilitation in improving the efficiency of diaphragm on a long term basis, thereby reducing the risks associated with pulmonary complications.**

Keywords: Tetraplegia; Abdominal Weights; EMG

1. INTRODUCTION

Complete lesions of the spinal cord affect the respiratory inspiratory and expiratory muscles. The degree of impairment in respiratory function is related to the level of the lesion [1]. If the lesion is below the C3–C4 level, the diaphragm is intact but the loss of other respiratory muscles decreases the vital capacity (VC) to approximately 50% [2,3] and total lung capacity (TLC) to approximately 70% [3-5] of predicted normal values. Paralysis of the expiratory muscles reduces the ability to force expiration leading to an increased residual volume and reduced ability to huff (forcefully exhale) and cough. This may cause secretion to accumulate in the airways. In complete lesions above the Th6 level, the autonomic nervous system is injured, and bronchial hypersecretions occur, which further aggravates problems regarding secretions [6].

Currently a complete retrival of the muscle efficiency following spinal cord injury (SCI) is not known. Cases in which patients are unable to maintain adequate ventilation, long-term mechanical ventilator support is indicated [7]. Although this treatment is effective, it can also lead to serious medical complications such as infection, pneumonia, atelectasis, and even death [8-10]. In fact, the primary cause of death after SCI, regardless of the level of injury, is caused by respiratory insufficiency and complications associated with impaired respiratory function.

Training of these muscles may improve the daily activities. Respiratory muscle training in healthy subjects, COPD (Chronic Obstructive Pulmonary Disease) and other pulmonary patients has shown positive effects [11-17]. In individuals with tetraplegia, training of the inspiratory muscles has been applied by Gross *et al.* [18] Biering-Sorensen *et al.* [19] and Derrickson *et al.* [20]. Use of abdominal weights, resistive inspiratory muscle training, Abdominal binders [21], Trendlenbergs position and Incentive spirometry [22] have been reported in literature to have useful effects in improving the effi-

ciency of diaphragm among the target population. In the survey done we found a paucity of literature on the long term effects of these therapeutic modalites on the respiratory status of tetraplegic patients, thus we intended to observe the influence of abdominal weights on the efficiency of the available respiratory muscle on a tetraplegic patient over a period of 1 year.

2. CASE REPORT

A 30-yr-old male patient presented with complaints of weakness of arms and legs, with fecal and urinary incontinence, in March 2004. The patient's history revealed that a C5 vertebrate fracture caused SCI after a fall from height (mango tree) in December 2003. He had applied to a clinic and had been managed conservatively with bed rest for 3 months.

The patient's history included an internal fixator at his femur because of a fracture that occurred in another vehicle accident before 6 yrs. The patient's medical status was stable. He had no pressure ulcers, range of motion (ROM) of all four limbs were full and free. On clinical neurologic examination, he had spastic tetraplegia with loss of motor function below the C6 level and loss of all sensation below C7 dermatome. Spasticity was found to have grade 1+ in Modified Asworth scale (MAS) in all 4 limbs. Deep tendon reflexes were hyperactive, and plantar response was extensor bilaterally. The patient was classified as C6 American Spinal Injury Association grade A and admitted to a rehabilitation program. The rehabilitation program involves concurrent sessions of physiotherapy, occupational therapy, vocational training, prosthetics and orthostics and recreation classes. In the department of physiotherapy the patient was undergoing re-education exercises for the upperlimbs, progressive orientation to erect stance using tilt table to counteract postural hypotension, passive movement for the lowerlimbs, passive stretching for hamstrings and tendo achilles. In addition to this the patient was given strengthening to the diaphragm muscle using Abdominal weights as a part of respiratory rehabilitation.

3. RESPIRATORY ASSESSMENT

Patient had no history of COPD, TB or other chronic respiratory illnesses. Patient demonstrated a diaphragmatic breathing pattern. Chest expansion was 2 cms at the level of xiphisternum and nil at nipple and axilla. Cough was weak functional. Auscultatory findings revealed a good air entry in all lobes and no added sounds were recorded. Muscle strength of the Diaphragm was good. The strength of the diaphragm involves interpretation of the total neurological involvement, complete respiratory evaluation and observational techniques. It is best done in supine lying. “Poor power” is being graded if the subject is not able to expand his/her epigastric region fully on deep inspiration. “Fair power”, if the subject is able to expand his/her epigastric region fully on deep inspiration. “Good power” the therapist’s hands are placed over the epigastric region with fingers spread, and the subject is asked to inhale, while maximum manual resistance is applied. If the subject is able to complete a full epigastric raise against resistance then he\she can be graded as Good. The subjects who are able to take resistance but not able to hold can be graded as “Fair plus” [23]. Subjects with diaphragmatic power of fair plus and above are candidates for progressive resisted exercises.

4. MATERIALS AND METHODS

A prospective case study of a 30 year-old tetraplegic patient, with complete spinal cord section at C6 level, was considered. The subject gave written consent to participate in the trial, the study was approved by the research board of Christian Medical College, Vellore, India. Initial assessment was done in march 2004 and subsequent assessments were planned at an interval of three months for the next one year. The 2 outcome measures which were considered are peak amplitude of Electromyographic (EMG) readings of diaphragm, intercostals and sternocliedomastoid muscles and Inspired Lung Volume (ILV). The investigator who performed these tests was blind to the situation.

4.1. EMG Analysis

The EMG activity was measured using 3 pairs of Silver chloride bipolar surface electrode. The active electrodes were placed over T7 & T8 intercostal space, T4 & T5 intercostal space and mid portion of sternocleidomastoid to get the electrical activity of diaphragm (DIA), intercostals (INT) and sternocleidomastoid (SCM) muscles respectively as shown in **Figure 1**. Readings of all three groups of muscles were taken simultaneously [24]. Electrodes were secured to the skin using adhesive plaster after skin preparation. While taking the readings the subject was instructed to take 3 consecutive deep inspirations followed by expiration, readings were recorded for 10 seconds and the mean of peak amplititudes in the EMG were recorded. Three trials were done and the best response of peak amplitude was noted.

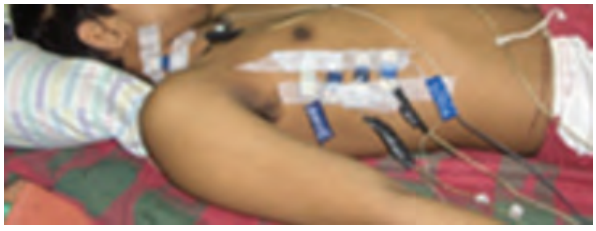


Figure 1. Placement of surface electrodes.

4.2. ILV Analysis

The ILV was assessed using a volume adjustable portable spirometer (Spectra Spirometer™) The spirometer has calibrations for adjusting the flow rate (100 cc, 200 cc,…800 cc) The device has a single chamber with 1 ball inside. Air flows into this channel on inspiration and raises the ball depending on the flow inhaled per second. This upward movement of the ball during inspiration provides the patient with visual feedback *i.e.* an indirect indicator of the inspired volume. The patient was instructed to perform a deep inspiration through the mouth piece and while inspiring he was required to keep the ball inflated for the maximum seconds possible. As shown in **Figure 2**.

ILV was measured using the formula: ILV = Flowrate × seconds

Flow rate: was kept constant at 400 *cc. Seconds: was a measure of patients ability to keep the spirometer ball inflated during inspiration and was calculated using a stop watch.*

4.3. Treatment Protocol

Diaphragm was strengthened using abdominal weights for 15 minutes/day for six days/week for a period of 1 year. An initial evaluation of weight to be placed over the abdomen was done. The subject was positioned in supine lying, then a minimal weight was placed over the epigastric region (weights starts with half a kilogram) and then the subject was allowed to breath (the weight should come up fully with each inspiration). If the subject showed any signs of fatigue or started using his accessory muscles, the weights were taken off immediately. Adequate rest was given, then the procedure was repeated using a lesser weight. If the patient is able to take up the weight comfortably for 15 minutes, a short break was given and the procedure was repeated by increasing the weight by half a kilogram, thus the appropriate weight for training the diaphragm was determined by trial and error method [23]. The evaluated weight was placed over the epigastric region with an isosceles triangular board. The board was placed in such a way that one of the corners touches the xiphisternum and the other two corners touching the anterior borders of the ribcage. Cushioning is provided with the under surface of the triangular board in order to prevent skin irritation, as shown in **Figure 3**. Evaluated weights were added to the triangular board using dish weights. During the training period the subject was instructed to perform the exercise in supine lying and do normal breathing with the weights on for 15 minutes. Progression in weights was done during each review. Subsequent assessments were planned during the routine medical screening and checkups of the client.

Figure 2. Analysis of ILV using spirometer.

Figure 3. Placement of abdominal weights.

5. RESULTS

Weights were progressively increased during each visit. Initial weight was 5 kgs and the patient progressed to 12 kgs by the end of the trial, as shown in **Table 1**.

Increment in the mean of peak amplitudes of EMG values were noted in DIA and SCM muscles, progress in the INT muscle did not show a consistent change (shown in **Figure 4**). Similarly the INV showed a gradual rise from 1772 ml to 2760 ml over the study period (shown in **Figure 5**).

6. DISCUSSION

The efficiency of respiratory muscles were studied over a period of 1 year, following administration of abdominal weighted training with the aim to determine the long term effects of the examined respiratory muscle training. The subjects respiratory muscle efficiency was assessed using recordings made over key respiratory muscles with the help of surface EMG. EMG was chosen because it captures the electrical activity happening within the muscle which is a direct measure of maximal contraction of the assessed muscle. Work done by Jennifer Beck et al on healthy individuals states there is no artifactual effect of lung volume on the diaphragm EMG signal strength during voluntary contractions [25]. Thus the EMG readings

Table 1. Progress of the study.

DATE	Weights used in Kgs	EMG in milli volts		INV in ml
		DIA	1.2453	
Day 1	3	INT	0.7237	1772
		SCM	1.5234	
		DIA	1.4356	
3 months	5	INT	0.5634	2080
		SCM	1.5735	
		DIA	1.5643	
6 months	8	INT	0.8675	2280
		SCM	1.6876	
		DIA	1.7536	
9 months	10	INT	0.7457	2440
		SCM	1.7453	
		DIA	1.9675	
12 months	12	INT	0.9656	2760
		SCM	1.8758	

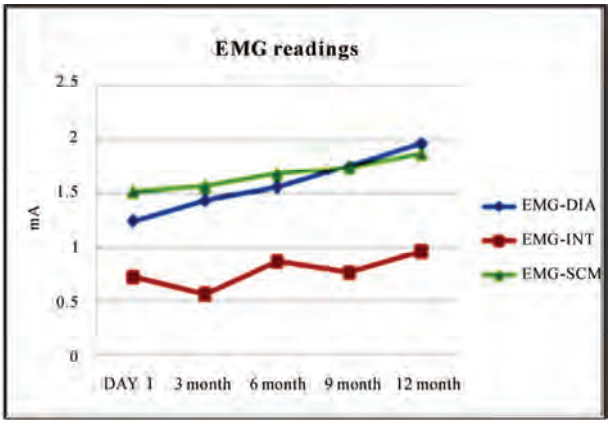


Figure 4. Peak amplitude of the analyzed muscles.

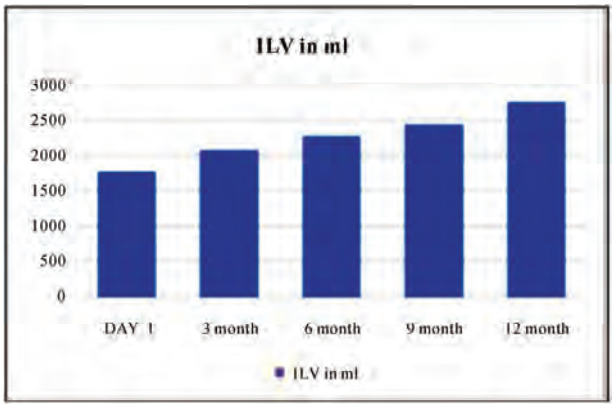


Figure 5. ILV over the study period.

can be considered as an objective measure of respiratory muscle efficiency. ILV was considered as the secondary outcome measure because of the ease of administration. Though the objectivity of the test has not been tested, the results obtained are directly related to strength of the respiratory muscles and pulmonary compliance.

Our study shows the following findings:

1) Measures of EMG of the DIA and SCM muscles as well as the ILV have showed a gradual rise which implies: Use of abdominal weights as a part of respiratory rehabilitation has beneficial long term effects; 2) In patients with tetraplegia, respiratory muscles in particular, the diaphragm is trainable in terms of muscle efficiency, provided that the muscle performance is not severely compromised as in cases of ultra high lesions who require ventilator assistance; 3) The improvement in the muscle efficiency obtained during the early rehabilitation can be maintained or improved using simple non sophisticated exercises like abdominal weights. Clinically we did not observe any deterioration in the patients respiratory as well as functional status which is a good sign for a tetraplegic.

We hypothesized a reduction in the EMG activity of the sternocleidomastoid muscle following training, due to an improvement in the efficiency of diaphragm, which is the primary respiratory muscle. We found an increase in the activity of sternocleidomastoid muscle on the subject along with the increase in activity of the diaphragm. However we could not explain the reason for this phenomenon.

There were few limitations in our study which includes spasticity and adherence of the subject to the training program. There are possibilities that disturbances from the abnormally activated trunk muscles could have reduced the accuracy of EMG activity of the diaphragm and other respiratory muscles. Outcome demonstrated by the ILV has shown a consistent rise which can be considered to overcome the limitation of misleading values from the EMG if any. Though we used a log book for tracking the adherence to the treatment protocol a direct supervision of the subject on a regular basis at community level can be considered for upcoming studies, for more accurate results.

7. CONCLUSIONS

The peak EMG amplitude and ILV showed a consistent rise on the subject who was trained using abdominal weights. This is an apparent indication of improved performance of the target muscle with regards to strength and pulmonary function. The improvement of the muscle efficiency was maintained throughout the study period which is a positive sign of long term effects of the technique. Cost effectiveness, ease of administration of this technique ensures its frequent usage by clinical practi-

tioners. With adequate instructions and proper training, this technique does not require direct supervision by a therapist and so is useful for community based rehabilitation. Thus we conclude stating Abdominal weights can be used as an effective adjunct to pulmonary rehabilitation in improving the efficiency of diaphragm on a long term basis, thereby reducing the risks associated with pulmonary complications.

REFERENCES

[1] Zimmer, M.B., Nantwi, K. and Goshgarian, H.G. (2007) Effect of spinal cord injury on the respiratory system: Basic research and current clinical treatment options. *Journal of Spinal Cord Medicine*, **30(4)**, 319-330.

[2] Forner, J.V. (1980) Lung volumes and mechanics of breathing in tetraplegics. *Paraplegia*, 1**8(4)**, 258-266.

[3] Rutchik, A., *et al.* (1998) Resistive inspiratory muscle training in subjects with chronic cervical spinal cord injury. *Archives of Physical Medicine and Rehabilitation*, **79(3)**, 293-297.

[4] Bake, B., Fugl-Meyer, A.R. and Grimby, G. (1972) Breathing patterns and regional ventilation distribution in tetraplegic patients and in normal subjects. *Clinical Science*, **42**, 117-128.

[5] De Troyer, A. and Estenne, M. (1991) Review article: The expiratory muscles in tetraplegia. *Paraplegia*, **29(6)**, 359-363.

[6] Bhaskar, K.R., *et al.* (1991) Bronchial mucus hypersecretion in acute quadriplegia. Macromolecular yields and glycoconjugate composition. *American Review of Disease*, **143(3)**, 640-648.

[7] Jackson, A.B. and Groomes, T.E. (1994) Incidence of respiratory complications following spinal cord injury. *Archives of Physical Medicine and Rehabilitation*, **75(3)**, 270-275.

[8] Claxton, A.R., Wong, D.T., Chung, F. and Fehlings, M.G. (1998) Predictors of hospital mortality and mechanical ventilation in patients with cervical spinal cord injury. *Canadian Journal of Anesthesia*, **45(2)**, 144-149.

[9] Fishburn, M.J., Marino, R.J. and Ditunno, J.F. Jr. (1990) Atelectasis and pneumonia in acute spinal cord injury. *Archives of Physical Medicine and Rehabilitation*, **71(3)**, 197-200.

[10] Frankel, H.L., Coll, J.R., Charlifue, S.W., *et al.* (1998) Long term survival in spinal cord injury: A fifty year investigation. *Spinal Cord*, **36(4)**, 266-274.

[11] Akabas, S.R., Bazzy, A.R., DiMauro, S. and Haddad, G.G. (1989) Metabolic and functional adaptation of the diaphragm to training with resistive loads. *Journal of Applied Physiology*, **66(2)**, 529-535.

[12] Belman, M.J. and Shadmehr, R. (1988) Targeted resistive ventilatory muscle training in chronic obstructive pulmonary disease. *Journal of Applied Physiology*, **65(6)**, 2726-2735.

[13] Boutellier, U., BuÈ chel, R., Kundert, A. and Spengler, C. (1992) The respiratory system as an exercise limiting factor in normal trained subjects. *European Journal of Applied Physiology*, **65(4)**, 347-353.

[14] Grassino, A. (1989) Inspiratory muscle training in COPD patients. *European Respiratory Journal*, Supplement **7**, 581s-586s.

[15] Leith, D.E. and Bradley, M. (1976) Ventilatory muscle strength and endurance training. *Journal of Applied Physiology*, **41(4)**, 508-516.

[16] Morgan, D.W., Kohrt, W.M., Bates, B.J. and Skinner, J.S. (1987) E€ects of respiratory muscle endurance training on ventilatory and endurance performance of moderately trained cyclists. *International Journal of Sports Medicine*, **8(2)**, 88-93.

[17] Noseda, A., *et al.* (1987) Resistive inspiratory muscle training and exercise performance in COPD patients. A comperative study with conventional breathing retraining. *Bulletin European Physiopathologie Respiratoire*, **23(5)**, 457-463.

[18] Gross, D., *et al.* (1980) The e€ect of training on strength and endurance of the diaphragm in quadriplegia. *American Journal of Medicine*, **68(1)**, 27-35.

[19] Biering-Sorensen, F., Lehmann Knudsen, J., Schmidt, A., Bund-gaard, A. and Christensen, I. (1991) Effect of respiratory training with a mouth-nose-mask in tetraplegics. *Paraplegia*, **29(2)**, 113-119.

[20] Derrickson, J., Ciesla, N., Simpson, N. and Imle, P.C. (1992) A comparison of two breathing exercise programs for patients with quadriplegia. *Physical Therapy*, **72(11)**, 763-769.

[21] Bodin, P., Fagevik Olsen, M. and Bake, B. (2005) Effects of abdominal binding on breathing pattern during breathing exercises in persons with tetraplegia. *Spinal Cord*, **43**, 117-122.

[22] Kisner, C. and Colby, L.A. In: Management of pulmonary conditions. Therapeutic Exercises: Foundation and techniques, 3rd Edition, FA. Davis Company Publishers, 665-672.

[23] Wetzel, J.L. and Lunsford, B.R. (1995) In: Scot Irwin, T., Ed., Management of pulmonary conditions. Cardio pulmonary physical therapy: A guide to practice, 3rd Edition, Mosby publisher, St. Louis, 584-586.

[24] Lin, H. and Chung, C.-C. (1999) Abdominal weight and Inspiratory resistance: Their immediate effects on inspiratory muscle functions during maximal voluntary breathing in chronic tertraplegic patients. *Archives of Physical Medicine and Rehabilitation*, **80(7)**, 741-745.

[25] Jennifer, B., Christer, S., Lars, L. and Alex, G. (1998) Effects of lung volume on diaphragm EMG signal strength during voluntary contractions, *Journal of Applied Physiology*, **85(3)**, 1123-1134.

Vol.2, No.5, 412-417 (2010)
doi:10.4236/health.2010.25062

DNA damage and cell death assessment in patients with severe multiple trauma using comet assay

Aliy K. Zhanataev[1]*, Victor V. Moroz[2], Andrey D. Durnev[1], Maria Yu. Muravyeva[2], Vasiliy I. Reshetnyak[2]

[1]Science Research Institute of Pharmacology, Russian Academy of Medical Sciences, Moscow, Russia; *Corresponding Author: azhanataev@yandex.ru
[2]Science Research Institute of General Reanimatology, Russian Academy of Medical Sciences, Moscow, Russia; v_reshetnyak@yahoo.com

Received 9 December 2009; revised 20 February 2010; accepted 23 February 2010.

ABSTRACT

Purpose: To determine the DNA strand breaks, oxidative DNA damage and cell death in blood and plasma total antioxidant status (TAOS) in 22 patients with severe multiple trauma. Materials and methods: The DNA comet assay was used to measure DNA strand breakage, 8-oxoguanine levels and apoptotic and necrotic nuclei in after admission (day 0) and on days 3, 5, 7 and 15. TAOS was determined by colorimetric method. Results: Trauma patients had high DNA damage at admission ($p < 0.01$), that further increased with maximum value on day 5 ($p < 0.001$). On day 15 the degree of DNA damage remained significantly elevated ($p < 0.01$). No significant difference in the 8-oxoguanine levels at all days examined was found. Patients had a high percentage of apoptotic and necrotic comets at admission, with maximum values on days 3 and 5. A significantly lower TAOS was observed in patients on admission and days 3, 5, 7 and 15 ($p < 0.001$ in all cases). A decreasing of TAOS on days 7 and 15 compared to that on admission ($p < 0.05$) was observed. Conclusions: Blood cells from severe trauma patients' display increased DNA damage associated with apoptosis and necrosis. Reduced plasma TAOS and a tendency to increase of 8-oxoguanine in DNA was observed.

Keywords: Apoptosis; Necrosis; Multiple Trauma; DNA-Comet Assay; 8-Oxoguanine; Total Antioxidant Status

1. INTRODUCTION

Severe multiple trauma remains as one of the major problems of contemporary medicine and society throughout the world. Advances in critical care of the trauma patient have resulted in improved outcome, but despite these efforts, up to half of the patients with traumatic injury die or are left with severe disability [1-3].

Multiple organ failure (MOF) is the major complication after sever multiple trauma [4-7]. Experimental evidences suggest that MOF in the event of trauma might be relates to stress-induced cell death by apoptosis, but its exact mechanisms is not fully understood [8-12]. DNA damage including oxidative DNA bases modification is one of the intrinsic signals initiating apoptosis [13]. At present, data about DNA damage in critical illness are lacking. The Single Cell Gel Electrophoresis assay (SCGE), or comet assay are sensitive method for the evaluation of DNA damage from individual cells based on the migration of denatured DNA through an electrophoretic field [14]. The aim of this pilot study was an assessment DNA strand breakage, 8-oxoguanine levels and cellular death of white blood cells and their possible connection with total antioxidant status in patients with severe multiple trauma.

2. MATERIALS AND METHODS

The study covered 22 patients who were admitted with severe multiple traumas (SMT) (**Table 1**). The study protocol was approved by ethics committee and informed consent was obtained from all patients or their relatives. Inclusion criteria were age older than 18 years, admission to the trauma intensive care unit and an Acute Physiology and Chronic Health Evaluation (APACHE II) score higher than 12. Patients with traumatic brain injury were excluded. The age ranged from 21 to 68 years. Body weight ranged from 50 to 95 kg. Clinical characteristics of the patients with severe multiple trauma on admission is presented to **Table 2**. The length of stay in the unit ranged from 3 to 32 days, with a mean of 10.5 ±

5.1 days. The patient's condition on admission was an APACHE II of 19.1 ± 5.4, blood loss 22-45 ml/kg. Blood loss was calculated using indirect method (vital signs, serial hematocrits measurement) and by direct intraoperative estimation of blood loss. Optimization of hemodynamic (systolic blood pressure > 90 mm Hg) and oxygenation (arterial oxygen saturation > 90%) was reached during the first hours after the admission in the intensive care unit. Single organ failure (SOF) occurred in 12 patients (54.5%) and multiple organ failure occurred in 6 patients (27.3%). All SOF was caused by respiratory failure. Respiratory failure occurred first in the majority of patients with multiple organ failure, followed usually by cardiovascular insufficiency. The mortality level was 23% (5 patients). Blood was collected after admission (day 0) within 4 hours ($n_0 = 22$), and on days 3 ($n_3 = 22$), 5 ($n_5 = 13$), 7 ($n_7 = 11$) and 15 ($n_{15} = 5$) of admission. Blood samples (2 ml) were taken into EDTA tubes, mixed (1:1) with RPMI-1640 medium containing 20% DMSO as cryoprotectant and immediately frozen to –20℃. Samples stored until analysis no more 20 days avoiding repeated freeze-thaw cycles. Twelve subjects without acute or chronic disease were used as control group (**Table 1**). Blood samples from controls handled and stored using identical procedures.

2.1. Single Cell Gel Electrophoresis Assay

DNA damage was evaluated using alkaline comet assay [15]. Briefly, after quick (within 1-2 min) thawing in a water-bath at 37℃, 50 µl of whole blood samples were mixed with 500 µl of 1% melted agarose (low melting point) and layered (70 µl) onto a microscopy slide. The slides with the agarose-embedded cells were subjected to a lysis step (1 h at 40C in 1% N-lauroylsarcosine, 2.5M NaCl, 100 mM Na_2EDTA, 1% Triton X-100, 10% DMSO, pH 10.0). After the lysis step slides were placed in an ice-cold electrophoresis chamber containing alkaline electrophoresis solution (300 mM NaOH, 1mM Na_2EDTA, pH > 13.0) for 20 min to allow DNA unwinding. The electrophoresis was subsequently conducted for 20 min at 1 V/cm and ~300 mA. At the end of the electrophoresis the slides were fixed in 70% ethanol, air-dried and stored in the dark at room temperature until scored. Just prior to scoring, slides were stained with SYBR Green I (1:10000 in TE-buffer, pH 7.4) for 20 min. Microscopical analysis was carried out at × 200 magnification using MIKMED-2 microscope (LOMO, Russia) provided with epifluorescence and equipped with a FITC filters. Images of 100 randomly selected comets were captured using CCD camera (VEC-335, EVS, Russia) and analyzed with CASP 1.1.2 image analyzer software [16] to evaluate %DNA in tail (the percentage of total fluorescence migrated in the tail for each nucleus) used as a measure of DNA damage. In parallel analysis levels of apoptotic and necrotic comets was estimated. Comet assay in the standard alkaline version has a fragment size resolution of 10-100 kb and early to middle chromatin fragmentation during apoptosis can be monitored by the technique [17]. Thus, DNA-comets of apoptotic cells on slides clearly differ from damaged or undamaged cells by forming comet- like structures with spread tail and small almost invisible heads (**Figure 1(b)**) [14,17]. Necrotic cells displayed a characteristic view with large nuclear remnants which reflected random DNA degradation (**Figure 1(c)**).

Table 1. Demographic data of the investigated groups.

Parameter	Trauma patients (n = 22)	Healthy subjects (n = 12)
M/F, n (%)	14 (63,6%)/8 (36,4%)	5 (41,7%)/7 (58,3%)
Age, years	41.9 ± 13.3	37.9 ± 9.6
Weight, kg	75.7 ± 6.4	67.8 ± 7.5
Motor vehicle accident	18 (81,8%)	-
Fall	2 (9,1%)	-
Other mechanism of injury	2 (9,1%)	-
Blunt trauma	16 (72,7%)	-
Penetrating trauma	6 (27,3%)	-

2.2. Detection of 8-Oxoguanine and TAOS

Human 8-oxoguanine DNA Glycosylase (hOGG1) FLARE Assay Kit (R&D Systems, Minneapolis, USA) was used to evaluate oxidative DNA damage in blood cells. This test uses the hOGG1 enzyme, a glycosylase that recognizes and specifically cuts the oxidized bases principally 8-oxoguanine (8-oxoG) from DNA, producing apurinic sites converted in breaks, which can be detected by comet assay. The comet assay was carried out as described above, with the exception that after lysis of cells the slides were washed three times for 15 min with FLARE™ buffer. After this time samples were incubated with 100 µl of hOGG1 enzyme (1:400 in REC dilution buffer) or with 100 µl of REC dilution buffer only. Slides incubated in humidity chamber at 37℃ for 1 h. DNA unwinding, electrophoresis, microscopic analysis and comet scoring were then completed as described above. For each blood sample the mean values of the %DNA in tail for 100 comets from hOGG1-treated cells (%DNAenz) and hOGG1-untreated cells (%DNAbuf) was calculated. %DNAenz/%DNAbuf ratio (in a.u.) was used as measure of 8-oxoG content in DNA of blood cells.

Among the methodologies used to evaluate TAOS, the most widely used colorimetric method are 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation (ABTS +) based methods. TAOS of plasma samples was assayed by using a commercially available kit (Randox Laboratories Ltd., Crumlin, UK) on an automatic Cobas

Mira Plus Chemistry analyzer (Roche Diagnostics Ltd., USA). The results were expressed as mmol/l Trolox equivalent.

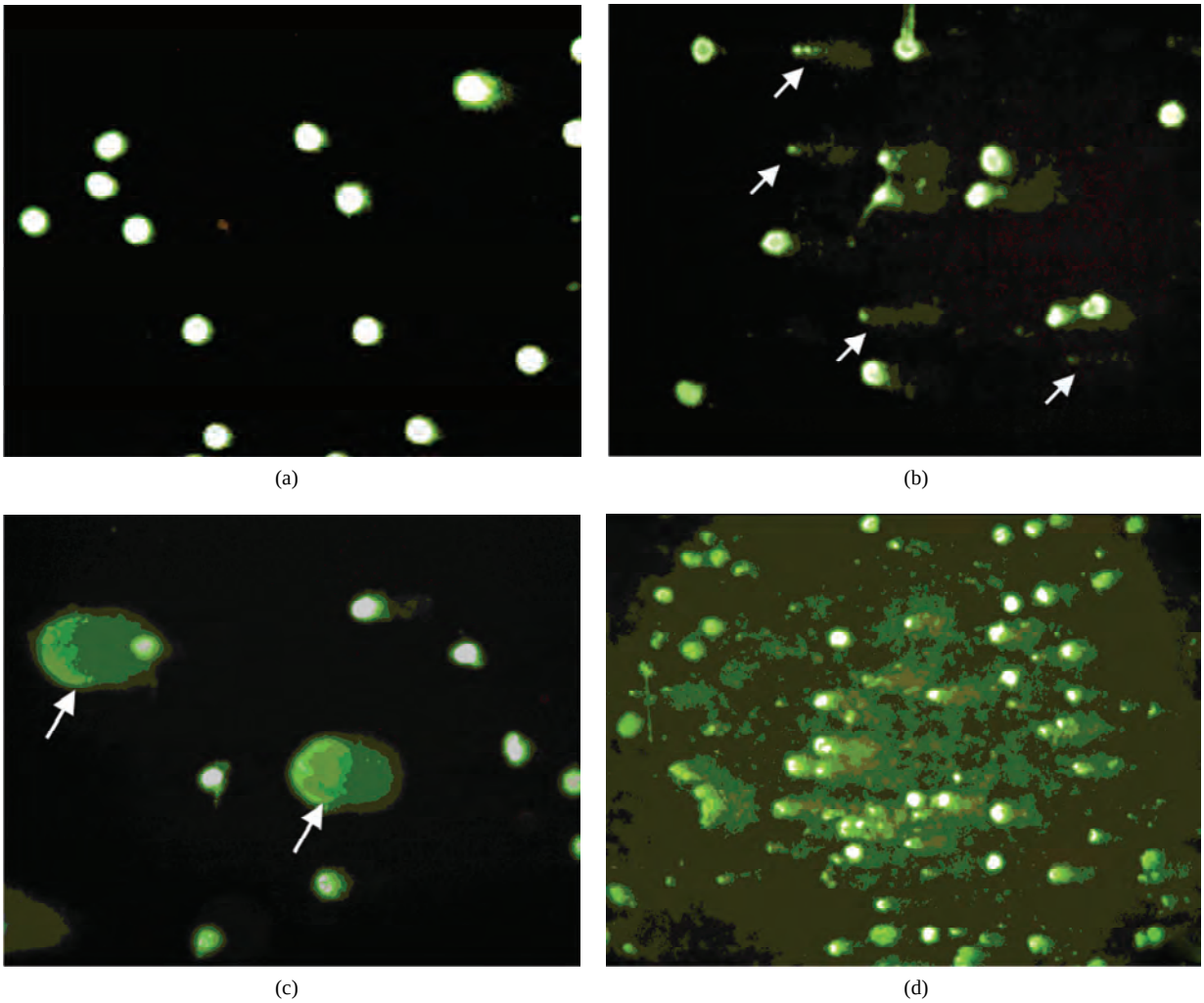
2.3. Statistical Analysis

Data are presented as mean ± SD. Statistical comparisons between controls and patients were performed by Student's t-test for independent samples. A Wilcoxon matched pairs signed rank test was used to compare paired samples. Correlations were evaluated using Spearman's rank correlation test. A value of $p < 0.05$ was considered as statistically significant.

3. RESULTS

Typical DNA-comet images of blood cells taken from healthy individuals and patients with SMT are presented in **Figure 1**. Comparison of DNA damage in blood cells from patients with SMT and healthy controls is presented in **Figure 2(a)**. Tail DNA percentage in blood cells from healthy controls ranged from 2.4 to 10.4 with mean value 6.1 ± 2.2%. A significantly higher level of DNA damage was seen in patients on the day of admission (10.6 ± 5.9%; $p < 0.01$). The further increase in DNA damage levels was observed up to day 7 with maximum value on day 5 (14.2 ± 4.2% DNA in tail; $p < 0.001$). On day 15 the degree of DNA damage returned to the admission value, but remained significantly elevated in comparison with healthy controls (6.1 ± 2.2% vs. 10.3 ± 2.4; $p < 0.01$).

There was no significant difference in the levels of 8-oxoG between controls and trauma patients at all days examined (**Figure 2(b)**). A marked increase in 8-oxoG levels was observed on day 7 (up to 2.1 ± 0.6 a.u.). Pair wise comparison indicated that this increase was not significant.

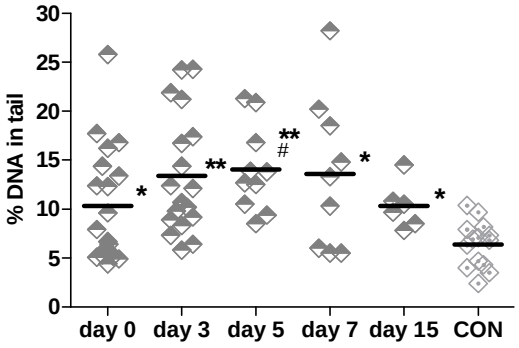


(a) (b) (c) (d)

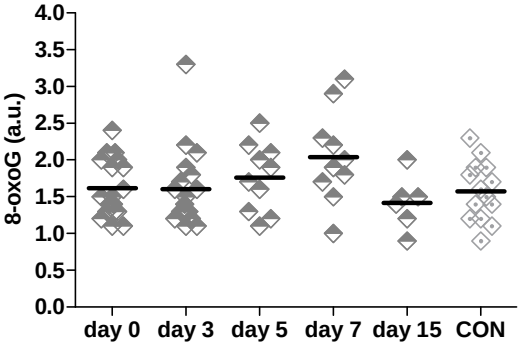
Figure 1. Typical DNA-comet images of blood cells taken from a control subject (a) and patients with severe multiple trauma (b,c,d). Arrows indicates DNA-comets of apoptotic (b) and necrotic (c) nuclei. Image from blood sample with high-fragmented DNA are showed (d). Magnification × 200.

Healthy individuals showed low level of spontaneously apoptosis of blood cells (0.9 ± 0.3% of apoptotic comets; **Figure 2(c)**). In contrast, percentage of apoptotic comets was found elevated in patients at day of admission (2.7 ± 2.6%, range from 0.4 to 10.3; $p < 0.01$). By day 3 mean apoptosis rate increased up to 6.9 ± 7.7% ($p < 0.001$) with widely varied values from 0.7 to 32%. From day 5 apoptotic comets score gradually decreased to value 2.3 ± 1.1% on day 15. No necrotic DNA-comets were found in slides from controls' blood samples. The mean percentage of necrotic comets in patients was 4.9 ± 5.9% at day 0 (**Figure 2(d)**). Further increasing to maximum value of 9.3 ± 7.1% on day 5 was observed, but from day 7 to day 15 necrotic DNA-comets reduced to 5.0 ± 3.6%. No any statistical differences at pairwise comparison were found due to high interindividual diversity and insufficient number of patients.

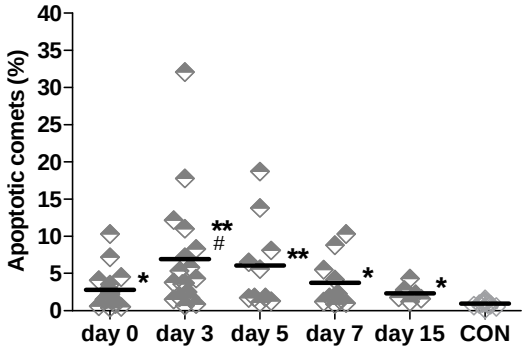
A statistically significantly lower TAOS values (**Figure 2(e)**) was observed in patients on admission (0.84 ± 0.22 mmol/l) and days 3, 5, 7 and 15 (0.74 ± 0.23; 0.76 ± 0.26; 0.68 ± 0.25 and 0.59 ± 0.16 mmol/l, respectively) as compared to healthy subjects (1.34 ± 0.25 mmol/l; $p < 0.001$ in all cases). A statistically significant decreasing of TAOS on days 7 and 15 was also observed compared to that on admission ($p < 0.05$).



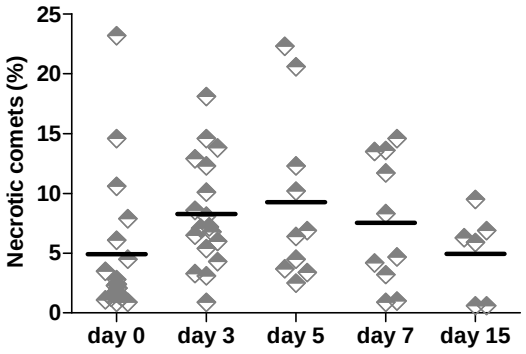
(a)



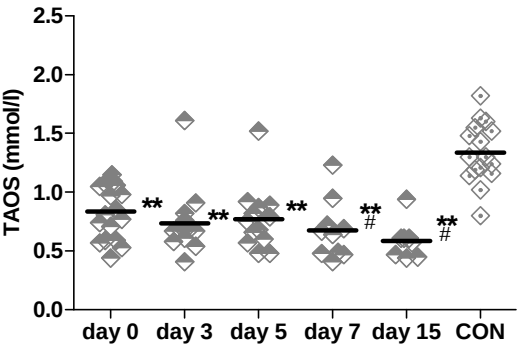
(b)



(c)



(d)



(e)

** - p < 0.01 as compared with controls; **- p < 0.001 as compared with controls; # - p < 0.05 as compared with value at day 0.*

Figure 2. DNA damage (a), 8-oxoG levels (b), apoptotic (c) and necrotic (d) comets percentage in blood cells and plasma total antioxidant status (e) in healthy controls (CON) and patients with severe multiple trauma at different days of analysis.

Correlation analysis demonstrated that there is a significant direct relationship between %DNA in tail and percentage of necrotic comets on day 0 ($r = 0.67$; $p < 0.001$) and day 3 ($r = 0.54$; $p < 0.01$) and between %DNA in tail and percentage of apoptotic comets on day 7 ($r = 0.69$; $p < 0.001$). A significant negative correlation

between %DNA in tail and 8-oxoG levels on day 0 (r = –0.71; $p < 0.001$), day 3 (r = –0.62; $p < 0.001$) and day 5 (r = –0.74; $p < 0.001$) was found. No any correlations between TAOS and DNA damage, 8-oxoG, apoptotic or necrotic comets were found

4. DISCUSSION

The DNA comet assay is sensitive method for the evaluation of DNA damage from individual cells based on the migration of denatured DNA through an electrophoretic field [14]. Under alkaline conditions, comet assay detects single and double strand breaks, alkali-labile and abasic sites, and DNA cross-links. By adding modifying enzymes can be investigated specific types of DNA damage, such as oxidative damage of DNA bases [14]. Also, comet assay enable the discrimination of apoptotic and necrotic nuclei on the basis of their characteristic signature of DNA fragmentation patterns [14, 17]. Our data represent the first examination of the DNA damage in blood cells of patients with severe multiple trauma. A significantly higher level of DNA strand breaks, apoptotic and necrotic nuclei in blood cells of patients versus controls, beginning from day of admission were found. The analysis of the data allows assuming that the main processes connected with DNA damage and cell death at trauma occur from day 3 to day 5. In our trial trauma has served as a model for investigation because the interval between triggering event and development of organ failure can be assessed precisely and primary organ injury can be easily separated from remote organ dysfunctions.

Moderate hypoxia and hypoperfusion (**Table 2**), organ and soft tissue injuries, fractures, as well as ischemia/reperfusion probably induced disbalance in pro- and antioxidative system and as result oxidative stress in patients. The low level of TAOS in patients beginning from day of admission with a progressive 42% reduction during the next two weeks was observed. Several studies indicate that oxidative stress occurs in critical illness [9,19,20]. The presence of 8-oxoG in DNA is considered a marker of oxidative DNA damage. Using HPLC with electrochemical detection elevated oxidative DNA damage in trauma patients as measured by estimating 8-oxodG:dG ratios in DNA of blood cells was shown [20]. However, in this study using comet FLARE assay no differences in 8-oxoG levels were observed, despite a markedly reduced TAOS. On the one hand it might be connected with intensive DNA repair. Alkaline comet assay detects strand breaks, alkali-labile sites, as well as abasic sites-excision repair sites missing either a pyrimidine or purine nucleotide, and the observed high level of DNA breaks might reflect the high repair activity. The presence of an inverse relationship between DNA breaks and 8-oxoG levels observed on days 0, 3 and 5 may support this hypothesis. On the other hand elimination of cells with oxidatively damaged DNA through apoptosis/necrosis can lead to an underestimation of 8-oxoG. So, necrotic and apoptotic comets have been excluded from 8-oxoG analysis due to methodological features. Data will allow assuming that evaluation of repair product of 8-oxoG-8-hydroxy-2-deoxyguanosine (8-oxodG) in plasma and/or 8-oxoG content in circulating plasma cell-free DNA in future may be a more useful approach for estimating oxidative DNA damage in critical ill patients. Also we found that there were no significant correlations between DNA damage and TAOS. These results indicate that DNA damage at trauma may be caused not only by oxidative stress but also by other pathways.

In eight patients for days 3 and/or 5 the massive DNA fragmentation revealed on slides as a diffuse distributed high-fragmented DNA was observed (**Figure 1(d)**), complicating the analysis and a scoring of DNA comets. It is not clear, whether it is a DNA of necrotic or apoptotic cells. *Pachl et al.* showed that in critically ill patients both apoptotic and necrotic DNA contributed to total plasma DNA and its increase predicted future development of multiple organ failure and death [21]. The question remains open with regard to whether is this high-fragmented DNA a consequence apoptosis/necrosis of blood cells or this DNA eliminated in blood from damaged tissues and/or organs due to intensive cell death. Apoptosis in thymus, liver, lung, intestine and spleen after major trauma combined with shock has been demonstrated in animal experiments [22]. Non-lethal mechanical trauma causes significant TNF-alpha production that in turn stimulates myocardial apoptosis via oxidative/nitrative stress [23]. In patients with major trauma, early apoptosis was detected in lymphoid tissues [12]. It is necessary to notice that the exception of the analysis of the samples with high-fragmented DNA can explain absence of statistically significant differences at

Table 2. Clinical characteristics of the patients with severe multiple trauma on admission.

Parameter	Value
PaO_2/FiO_2	267.54 ± 44.32
Glasgo coma score	13.5 ± 1.3
Mean arterial pressure, mm Hg	64.1 ± 23.2
Crystalloid infusion in 4 hours, ml	4826 ± 259
Lactate, mmol/l	3.7 ± 1.45
Creatinin, mg/dl	0.82 ± 0.48
Bilirubin, mg/dl	0.96 ± 0.32
Initial hemoglobin level, g/l	112.3 ± 15.4
Platelets, × 10^3/ml	210.7 ± 38.2

the analysis, despite obvious tendencies.

Continuing studies with a larger number of patients are required. Our results underscore the need to use the comet assay in two variants, the alkali and the neutral assays that allows a more complete study of DNA damage. Also, parallel analysis of plasma/serum DNA and DNA repair rate of oxidative DNA damage may help evaluate interrelations of different factors and its role in cellular death mechanisms and development MOF.

In conclusion, blood cells from severe trauma patients' display increased DNA damage associated with apoptosis and necrosis. Reduced plasma TAOS and a tendency to increase of 8-oxoguanine in DNA was observed.

REFERENCES

[1] Holtslag, H.R., van Beeck, E.F., Lindeman, E., *et al.* (2007) Determinants of long-term functional consequences after major trauma. *Journal of Trauma*, **62(4)**, 919-927.

[2] Soberg, H.L., Bautz-Holter, E., Roise, O., *et al.* (2007) Long-term multidimensional functional consequences of severe multiple injuries two years after trauma: A prospective longitudinal cohort study. *Journal of Trauma*, **62(2)**, 461-470.

[3] Stalp, M., Koch, C., Ruchholtz, S., *et al.* (2002) Standardized outcome evaluation after blunt multiple injuries by scoring systems: A clinical follow-up investigation 2 years after injury. *Journal of Trauma*, **52(6)**, 1160-1168.

[4] Nast-Kolb, D., Aufmkolk, M., Rucholtz, S., *et al.* (2001) Multiple organ failure still a major cause of morbidity but not mortality in blunt multiple trauma. *Journal of Trauma*, **51(5)**, 835-842.

[5] Durham, R.M., Moran, J.J., Mazuski, J.E., *et al.* (2003) Multiple organ failure in trauma patients. *Journal of Trauma*, **55(4)**, 608-616.

[6] Antonelli, M. and Caricato, A. (2007) Post-injury multiple organ failure and late outcome. Is it just an association? *Critical Care*, **11(5)**, 166.

[7] Walsh, C.R. (2005) Multiple organ dysfunction syndrome after multiple trauma. *Orthopedic Nursing*, **25(5)**, 324-333.

[8] Keel, M. and Trentz, O. (2005) Pathophysiology of polytrauma. *Injury*, **36(6)**, 691-709.

[9] Goodyear-Brunch, C. and Pierce, J.D. (2002) Oxidative stress in critically ill patients. *American Journal of Critical Care*, **11**, 543-553.

[10] Cobb, P.J., Buchman, T.G., Karl, I.E., *et al.* (2000) Molecular biology of multiple organ dysfunction syndrome: Injury, adaptation and apoptosis. *Surgical Infection* (*Larchmt*), **1(3)**, 207-215.

[11] Papathanassoglou, E.D., Moynihan, J.A. and Ackerman, M.H. (2000) Does programmed cell death (apoptosis) play a role in the development of multiple organ dysfunction in critically ill patients? A review and a theoretical framework. *Critical Care Medicine*, **28(2)**, 537-549.

[12] Hotchkiss, R.S., Schmieg, R.E., Swanson, P.E., *et al.* (2000) Rapid onset of intestinal epithelial and lymphocyte apoptotic cell death in patients with trauma and shock. *Critical Care Medicine*, **28(9)**, 3207-3217.

[13] Norbury, C.J. and Zhivotovsky, B. (2004) DNA damage-induced apoptosis. *Oncogene*, **23**, 2797-2808.

[14] Collins, A.R. (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Molecular Biotechnology*, **26(3)**, 249-261.

[15] Tice, R.R., Agurell, E., Anderson, D., *et al.* (2000) Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis*, **35(3)**, 206-221.

[16] Konca, K., Lankoff, A. and Banasik, A. (2003) A cross-platform public domain PC image-analysis program for the comet assay. *Mutation Research*, **534(1-2)**, 15-20.

[17] Morley, N., Rapp, A., Dittmar, H., *et al.* (2006) UVA-induced apoptosis studied by the new apo/necro-Comet-assay which distinguishes viable, apoptotic and necrotic cells. *Mutagenesis*, **21(2)**, 105-114.

[18] Barzilai, A. and Yamamoto, K. (2004) DNA damage responses to oxidative stress. *DNA Repair* (*Amst*), **3(8-9)**, 1109-1115.

[19] Valko, M., Leibfritz, D., Moncol, J., *et al.* (2007) Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry & Cell Biology*, **39(1)**, 44-84.

[20] Oldham, K.M., Wise, S.R., Chen, L., *et al.* (2002) A longitudinal evaluation of oxidative stress in trauma patients. *Journal of Parenteral and Enteral Nutrition*, **26(3)**, 189-197.

[21] Pachl, J., Duska, F., Waldauf, P., *et al.* (2005) Apoptosis as an early event in the development of multiple organ failure? *Physiological Research*, **54**, 697-699.

[22] Guan, J., Jin, D.D., Jin, L.J., *et al.* (2002) Apoptosis in organs of rats in early stage after polytrauma combined with shock. *Journal of Trauma*, **52(1)**, 104-111.

[23] Li, S., Tao, L., Jiao, X., *et al.* (2007) TNFalpha-initiated oxidative/nitrative stress mediates cardiomyocyte apoptosis in traumatic animals. *Apoptosis*, **12(10)**, 1795-1802.

Vol.2, No.5, 418-428 (2010)
doi:10.4236/health.2010.25063

Mapping out the social experience of cancer patients with facial disfigurement

Alessandro Bonanno*, Jin Young Choi

Department of Sociology Sam Houston State University, Huntsville, USA; *Corresponding Author: SOC_AAB@SHSU.EDU

Received 25 November 2009; revised 14 January 2010; accepted 16 January 2010.

ABSTRACT

This article contributes to the limited literature on the social consequences of cancer generated facial disfigurement by reporting the result of an exploratory analysis of interaction between facially disfigured cancer patients and strangers and acquaintances (secondary groups). Secondary groups are those in which membership occurs due to performance of formal and/or non-intimate roles. Interaction is studied as it takes place in different social settings. Individuals who are affected by cancer of the head and neck region can now expect to survive for many years after the cancer is detected and later surgically removed. Because of surgery, these survivors live the rest of their lives with facial disfigurement and are stigmatized and socially excluded. It follows that a new and socially relevant situation has emerged: as medicine develops and allows more patients to survive, it forces them to spend significant portions of their lives dealing with the stigma associated with facial disfigurement. Research on social issues pertaining to facially disfigured cancer patients remains sparse. Limited knowledge has been produced on the "social context" within which interaction between the disfigured and relevant social groups takes place. To date most research has focused on the individual and his/her ability to adapt to the condition of facially disfigured. To address this scientific gap and document the manner through which the interaction process is socially created and evolves, interviews with fourteen facially disfigured cancer patients were carried out. These interviews were designed to reconstruct the interaction experiences of these individuals in different social contexts. Data were analyzed through the qualitative approach of grounded theory. Results indicate that patients can be divided into two groups: *Occasionally Comfortable Patients* and *Always Comfortable Patients*. Occasionally comfortable patients are individuals who experience different levels of comfort in interaction. In some situations they do not feel stigmatized, but other interactions constitute the contexts within which this discomfort emerges. Discomfort in interaction was employed as an indicator of stigmatization. Interacting groups were divided into small and large. Intrusion (unsolicited attention to patients) in interaction in large and small groups always generates uncomfortable situations. Sympathy (unsolicited comments and/or actions in support of patients) is associated with comfort in interaction in small groups and produces varying patterns in the case of large groups. Benign neglect (a situation in which interacting individuals do not pay particular attention to patients) produces comfort in interaction within large groups and varying outcomes in the case of small groups. Always comfortable patients are those who do not experience discomfort in interaction regardless of the size and characteristics of the interacting group. The article concludes by stressing that facially disfigured cancer patients should be prepared to face different interaction patterns. Simultaneously, efforts should be made to educate patients and the general public about these interaction patterns.

Keywords: Facial Disfigurement; Cancer; Stigma; Social Interaction

1. INTRODUCTION

The objective of this article is to illustrate the results of an exploratory analysis on the patterns of social interaction experienced by individuals who are facially disfigured because of cancer. Individuals who are affected by cancer can expect to be cured and/or survive for a number of years after the cancer is detected and later surgically removed [1,2]. This is also the case of patients with head and neck cancer who remained facially disfigured

because of it [3,4]. In this particular instance, surgical intervention signifies the removal of portions of the face that are affected by the malignancy. Additional consequences are possible and they often include the collapse of parts of the face that may not be directly touched by surgical intervention [5]. Accordingly, patients' facial disfigurement may involve a larger portion of the face than that originally affected by cancer [6]. Surgical procedures to correct these alterations are common along with the availability of increasingly sophisticated prostheses [3]. Yet, results often do not rectify notable differences from the "normal" face. Accordingly survivors typically live the rest of their lives with major alterations of their normal facial appearance.

In contemporary society, the face represents one of the most notable items employed to determine identity and participate in social interaction [7-9]. It is a central element of communication [10,11], an item employed to attribute both "normality" and ownership of socially desirable characteristics [12-16] and a feature that defines interaction as individuals endowed with a pleasant face are better treated than other and less attractive members of society [17,18]. Owing to the social importance of the face, facially disfigured individuals are stigmatized and experience difficulties when interacting with other segments of society [11,12,14-16,19-21]. It follows that a new and socially relevant situation has emerged. As medicine develops and allows more patients to survive, it also makes them spend significant portions of their lives dealing with the stigma associated with facial disfigurement.

Research on social issues pertaining to facially disfigured cancer patients remains sparse and attracts even less attention than the already limited research associated with other forms of acquired and congenital disfigurement [10,15,22-24]. Additionally, analytical problems have limited the accuracy of available research results [25]. In this context, limited knowledge has been produced on the "social context" within which interaction between the disfigured and relevant social groups takes place [21,25]. Throught this paper we would like to contribute to this limited literature. In particular, this article offers the results on an exploratory analysis on patterns of interaction between disfigured patients and members of secondary groups (acquaintances and strangers) as interaction occurs in different social settings. Interaction with other groups, such as family members and friends (primary groups), is important. However, in this work we focus exclusively on interaction between patients and members of secondary groups. The study of the interaction with other groups remains a relevant topic to be further explored.

Methodologically, the study consists of in-depth interviews with fourteen cancer patients who underwent surgery to treat head and neck cancer and remained facially disfigured because of it. Patients were enrolled following a protocol that excluded minors and those who could not express themselves in English. Given the relative limited size of this patient population, the adoption of a qualitative approach was recommended. A semi-structured questionnaire was employed and data were analyzed employing *Grounded Theory* [26]. Grounded theory is a technique of qualitative data analysis that allows the identification of patterns directly from the data. Rather than hypothesis verification (pre-existing ideas to be verified in reality), grounded theory allows the development of categories (variables) and their relationships (patterns) that are not established a priori. These categories and patterns emerge directly from the data and can be always traced back to the data themselves. Because there are no pre-conceived hypotheses to be tested, this technique is particularly appropriate for exploratory analyses [26]. This is a qualitative study as it is aimed at illustrating the manner though which the patterns of interaction unfold. It explains the "how" these patterns manifest themselves and develop rather than how often they occur in reality. It also stresses the process through which the interaction is created and grows rather than its outcomes. Given the small size of the sample, this approach has been considered more appropriate than quantitative statistical analysis.

The statements concerning the result have been verified through established data verification techniques. In particular the techniques of "saturation" and "negative cases" were employed. In the case of saturation, the creation of categories is verified by searching for possible instances in which data cannot be explained by the category in question. When no more instances to be explained by the category in question are found in the data, the category is said to be saturated and therefore verified. In the instance of negative cases, categories are compared with situations in which their attributes are contradicted by evidence. As categories that have negative cases are eliminated, the remaining ones are considered verified. These techniques were applied to the construction of the categories and patterns employed in this article.

The objective of the article is to identify patterns of interaction between facially disfigured individuals and members of secondary groups. Secondary groups were defined as non-intimate, formal groups consisting of acquaintance and strangers. Results indicate that facial disfigured patients feel stigmatized when interacting within small and large groups and the interacting counterpart(s) shows "intrusion" (*i.e.*, unrequested attention to patients including unwanted questions, remarks and stares). Patients respond in varying manner when "sympathy" (*i.e.*, unsolicited comments and/or actions showing support) and "benign neglect" (*i.e.*, no particular attention paid to patients) characterized the interaction. A group of patients that do not feel stigmatized during various types of interaction was indentified. The paper

concludes by stressing the importance of studying stigmatization through a sociological approach and by pointing out that cancer survivors and the general public alike could benefit from education about these interaction outcomes.

2. CANCER AND FACIAL DISFIGUREMENT: SALIENT LITERATURE

Stigma is a mark of disgrace attached to people who are considered different. As indicated by Goffman [13], difference is socially constructed and is the outcome of discrepancies between an individual virtual social identity (expectations about what that individual ought to be) and his/her actual social identity (the attributes he/she actually posses) [13]. When the actual social identity is perceived as departing from normality, the individual is "reduced in our minds from a whole and usual person to a tainted, discounted one. "Such an attribute is a stigma" [13]. Stigma is attached to an individual's feature "that is deeply discrediting" and that separates that person from the group of the normals. However, its actual genesis should not be linked to attributes but, rather, to the interaction between the stigmatized person and other members of society. "A language of relationships, not attributes, is really needed" Goffman states [13]. Stigma is generated by the existence of a number of blemishes. There are those of individual character such as homosexuality, dishonesty, imprisonment, radical political behavior, and addiction. There are those of tribal stigma that are related to a person's religion, ethnicity or race. And there are those of "abominations of the body" that refer to physical abnormalities. Goffman includes facial disfigurement in this category [13].

Following the seminal work of Erving Goffman [13], social stigma has been widely studied and this production includes works such as those on stigma generated from diseases (*i.e.*, cancer and AIDS) [27], physical disabilities [28,29], and mental health[1] [30,31].

In spite of this wealth of contributions, the stigma caused by facial disfigurement has been the subject of only a relatively small number of works [10,15,21,22, 24,32]. These analyses stressed the social importance of the face and the problems that affect those who display visible facial blemishes [12-16]. In a society in which individuals are fully clothed for virtually all of their social activities, the face represents one of the most notable physical attributes and a significant source of social information prior to, and during, social interaction [8,9, 30,33]. Accordingly, people possessing an attractive face[2] are not only considered physically pleasing[3], but they are often viewed as endowed with intellectual and emotional characteristics that are unrelated to their physical appearance [10,11,36]. Intelligence, kindness, likableness, and high morality are frequently associated with an attractive appearance. Unsurprisingly, these individuals are also better treated by others than less attractive members of society [17,18]. Lacking some of these physical attributes, facially disfigured individuals commonly engender negative responses by other members of society. Stigmatized and socially excluded, their ability to interact is often distorted and interaction is the source of problems including verbal and physical abuse, ridicule, hostile behavior, and isolation [10,12,37].

The literature identifies two types of facial disfigurement: congenital and acquired. Acquired disfigurement received less scientific attention than the already limited literature on congenital disfigurement [24]. Acquired disfigurement is further divided into trauma- and cancer-generated disfigurement. Research demonstrates that individuals with acquired facial disfigurement suffer psycho-social consequences that are different and, at least to some degree, more pronounced than those experienced by individuals with congenital disfigurement [18,24]. Among individuals with acquire facial disfigurement, cancer patients[4] experience less social and psychological problems than individuals who have been disfigured because of trauma [38]. However, cancer patients' "fear of dying is immense" [39]. And this situation affects these individuals and their family members' perception of disfigurement [40]. In this context, patients are more preoccupied with the evolution of their cancer than with the social consequences of the scars that the disease left on their faces [24,40]. As this fear of dying diminishes, however, the process of dealing with the deformity affects both patients and family members

[1]This abundant literature has also underscored important limits of the use of the concept of stigma. For instance, stigma has been studied with a strong individualistic focus, it is often employed by people who do not belong to stigmatized groups, there is no consensus on a common definition, and the existence of these multiple definitions allow the charge that this concept is too inclusive to be actually informative ([28] Cahill and Eggleston, 1995:682; [34] Link and Phelan, 2001: 365-366). There are also uncertainties about its manifestations as "felt" stigma—the individual's "shame" associated with the blemish—is much more common than the rare "enacted" stigma—or the existence of overt episodes of discrimination ([35] Jacobi, 1994:269).

[2]Early research on physical appearance paid more attention to the face than the rest of the body. It was stressed that the face was the most visible part of the body, its stability of appearance was grater than body appearance both in terms of the individual's lifespan and developmental stages, and physical attractiveness was predominantly determined by the face. Recent research, however, while still emphasizing the face [4], has underscored the growing importance of the body in the determination of beauty and attractiveness [9,20].

[3]"…Beauty is perceived as residing principally in the face." [33].

[4]While the social perception of cancer has changed in recent decades, this disease engenders a wide variety of attitudes and responses that differentiate it from other pathological situations [46]. Often, these attitudes and responses are stigmatizing [47,48]. However, differences have been recorded between reactions to forms of cancer that are perceived as "uncontrollable"—such as breast cancer – and those that are perceived as "controllable"—such as lung cancer due to smoking. Because the latter are seen as deriving from the patient's voluntary actions, more stigmatizing reactions are expected [49].

[39,40]. The association of cancer and disfigurement is persistent. In effect, therapy almost inevitably mandates surgical removal of cancer-affected parts of the face making it an undesirable consequence of successful medical intervention [20,41,42].

Among the limits of this otherwise important literature is the lack of attention paid to the social context within which the consequences of facially disfigurement emerge [12,21,25,32]. Emphasis is placed on the individual, his/her characteristics, and efforts to adapt to his/her condition of disfigured [4,39]. This posture downplays "the everyday experience of the disfigured population in social settings" [17,21,25,32,37,43]. In this context, the "interaction process" through which disfigurement is experienced is viewed almost exclusively in terms of the significance of individual behavior as a predictor of social outcomes [19,32,43]. This is also the case when other social actors—such as partners—are studied [37,44]. In particular, limited attention is paid to the fact that disfigurement is socially constructed and generated through processes of interaction that involve multiple actors and take different forms according to the settings in which they unfold [21].

The individual remains the research focus also in studies on the relationship between cancer-generated facial disfigurement and stigmatization [45]. Indeed, research on stigma has been "decidedly individualistic in focus" [34]. Accordingly, the ways in which interaction between the facially disfigured and the "normals" unfolds in different realms of society has been understudied. As Thompson and Kent put it "Most studies have examined the 'view form the inside," with little work [carried out] on the social and the 'view form the outside'" [25].

In this literature, the interaction of disfigured individuals within primary groups has been privileged over than within secondary groups [15,16,45]. In particular, the spheres of the "family" and "caregivers" are portrayed as social settings in which the disfigured find more comfortable terms of interaction. The family is seen both as a "safer place" in which the disfigured feel protected and supported and the institution that provides them with alternative messages than the stigmatizing ones coming from society and its strangers [15,16]. Interaction with caregivers, and in particular those between surgeon, social workers and disfigured patients, is also viewed as comfortable [15]. To be sure, race-, ethnicity-, and culture-based forms of discrimination in the hospital are documented [16] and improvement in the manner in which health care professionals deal with those who are facially disfigured is frequently indentified as a primary programmatic objective. Nevertheless, the hospital is often identified as one of the settings in which patients achieve comfort in interaction [15].

The same literature indentifies secondary groups in terms of "strangers" who populate "public places" where they not only intrude into the lives of the facially deformed through "staring, remarks, and questions, or obvious eye avoidance [16], but also negate that "civil inattention" normally granted to other members of society [16]. These groups constitute the "popular mind" [16] that produces "generalized prejudice" [16]. The social settings where secondary groups exist are seen as generating stigmatization as people deal "differently with those who have undergone facial surgery" [15] and engender "fear" to the disfigured [15]. Ultimately, society is the source of generalized stigmatization through widespread stereotyping that allows the "…unsightly face [to be] utilized as a visible symbol or a personification of evil, disease, criminality, or mental deficiency" [16]. This process of stigmatization is so pervasive that it is viewed as if it is conducted uniformly throughout society regardless of actors and settings [15,16]. While evidence indicates that society is the primary source of stigmatization [10,20,24,37,39], the manner thought which facially deformed cancer patients experience stigmatization in different spheres of society is understudied [45]. van Doorne and his associates [39], share the view that members of the immediate family provide strong support to cancer patients who are facially disfigured. They also stress that strangers are consistently the source of stigmatization. In their view, further investigation is needed to ascertain the manner in which non-immediate family members and acquaintances react to facially disfigured patients.

In essence, the study of stigmatization of the facially disfigured is couched in approaches that stress the actions and adaptability of the individual, on the one hand, and the homogenous dimension through which stigmatization is created in society, on the other. In this context, the manner in which facially disfigured cancer patients experience stigmatization while interacting with secondary groups in different spheres of society has not been adequately explored. This literature does not differentiate between the many settings that form the sphere of society and that are the contexts in which interaction with secondary groups takes place [45]. Owing this gap in the literature, the objective of this paper is to provide an exploratory analysis of the patterns of social interaction experienced by individuals who are facially disfigured because of cancer as they interact with members of secondary groups.

3. METHODS

This exploratory research employed in-depth interviews with fourteen individuals who were facially disfigured because of head and neck cancer. A purposive sample was selected from a list of patients who were registered at MD Anderson Cancer Center in Houston, Texas. This

particular group of patients had cancer of the upper face area and specifically orbital and periorbital cancer. Potential participants were identified following the recruitment guidelines. They were adult patients (18 years old and older), who were diagnosed with various forms of head and neck cancer, underwent facial surgery to remove the malignancy and, in the process, acquired observable changes in their facial appearance. Additionally, they were required to be able to communicate in a meaningful manner with investigators.

In-depth individual interviews were conducted by phone between January 2008 and April 2009. Twenty potential participants were contacted. Three of them could not participate for scheduling problems and three refused to be part of the project. Finally, a total of eight men and six women were interviewed. The median age of these patients was 66 years and the youngest patient was 31 and the oldest 81. At the time of the interview, the post-surgical period ranged from ten months to thirty five years and the median was five years. Some patients underwent additional reconstructive and plastic surgical procedures. While the extent of disfigurement varied, all of the patients were left with significant alterations in their facial appearance.

A semi-structured questionnaire was employed. Questions covered topics such as the individual's demographic and socio-economic characteristics; experience with cancer and treatment; perceived attitudes and actions of others; and interaction in various events and places. Patients were interviewed by a single research team member. This procedure was adopted to assure consistency in the interview procedure. With the patient's consent, interviews were audio recorded and written notes were also taken.

The transcribed texts were analyzed through grounded theory methodology [26,50]. This methodology permits the identification of conceptual categories and patterns among them from the text. Categories and patterns were verified through the techniques of "saturation" and "negative cases." Secondary groups were defined as nonintimate, formal groups formed by strangers and acquaintances. Following data analysis, salient interaction patterns were identified and patients were divided into those feeling comfortable, uncomfortable or having varied or mixed feelings. The feeling of uncomfortable was considered an indicator of the patient being stigmatized either in terms of felt stigmatization or enacted stigmatization. Felt stigmatization refers to the perception of the patient. Enacted stigmatization refers to overt actions of stigmatization. Interaction patters were identified and divided into three categories. *Intrusion* indicates that people pay unsolicited attention to patients, ask unwanted questions, make unwelcome remarks and stare. It also indicates that they make their unspoken curiosity felt. *Sympathy* refers to unsolicited comments and/or actions showing support to patients and the desire to be of assistance. *Benign neglect* denotes a situation in which interacting individuals did not pay particular attention to patients. Size of interacting group was divided into small and large. These concepts indicate that the secondary group in which interaction took place was either large or small. The actual size of the group was determined through patients' own descriptions of the settings.

4. FINDINGS

Following grounded theory analysis of the data, two groups of patients were identified: *Occasionally Comfortable Patients* (N = 10) and *Always Comfortable Patients* (N = 4). Patterns of interaction with *Occasionally Comfortable Patients* are visually synthesized in **Figure 1** and basic descriptive statistics for this group are presented in **Table 1**. Basic descriptive statistics for *Always Comfortable Patients* are summarized in **Table 2**.

Over 71 percent of the patients studies suffer from some forms of stigmatization. In relative terms, men suffer from stigmatization slightly more than women (75% versus 66.7%). Yet, women over 55 years of age experience stigmatization in slightly higher terms that men of the same age group. Intrusion is the source of stigmatization for the majority of patients and impacts men and women equally. Older men experience stigmatization from sympathy more than women of the same

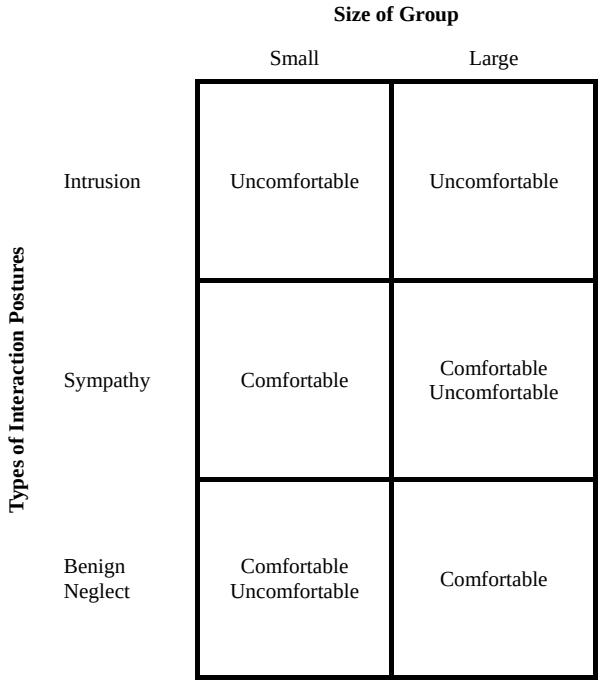


Figure 1. Patient-secondary group interaction by size of group

and types of responses to disfigurement for Occasionally Comfortable Patients.

Table 1. Basic characteristics and patterns of interaction of Occasionally Comfortable Patients, N = 10 (total patients studied N = 14).

Gender	**Men** 6 *75%*	**Women** 4 *66.7%*	**Total** 10 *71.4%*
Age and Gender	**Men > 55** 4 *66.7%* **Men < 55** 2 *33.3%*	**Women > 55** 3 *75%* **Women < 55** 1 *25%*	**Total** 7 *70%* **Total** 3 *30%*
Intrusion	3 *50%* **Men > 55** 2 *66.7 %* **Men < 55** 1 *33.3 %*	2 *50%* **Women > 55** 2 *100%* **Women < 55** 0 *0%*	5 50%
Sympathy	2 33.3% **Men > 55** 1 *50%* **Men < 55** 1 *50%*	1 *25%* **Women > 55** 0 *0%* **Women < 55** *1* *100%*	3 *30%*
Benign Neglect	1 *16.7%* **Men > 55** 1 *100%* **Men < 55** 0	1 *25%* **Women > 55** 1 *100%* **Women < 55** *0*	2 *20%*

Table 2. Basic characteristics of Always Comfortable Patients, N = 4 (total patients studied N = 14).

Gender	**Men** 2 *25%*	**Women** 2 *33.3%*	**Total** 4 *28.6%*
Age and Gender	**Men > 55** 2 100%	**Women > 55** 2 100%	**Total** 4 100%

age group while older women experience benign neglect relatively more than men. Due to the limited size of the sample and the qualitative design, these statistics are reported only as an illustration of possible quantitative trends. More pertinent findings are presented below in the discussion of the qualitative analysis.

4.1. Occasionally Comfortable Patients

Occasionally comfortable patients are individuals who experience different levels of comfort in interaction. In some situations they do not feel stigmatized, but other interactions constitute the contexts within which this discomfort emerges. Intrusion in interaction in large and small groups always generates uncomfortable outcomes. Here patients are stigmatized. Sympathy is associated with comfort in interaction in small groups and produces varying patterns in the case of large groups. Benign neglect produces comfort in interaction within large groups and varying patterns in the case of small groups.

4.1.1. Intrusion

This is a situation in which patients feel uncomfortable interacting within small and large groups alike. Members of interacting groups grant disfigured individuals the particular status of "different" and proceed to construct an interaction in which this condition is reinforced. Patients are noted because their different appearance. They are drawn in interaction patterns in which their status of different is constructed and reinforced through unwanted attention. People's questions, stares, remarks constitute elements that build a view of disfigurement that is stigmatizing. While varying, patients' responses are characterized by dissatisfaction with the interaction. Interaction in large and small groups alike provides the terrain for the development of this constructed stigmatization.

4.1.1.1. Small Groups

Joy provides an account of this type of interaction with small groups. She says:

"Sometimes I notice that people…can tell that this side of my face does not make the same movements [than the other side]. They are probably aware that there is something strange about that woman…The first time that I took my six year old to dancing—my six year old granddaughter, she dances every Wednesday and then she is at church afterwards. The children have special things going on after dancing at the church… My daughter works a lot and so that Wednesday is mine. I take them and do all of that kind of thing. Well, the first few times when I was taking her to dancing, I sit and wait… can't go off and leave her, wait in the room with the other parents and I could tell that some of the mothers were looking at me strangely, not in a mean way or anything like that but more curious, like there's something strange about that woman. What could it be? And generally, I have found that if I feel like somebody is making me feel uncomfortable… I'll go ahead and say something."

She adds:

"I went to a wedding shower for one of my daughter's friends who I didn't know very well and there were a lot of young women there that I did not know well… and I was uncomfortable there. I felt like the majority of them probably did not know that I had this problem [cancer generated facial disfigurement] and I'm sure they were wondering… that was an uncomfortable day…. I didn't

really enjoy that."

4.1.1.2. Large Groups

Arthur provides an instance of the interaction between patients and large secondary group. He says:

"I feel like everybody's always staring at me. It is strangers. [I have negative feelings] when dealing with strangers. Probably the worse is in restaurants. Because you are sitting there for a long time and the people around you are all eventually looking at you or you feel they are. [It is also the same] when you walk past somebody in a store or on the street or something… They stare at you… It is very crude and insensitive and it makes me skittish towards other people… it makes me feel very uncomfortable…I don't go out very much anymore."

Janet adds:

"When people keep staring at me and pretend they are not looking at me… It bothers me. Most adults—they will look at me. They all notice, I'm sure. A lot of them try to pretend… But it happens everywhere. The worst is at places like Super Centers. That where I found that people are most rude. It also bothers me that people let their kids be so rude, particularly big kids…. They run around you in front of me and go 'she doesn't have and eye! Look, look!' Then I want to smack them. I want to tell them 'you are very, very rude.'"

4.1.2. Sympathy

Sympathy creates interaction patterns that are dissimilar for small and large groups. In small groups, data reveal that sympathy engenders comfortable interaction between disfigured patients and acquaintances. The latter's statements and actions are intended and perceived as supportive and contribute to the construction of a situation in which disfigured patients find themselves at ease. The analysis indicates that varying patterns define the interaction between patient and members of large groups. Sympathy shapes positive interaction as people's support is employed to construct advantageous conditions for patients. Instrumentally, patients use this support even in situations in which it is not needed. Simultaneously, patients also feel uncomfortable when sympathy transcends the necessary. They feel that they their disfigurement is constructed as a grater disadvantage than it actually is and/or a situation that creates undeserved respect. In this instance, overt sympathy becomes a negative factor in the construction of interaction. It is important to note that sympathy is also associated with the existence of cancer. In some instances, interacting individuals display sympathy to patient primarily because of their status of survivors.

4.1.2.1. Small Group

Lisa provides evidence of a positive interaction with small groups. She says:

"Nobody tells me anything. The only thing is they ask me what happened, you know, what is wrong. That's the question. Then I have to tell them that I had surgery and I had cancer and I had a tumor… This is friends, friends that I haven't seen in a long time and I happen to meet in a store while I'm shopping, people that I haven't seen in a long time… they will ask questions… and when I explain to them what happened everybody is nice. They just want to know what had happened to me and they don't make [comments or remarks], not at all, everybody is nice."

4.1.2.2. Large Groups

Sympathy in interaction with large groups engenders a variety of results that include both comfortable and uncomfortable situations.

Tom states: "Depends on whether or not I'm wearing my prosthesis, the black patch or the medical patch and I get different reactions for each of those circumstances. When I'm wearing my prosthesis out in public… they do not know that I had surgery. If I wear it … on the street, in the store, in front of a group of people, at church or in a meeting, if they do not know I had surgery they still do not know I had surgery. [Because it is difficult to wear my prosthesis continuously][5], I wear my black patch. When I wear my black patch, I get better service. If I ride a plane… people treat me differently… they are sympathetic, overtly so… This is a person with a visual problem, we'll treat him differently and they do…. I think they feel sorry for me… They seem to be more sympathetic towards me and more ready to help me in stores, primarily in store settings or at the library or other public places. When I'm out in public service areas, the service that I receive appears to be somewhat more magnanimous… I'm going to take advantage of this thing as much as I can."

But he also indicates: "Sympathy is good, but at times I feel that I don't need it at all. I can do things on my own and do not need any help."

Mary says: "I'm very self-confident … around others because more and more people are very receptive of cancer survivors. So if you had cancer and you are a survivor, then people are very supportive…. I could be a horrible person (I'm not a horrible person) and they would be like 'oh, that's ok if you are a cancer survivor…. People at work have been very supportive… nobody has been negative about my appearance…. They simply ask what happened to you."

4.1.3. Benign Neglect

[5]This patient informs us that wearing the prosthesis creates a number of problems. There are problems associated with skin irritation where the prosthesis attaches to the face. When the skin is irritated, the discomfort forces the patient to wear either a black patch or a medical patch. Additionally, there are problems associated with the patients' activities. Wearing the prosthesis causes severe headaches when traveling by plane. Discomfort is also felt when pursuing outdoor activities or exercising.

Benign neglect refers to situation in which interacting individuals did not pay particular attention to patients. In the case of interaction with small groups, data show that varying patterns of interaction are possible. In some instances, lack of interests on the part of interacting individuals is felt and acted upon in positive terms. Disfigured patients do not feel stigmatized. Simultaneously, though, similar situations engender opposite interaction patterns as patients feel uncomfortable in the absence of attention paid to them. The fact that interacting individuals "ignore" patients is not necessary an indication of comfort in interaction. In the case of interaction with large groups, benign neglect generates comfortable interaction patterns. The size of the group is a condition that allows patients to pass unnoticed and this situation facilitates the manner through they interact with this group of strangers.

4.1.3.1. Small Groups

Following are a few instances of the consequences of benign neglect in interaction with small groups.

Carl says: "I have not noticed anything. I don't think people feel sorry for me any more or any less. I think that still they see me as Carl… When I'm with a small group of people, I don't see anyone doing anything differently for me… They treat me the same as always… When I go shopping people look at me… it doesn't feel good but I don't let it bother me, I don't feel like I'm less of a human. I don't have to look down like I'm ashamed to look them in the face, or like they are going to reject me or anything like that…. It bothers me though."

Ron states: "I generally try to avoid being seen in a prominent position… I really do believe that I have learned to accept it [my disfigurement], but I do realize that it's noticeable to people and that it's pretty obvious. … I felt good about myself before my cancer. Now, I look in the mirror and I'm missing a part that's very important to your looks…people at work tell me 'I don't even notice that your eye is missing, because I'm so used to you…. It bothers me, but I accept it."

4.1.3.2. Large Groups

Joy provides an instance of benign neglect in interaction with large groups. She says:

"We [my husband and I] go to [college] basketball games. We have a lot of those here. At the basketball game is different because everyone is so intent on what [the players] are doing what they are watching that they do not pay attention to anybody, because I mean, they are glued on what is going on on the court so I don't notice people reacting to me…. I'm pretty confident interacting with people except when I need to meet new people… I used to love meeting new people. I don't really enjoy that so much anymore… but I like to be in a large crowd when people don't notice you."

4.2. Always Comfortable Patients

Always comfortable patients are individuals who do not experience problems interacting in small and large groups alike. They are well adjusted to their disfigured status and, while they encounter behavior that could lead to enacted stigmatization, the interaction does not affected by them. As episodes of enacted stigmatization are quire rare, the lack of felt stigmatization denotes non-stigmatizing interaction patterns. Basic statistics about this group are summarized in **Table 2**. As it can bee seen from that table, no relevant differences seem to exist in terms of patients' age and gender.

Fred says: "I feel no difference the way that people treat me… I don't see any difference… when we go to the shopping mall, when we go to the restaurant, when we go anywhere. I see no difference in the way I'm treated now and the way I was treated five years ago [before surgery]…. I have no problems with the way I look whenever I'm meeting anyone. It's not something that I think about and it's something that I don't even consider…. I get some sympathy because I'm a cancer survivor, but this has very little to do with the way I look."

Frank continues: [when I interact with people I'm very confident] Oh, yeah, I think so. I went back to work on a full-time job where I used to travel a lot and go to a contractor's facility in [name of city]… all the time and meet with people, have meetings, give briefings and so, … I don't have any issue with it and there never seemed to be an issue with those people I deal with."

[When I go out] "the only people that tend to react are kids. And they'll look at you, and it is just like at my wife's church preschool class. I went to see her at her class today and went to lunch after that, and the kids came up and just looked and said, hey, how you doin'? And she said, oh, my husband's a pirate… they said, okay, great!... I haven't really had a negative situation so far…. When I had my knee operation the anesthesiologist that did the knee operation came to my house during Halloween and I answered the door and he was standing there with his child, and he said: you're really getting into the part, aren't you? Thinking that I had an eye patch on for Halloween. I said, nope, I really have to have an eye patch. He said: Oh, I'm sorry. That's the guy I had four months later to be my anesthesiologist on my knee surgery, and he mentioned how he had met someone with an eye patch. I said that was me. He said: Oh, I feel so bad about it. I said: don't worry, it happens all the time. You know, you get dressed up for something or whatever… but… it doesn't bother me and in fact I called the guy about a part on a pool the other day, told him my name, he said: yeah, you are the guy with the bad eye – wait, you don't mind that I said that, do you?' And I said, no, I don't mind that you said that."

5. CONCLUSIONS

In terms of the interaction between facially disfigured cancer survivors and secondary groups, this study identified the existence of two groups of patients. When interacting with strangers and acquaintances these cancer survivors can be grouped into *always comfortable patients* and *occasionally comfortable patients*. Always comfortable patients are patients who do not experience problems because of their disfigurement and their interaction with small and large groups does not lead to felt stigmatization. While episodes of enacted stigma may occur, they tend not to affect these well adjusted individuals, their counterparts, and interaction.

The group of patients classified as occasionally comfortable presents more complex patterns of interaction with secondary groups. This group experiences uncomfortable and stigmatizing interaction patterns in specific instances. At the same time, they experience non consistent or comfortable (*i.e.*, not stigmatizing) patterns of interaction in other situations. This is the group of patients that requires the most attention as their interaction with secondary groups varies according to the size of the group.

These findings add to the existing literature in a number of ways. First, they indicate that strangers do display varying patterns of interaction with the facially disfigured. Accordingly, the statement that "the reaction of people on the streets or in the neighborhood is …. consistent: they stare at most patient with facial defect" [39] (see also [15,16]) needs to be revised. This study demonstrates that the behavior of strangers is much more complex than previously recorded and it entails a number of distinct interaction patterns.

Second, this research clarifies questions raised by previous research on the interaction between facially disfigured cancer survivors and acquaintances [39]. Data on the various patterns of interaction within small groups demonstrate the complexity of interaction patterns existing between patients and acquaintances. Ultimately, acquaintances are the source of stigmatization and support alike as they intrude in the everyday existence of patients but also provide sympathy to cancer survivors.

Third, these findings contribute to the understanding the issue of the relationship between disfigurement and cancer. The existence of cancer mitigates the negative effects of facial disfigurement. It creates a process of interaction in which people display support to patients because they suffered from cancer. Once people learn that the patient's facial disfigurement is cancer related, they tend to be overtly supportive regardless of other conditions.

Finally, this research underscores the importance of the reading of the social consequences of cancer generated facial disfigurement in relational terms. The social consequences of facial disfigurement emerge as outcomes of interaction processes. As such, they involve patients and other segments of society as they interact in various social spheres. While the study of the manner through which individuals respond to disfigurement remains important, it is through the process of interaction that stigmatization or lack of it are constructed.

Three overall recommendations emerge from the analysis presented in this study. First, patients should be warned about the occurrence of possible difficulties when interacting in large and small groups alike. In this respect, they should be prepared to the possibility of experiencing episodes of intrusion, sympathy and benign neglect when interacting with small and large groups of acquaintances and strangers. Second, it appears desirable that interacting groups be sensitized about the patterns of interaction identified above. While complex, this solution should be considered as educational programs are discusses and implemented at various levels. Finally, the issue of the social consequences of cancer generated facial disfigurement should be studied with an interdisciplinary approach. In this context, it would be desirable to include a sociological component that would allow investigators to explore the collective dimension of the social construction of facial disfigurement and stigma.

REFERENCES

[1] Mood, D.W. (1997) Cancers of the head and neck. In: Varricchio, C., Ed., *A Cancer Source Book for Nurses*, Jones and Bartlett Publishers, Sudbury, 271-283.

[2] Davis, K., Wingo, P. and Parker, S. (1998) Cancer statistics by race and ethnicity. *CA*: *A Cancer Journal for Clinicians*, **48(1)**, 31-48.

[3] Davis, K., Roumanas, E.D. and Nishimura, R.D. (1997) Prosthetic-surgical collaboration in the rehabilitation of patients with head and neck defects. *Otolaryngologic Clinics of North America*, **30(4)**, 631-645.

[4] Dropkin, M.J. (1999) Body image and quality of life after head and neck cancer surgery. *Cancer Practice*, **7(6)**, 309-313.

[5] American Cancer Society. (2008) Detailed guide: Eye cancer. http://www.cancer.org

[6] Esmaeli, B. (ed.) (2009) *Ophthalmic Oncology*, Springer, Norwell.

[7] Anderson, R.C. and Franke, K.A. (2002) Psychological and psychosocial implications of head and neck cancer. *Internet Journal of Mental Health*, **1(2)**, 55-64.

[8] Cole, J. (1998) About face. The MIT Press, Cambridge.

[9] Jackson, L.A. (2002) Physical attractiveness: A sociostructural perspective. In: Cash, T.F. and Pruzinsky, T., Eds., *Body image. A handbook of theory, research, and clinical practice*. The Guilford Press, New York, 13-21.

[10] Kish, V. and Lansdown, R. (2000). Meeting the psychosocial impact of facial disfigurement: Developing a clinical service for children and families. *Clinical Child Psychology and Psychiatry*, **5(4)**, 497-512.

[11] Macgregor, F. (1990) Facial disfigurement: Problems and

management of social interaction and implication for mental health. *Aesthetic and Plastic Surgery*, **14(4)**, 249-257.
[12] Furness, P., Garrud, P., Faulder, A. and Swift, J. (2006) Coming to terms. A grounded theory of adaptation to facial surgery in adulthood. *Journal of Health Psychology*, **11(3)**, 453-466.
[13] Goffman, E. (1963) Stigma. Notes on the management of spoiled identity. Simon & Shuster, New York, 2-52.
[14] Hawkesworth, M. (2001) Disabling spatialities and the regulation of a visible secret. *Urban Studies*, **38(2)**, 299-318.
[15] Hughes, M. (1998) The social consequences of facial disfigurement. Ashgate, Aldershot, 24-279.
[16] Macgregor, F. (1974) Transformation and identity: The face and plastic surgery. Quadrangle/The New York Times Book Co, New York, 8-208.
[17] Bull, R. and Rumsey, N. (1988) The social psychology of facial appearance. Springer Vale, New York.
[18] Cash, T.F. and Pruzinsky, T. (eds.) (2002) Body image. A handbook of theory, research, and clinical practice. The Guilford Press, New York.
[19] Bull, R. and Stevens, J. (1981) The effects of facial disfigurement on helping behavior. *The Italian Journal of Psychology*, **8(1)**, 345-351.
[20] Callahan, C. (2004) Facial disfigurement and sense of self in head and neck cancer. *Social Work in Health Care*, **40(2)**, 73-87.
[21] Kent, G. (2000) Understanding the experiences of people with disfigurements: An integration of four models of social and psychological functioning. *Psychology, Health & Medicine*, **5(2)**, 117-129.
[22] Clarke, A., Rumsey, N., Collin, J.R.O. and Wyn-Williams, M. (2003) Psychological distress associated with disfiguring eye conditions. *Eye*, **17**, 35-40.
[23] Newell, R.J. (1999) Altered body image: A fear-avoidance model of psycho-social difficulties following disfigurement. *Journal of Advanced Nursing*, **30(5)**, 1230-1238.
[24] Pruzinsky, T., Levine, E., Persing, J.A., Barth, J.T. and Obrecht, R. (2006) Facial trauma and facial cancer. In: Sarwer, D.B., Pruzinsky, T., Cash, T., Goldwyn, R.M., and Persing, J.A., Eds., *Psychological aspects of reconstructive and cosmetic plastic surgery*: *Clinical, empirical and ethical perspectives*, Lippincott Williams, Philadelphia PA, 125-143
[25] Thompson, A. and Kent, G. (2001) Adjusting to disfigurement: process involved in dealing with being visibly different. *Clinical Psychology Review*, **21(5)**, 663-682.
[26] Strauss, A. (1987) Qualitative analysis for the social scientists. Cambridge University Press, New York.
[27] Fife, B.L. and Wright, E.R. (2000) The dimensionality of stigma: A comparison of its impact on the self of persons with HIV/AIDS and cancer. *Journal of Health Social Behavior*, **42**, 50-67.
[28] Cahill, S. and Eggleston, R. (1995) Reconsidering the stigma of physical disability: Wheelchair use and public kindness. *The Sociological Quarterly*, **36(4)**, 681-698.
[29] Susman, J. (1994) Disability, stigma and deviance. *Social Science and Medicine*, **38**, 15-22.
[30] Angermeyer, M. and Matschinger, H. (1994) Lay beliefs about schizophrenic disorder: The results of a population study in Germany. *Acta Psychiatrica Scandinavica*, **89**, 39-45.
[31] Corrigan, P.W. and Penn, D.L. (1999) Lessons from social psychology on discrediting psychiatric stigma. *American Psychologist*, **54**, 765-776.
[32] Clarke, A. (1999) Psychosocial aspects of facial disfigurement: problems, management and the role of a lay-led organization. *Psychology, Health and Medicine*, **4(2)**, 127-142.
[33] Synnott, A. (1989) Truth and goodness, mirrors and masks—Part I: A sociology of beauty and the face. *The British Journal of Sociology*, **40(4)**, 607-636.
[34] Link, B.G. and Phelan, J.C. (2001) Conceptualizing Stigma. *Annual Review of Sociology*, **27**, 363-385.
[35] Jacobi, A. (1994) Felt versus enacted stigma: A concept revisited. *Social Science and Medicine*, **38(2)**, 269-274.
[36] Feingold, A. (1992) Good looking people are not what we think. *Psychological Bulletin*, **111**, 304-341.
[37] Hagedoorm, M. and Molleman, E. (2006) Facial disfigurement in patients with head and neck cancer: The role of social self-efficiency. *Health Psychology*, **25(5)**, 643-647.
[38] Rybarczyk, B.D. and Behel, J.M. (2002) Rehabilitation medicine and body image. In: Cash, T.F. and Pruzinsky, T., Eds., *Body image. A handbook of theory, research, and clinical practice.* The Guilford Press, New York, 387-393.
[39] van Doorne, J.M., van Waas, M.A. and Bergsma, J. (1994) Facial disfigurement after cancer resection: A problem with an extra dimension. *Journal of Investigative Surgery*, **7(4)**, 321-326.
[40] Bonanno, A. and Choi, J.Y. (2009) Psychosocial aspects of orbitofacial disfigurement in cancer patients. In: Esmaeli, B., Ed., *Ophthalmic Oncology*, Norwell, Springer, 96-105.
[41] Millsopp, L., Brandom L., Humphris, G. and Lowe, D. (2006) Facial appearance after operations for oral and oropharyngeal cancer: A comparison of case notes and patient-completed questionnaire. *British Journal of Oral and Maxillofacial Surgery*, **44**, 358-363.
[42] Valente, S. (2004) Visual disfigurement and depression. *Plastic Surgical Nursing*, **24(4)**, 14-146.
[43] Partridge, J. (1998) Changing faces: Taking up Macgregor's challenge. *Journal of Burn Care and Rehabilitation*, **19**, 174-180.
[44] Vickery, L.E., Latchford, G., Hewinson, J., Bellew, M. and Faber, T. (2003) The impact of head and neck cancer and facial disfigurement on the quality of life of patients and their partners. *Head & Neck*, **25(4)**, 289-296.
[45] Bonanno, A., Choi, J.Y. and Esmaeli, B. (2008) The contradictions of medical sociology understanding of stigma in facially disfigured individuals. *Annual Meeting of the Southwest Social Science Association*, Las Vegas.
[46] Mosher, C. and Danoff-Burg, S. (2007) Death anxiety and cancer related stigma: A terror management analysis. *Death Studies*, **31**, 855-907.
[47] Berremberg, J.L. (1989) Attitudes towards cancer as a function of experience with the disease: A test of three models. *Psychology and Health*, **3**, 233-243.
[48] Bloom, J. and Kessler L. (1994) Emotional support fol-

lowing cancer: A test of the stigma and social activity hypothesis. *Journal of Health and Social Behavior*, **35**, 118-133.

[49] Weiner, B., Perry, R.P. and Magnusson, J. (1988) An attributional analysis of reaction to stigma. *Journal of Social Issues*, **35(1)**, 120-155.

[50] Glaser, B. and Strauss, A. (1987) The discovery of grounded theory. Aldine Transaction New Brunswick, NJ.

Vol.2, No.5, 429-434 (2010)
doi:10.4236/health.2010.25064

Health

Coping styles as predictors of survival time in bladder cancer

Jochen Hardt[1]*, Rolf Gillitzer[2], Susanna Schneider[1], Sabine Fischbeck[1], Joachim W. Thüroff[2]

[1]Department of Medical Psychology and Medical Sociology, University of Mainz, Mainz, Germany; *Corresponding Author: jochen.hardt@gmx.de
[2]Clinic for Urology, University of Mainz, Mainz, Germany

Received 2 December 2009; revised 8 January 2010; accepted 15 January 2010.

ABSTRACT

The role of coping in the survival of cancer is a controversial topic. To specifiy the influence of coping on survival time, we conducted a longitudinal, prospective and observational study. In a preoperative interview, 105 patients with primary bladder cancer were asked about their active and depressive coping strategies. Ten years later, the survival rate was recorded; in cases of death, it was noted whether or not it was in consequence of the bladder cancer. Kaplan-Meier analyses of the collected data revealed a mean survival rate of about 60% after 10 years. Cox regression demonstrated no significant effect for active or depressive coping when tumour stage was controlled for. Patients who presented with high values for either of the coping strategies lived only slightly longer than those with low values. Therefore, it can be concluded that preoperative coping does not seem to demonstrate an important role for survival in bladder cancer.

Keywords: Oncology; Coping; Bladder Cancer; Survival; Longitudinal Study

1. INTRODUCTION

With respect to survival time of patients with cancer, the role of coping is a controversial topic. Early intervention studies provided some evidence that active coping and the development of a fighting spirit against the cancer were associated with longer survival times [1-3]. In these studies, a usually randomly selected portion of the patients received some sort of psychotherapy or psychosocial support, whereas patients in a control group did not. Typically, patients who were treated displayed longer survival times than controls [4]. These studies have been criticised as having flaws in design and analyses [5]. For example, in the study by Spiegel *et al.* [6], the control group showed an unusually strong decline in survival, *i.e.* an atypical form for a survival curve. The intervention group, however, displayed a rather normal survival curve, as would be expected for the given type of cancer. Furthermore, the sample sizes in these studies have been criticised as being small on average-often fewer than 40 subjects. In 2007, three replication studies of classical psychosocial interventions were published-none showing a positive effect on survival time [7]. Most of the survival studies were carried out on patients with breast cancer, therefore the results might not necessarily be representative for other types of cancer. A recent review concluded: "Some researchers view the mind-cancer survival question as resolved and negative, whereas others identify conceptual and methodological challenges and view the possible impact of psychosocial factors on survival as simply unproven" [8].

A study that bolstered the latter supposition demonstrated that giving psychosocial support did lead to prolonged survival [9] in breast cancer. Additionally, a further study proved that giving even very limited psychosocial support to patients with gastrointestinal cancer led to a doubled survival rate after 10 years when compared with controls without psychosocial support [10].

In general, observational studies have added to the controversy rather than confirming one specific theory. Similar to intervention studies, observational studies yielded some evidence for better survival in patients with active versus depressive coping, but also some evidence that psychosocial factors do not have an effect on survival rates [2]. These observational studies often suffered from the fact that many variables with a potential impact on the development of cancer were examined in small samples. For example, Gruhlke *et al.* [11] examined a total of 34 coping strategies in a sample of about 60 patients who were receiving bone marrow transplants. In a design like this, there is an increased risk of finding significant predictors even if no associations are actually

present. Another study found effects of traumatic stress experience on survival in breast cancer [12] in addition to effects of mature and immature defence styles in various patients with late-stage cancer [13]. Other results from observational studies appear to be sounder and could be replicated. In an early study, for example, slightly higher cancer rates in widowed people than in the rest of the population were reported [14]. This result was confirmed later, based on the Danish registries [15]. Faller *et al.* [16] demonstrated that depressive coping was associated with significantly shorter survival times in patients with lung cancer. In this study, various coping styles were measured in 107 patients before surgery; one of these styles proved to be associated with survival time assessed up to 10 years after surgery, *i.e.* depressive coping predicted shorter survival. The association remained significant even after controlling for cancer staging and the Karnofski performance status. Moreover, it was found that a majority of patients themselves believe that stress and emotional problems are associated with the development of cancer. Active coping did not predict a better survival, a result that was surprising because it was a main predictor in other studies [17]. Active and depressive coping differ on account of the fact that active coping focuses on finding solutions for the problems directly caused by the stressor itself whereas depressive coping concentrates on the negative emotions evoked by the stress. On the other hand, Akechi *et al.* [18] could not find any psychosocial predictor for survival time in patients with lung cancer. A study by Johansen et al. indicated that not even psychosocial distress was associated with survival in various forms of cancer [19]. Based on the Danish health registries, the authors compared the national incidence rate of cancer with the incidence rate in over 10 000 parents who had experienced the diagnosis of cancer in their child. The rationale of this study was that such an experience constitutes an extraordinary stress factor for a parent, and if psychosocial stress were associated with cancer, this stress would produce some effects. On finding that both rates were the same over a period of 50 years for breast cancer, lymphoma, leukaemia and other tumours, the authors concluded that psychosocial stress does not play a role in the incidence of cancer.

The present study was conducted to explore the role of coping in a rarely examined form of cancer, *i.e.* bladder cancer. The estimated yearly incidence rate of bladder cancer in Germany is about 40 per 100 000 in men and about 10 per 100 000 in women; in countries where schistosomiasis occurs, the rate is higher. At present, non-invasive forms of bladder cancer have been seen in about 80% of primary diagnoses and have usually been treated conservatively; invasive forms have affected about 15% of patients and lead to either bladder extraction, necessitating an artificial urinary diversion, or to radiation therapy. Even for invasive bladder cancer, the prognosis for patients is not bad. Leissner *et al.* [20] estimated 10-year survival rates of about 50% after radical surgery; for patients with low-stage tumours, survival rates were even better.

2. MATERIALS AND METHODS

2.1. Design

The design of the study was prospective and observational. In 1999, data collection for a one-year follow-up study about quality of life after urinary diversion was finished and data were analysed [21]. At that time, an article by Faller *et al.* [16] was published that showed differences in survival time of lung cancer patients depending on a preoperative coping strategy. Due to the fact that 1) the instrument used to assess self-rated coping was the same in the Faller *et al.* study and in ours; 2) the times of assessments were similar; 3) various background variables were assessed similarly; and 4) the sample sizes were almost identical, we decided to set up a 10-year follow-up of our sample in an attempt to cross-validate the results of Faller *et al*..

2.2. Subjects

Utilizing a consecutive recruitment strategy, we examined a total of 108 patients who were treated in the urological clinic in Mainz between January 1996 and April 1998 because of a primary bladder tumour. The patients were examined one to three days preoperatively by means of an interview and a questionnaire booklet. At that time, patients believed that their bladder was going to be removed and replaced by an artificial urinary diversion. A total of 105 of the patients received an artificial diversion, in three patients the bladder was retained. All patients gave written, informed consent to participate in a study about quality of life. They were informed that their participation in the study included their willingness to be contacted one year after surgery and asked to fill out another questionnaire about quality of life. Three patients withdrew their participation after surgery, and no further information was gathered about them. The remaining 105 patients were contacted one year after surgery; at this time, 81 patients reported about their quality of life. One year after surgery, patients showed a stronger decrease in physical than in mental quality of life; in addition, there were complex interactions between coping and quality of life [21]. At that time, 19 patients were deceased; out of the remaining five, three felt too sick to fill out the questionnaire and two did not respond to our letters. For the 19 patients who were deceased, we recorded when they had died and whether or not death was a consequence of the bladder tumour. In cases of uncertainty regarding the latter point, a urologist

was contacted to make a decision.

In 2007, all 81 patients who had responded one year after surgery, in addition to the five surviving patients who had failed to respond, were contacted again via a letter reminding them about their stay at the urological department of the University of Mainz and their participation in a study 10 years previously. The letter indicated that in the next few days they would receive a phone call query regarding their current quality of life. During the phone call, patients were first asked how they were coming along with their artificial urinary diversion; in case of problems, a visit to the urological department in Mainz was offered. For patients who were interested, an appointment with a urologist (RG) was set outside the regular patient schedule. Then, a quality of life questionnaire was sent to the surviving patients. For deceased patients, relatives were asked about the time of death and whether the death was a consequence of the bladder tumour or of another disease or circumstance. Several measures were taken to collect information about those patients who could not be reached in this way. Local authorities were contacted to find out whether a patient had moved or died. Those who had moved were contacted again at their new address; for the deceased, the local registries of death were consulted to find out whether or not the death was due to the bladder tumour. When no data were available, patients' family doctors, general practitioners, or local urologists were contacted.

2.3. Instruments

Coping was assessed by the Freiburger Fragebogen zur Krankheitsverarbeitung [FKV-LIS SE: 22], a widely used questionnaire in Germany. The questionnaire contains 35 items and is designed to measure five dimensions: active coping (5 items), depressive coping (5 items), distraction (5 items), self-affirmation (5 items), and trivializing (3 items; the remaining 12 items were not utilzed). Since we were interested only in replicating the results of Faller *et al.* [16] and not in undertaking any exploratory analyses, we considered only two dimensions: active and depressive coping. Neither dimension represents the end of a continuum; rather, each reflects a distinctive dimension [22]. The reliabilities of the scales were moderate within the present sample, *i.e.* Cronbach's $\alpha = 0.72$ for active coping and $\alpha = 0.69$ for depress- sive coping. The correlation between the two dimensions was slightly positive, *i.e.* $r = 0.27$, $p < 0.01$. Examples of items for active coping were "gathering information about the disease and its treatment" and "deciding to actively fight the disease". For depressive coping, examples were "self-pity" and "withdrawal from others". A significant background variable that was considered in the Faller *et al.* study was tumour stage; hence, we also included it in our analysis. Tumour stage and grade were coded according to the TNM classification. In addition, gender and age at surgery were included.

2.4. Statistics

A few missing data were substituted by the mean or a value close to the mean (**Table 1**). This method has been proven to be superior to analysis of complete data in various simulation studies and a complex method of substituting missings such as multiple imputation did not seem justified given the small amount of missing data in the present sample. Bivariate associations were calculated as Pearson correlation coefficients, except for survival time. Because a substantial proportion of patients in this sample died from reasons other than bladder cancer, multivariate as well as bivariate associations with survival time were calculated using Cox regressions, defining death from bladder cancer as the response. This procedure ensured that patients who were still alive and those who died due to disorders other than a bladder tumour were treated appropriately. Survival curves were presented by Kaplan-Meier estimates. Statistical analyses were performed by R [23].

3. RESULTS

Ten years after surgery, 41 patients were still alive; 46 were deceased due to the tumour progression and 17 patients had died from other diseases. Regarding the deceased patients, dates of death and information about the reasons for death were recorded for all 46 patients. Again, in cases of unclear information, a urologist (RG) was contacted to decide whether or not the death was tumour-related. Thus, a total of 105 patients (97% of the intended sample) were analysed. Additional relevant patient characteristics are summarised in **Table 1**.

In the bivariate analysis, there was a significant effect of active coping (hazard ratio = 0.70, $p < 0.05$) on survival in the assumed direction, depressive coping showed no significant effect (hazard ratio = 0.70, $p < 0.14$; **Figure 1**, **Table 2**). In the multivariate analyses, *i.e.* controlling for various confounders, tumour stage (hazard ratio = 1.51, $p < 0.01$) and age at surgery (hazard ratio = 1.04, $p < 0.05$) were the only significant predictors of survival time (**Table 3**). Active as well as depressive coping became non-significant.

4. DISCUSSION

The present study was designed to replicate a finding of Faller *et al.* that depressive coping is associated with shorter survival time and active coping may be associated with longer survival time in cancer patients. The hypothesis for this study was not formulated very specifically because the Faller *et al.* study did not prove a significant effect for active coping. However, the hypothesis had to be rejected for both coping strategies. Controlling for confounders moved all effects for either

Table 1. Description of variables.

	Variable	possible range	Valid N	Min	Max	Mean	Standard deviation	Missings substituted by
Y	Survival time (years)	0-11	105	0.01	11.17	5.98	4.19	-
A	Active coping (FKV)	0-5	104	1.00	3.20	3.36	0.88	3.36
D	Depressive coping (FKV)	0-5	104	1.00	5.00	1.82	0.64	1.82
S	Tumour stage	0-4	101	0.00	4.00	2.15	1.23	2
G	Tumour grade	0-4	105	0.00	4.00	2.36	1.07	2
E	Age at surgery	18-90	105	41.77	82.53	64.42	8.93	-
X	Sex		105	0 female	1 male	75% male	-	-

Table 2. Bivariate associations.

Variable	Survival time*	Active	Depressive	Stage	Grade	Age
Survival time						
Active coping	**0.69 (0.12)** p < 0.035					
Depressive coping	0.70 (0.17) p < 0.136	**0.27** 0.005				
Tumour stage	**1.52 (0.20)** p < 0.002	**–0.30** 0.002	–0.16 0.096			
Tumour grade	1.07 (0.15) p < 0.647	–0.02 0.814	0.03 0.760	**0.44** 0.000		
Age at surgery	**1.04 (0.22)** p < 0.029	–0.16 0.106	**–0.37** 0.000	0.03 0.729	–0.03 0.783	
Sex	1.50 (0.56) p < 0.274	0.07 0.474	–0.11 0.281	–0.05 0.579	0.07 0.472	**0.34** 0.000

* Hazard ratio (std err), all others Pearson correlations and p-values.

Table 3. Prediction of survival time.

Response variable: Y, survival time							
	Starting model			Selected model			Excluded variables
Explanatory variable	Estim. coeff.	Stand. error	z-value	Estim. coeff.	Stand. error	z-value	z-value
Active coping	–0.18	0.20	0.93	–0.27	0.10	–2.67	-
Depressive coping	0.01	0.27	0.05	-	-	-	
Tumour stage	0.41	0.15	2.70	0.41	0.13	3.07	
Tumour grade	–0.11	0.17	–0.63	-	-	-	
Age	0.03	0.02	1.62	0.04	0.02	2.09	
Sex	0.25	0.40	0.63	-	-	-	
Total model				$Chi^2_{(2)} = 14.12$, $p < 0.001$			

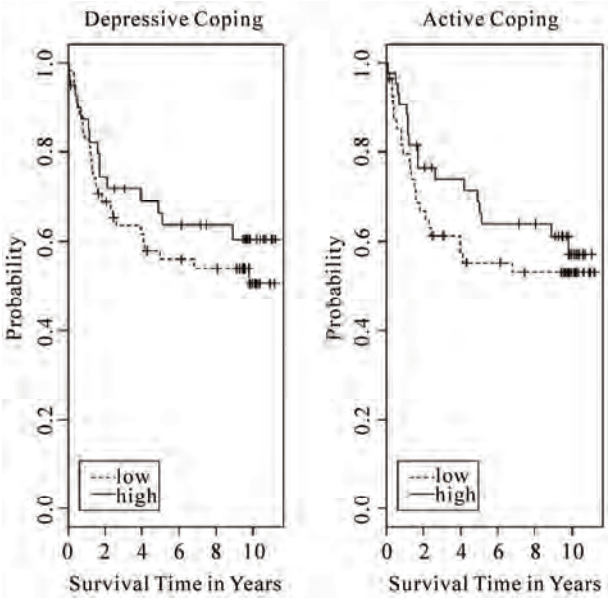


Figure 1. Kaplan-Meier estimates for survival as a function of active and depressive coping.

of the coping strategies well into the range of random variation.

When interpreting these results, it should be taken into consideration that our study focused on a rarely examind form of cancer, *i.e.* bladder cancer. Well-identified risk factors for bladder cancer are chemicals used in the rubber industry, smoking, and the use of certain drugs [24]. The Faller *et al.* study dealt with lung cancer and the Küchler *et al.* [10] study with gastrointestinal cancers; most other research concerning psychosocial risk factors has dealt with breast cancer. A possible explanation for the disparity of the results is that different forms of cancer may have different origins; it is possible that only some forms are associated with psychosocial factors. An alternative explanation takes unhealthy patient habits or behaviour into consideration, e.g. smoking in the case of lung cancer. Smoking is not only a risk factor for the development of lung cancer, continued smoking also contributes to a poor prognosis for those who have already developed cancer. It is possible that depressive coping is associated with more smoking than active coping, a theory that may explain the association observed in the Faller *et al.* study. Even though smoking is also discussed as a risk factor in bladder cancer, the association is weaker and probably works over a longer time period. So far, no unhealthy patient behaviour has been directly associated with bladder cancer.

The present study has the following limitations. 1) Bladder cancer has typically been present for at least 10 years when it is diagnosed. In patients who have severe bladder cancer, such as those in our study, it is probably longer-established for the majority of patients. Hence, a study beginning observation at the time of surgery relies on left-truncated data [25]. For this reason, it is possible that the time period during which psychosocial variables could have influenced the genesis of bladder cancer was not captured by our time of observation; 2) A sample size of about 100 may be too small to detect an effect; 3) Controlling all relevant confounders, for example smoking, was not possible in this study.

Given these limitations, the present study demonstrates an absence of any psychosocial effect on the course of cancer rather than support for a positive effect. It was active coping that was predictive in our unadjusted analysis rather than depressive coping as in the Faller *et al.* study. In addition, the effect of depressive coping tended towards a counterintuitive direction, despite its being non-significant. It is either possible that the effects of coping on survival in bladder cancer are small, or that there is no effect. This finding applies to a form of cancer that has not been studied before in this respect. Hence, further investigations are needed.

REFERENCES

[1] Spiegel, D. (1997) Psychosocial aspects of breast cancer treatment. *Seminars in Oncology*, **24**, 36-47.

[2] Petticrew, M., Bell, R. and Hunter, D. (2002) Influence of psychological coping on survival and recurrence in people with cancer: Systematic review. *British Medical Journal*, **325**, 1066-1075.

[3] Fawzy, F.I., Fawzy, N.W., Hyun, C.S., Elashoff, R., Guthrie, D., Fahey, J.L. and Morton, D.L. (1993) Malignant melanoma. Effects of an early structured psychiatric intervention, coping, and affective state on recurrence and survival 6 years later. *Archives of General Psychiatry*, **50(9)**, 681-689.

[4] Blake-Mortimer, J., Gore-Felton, C., Turner-Cobb, J.M. and Spiegel, D. (1999) Improving the quality and quantity of life among patients with cancer: A review of the effectiveness of group psychotherapy. *European Journal of Cancer*, **35(11)**, 1581-1586.

[5] Fox, B.H. (1998) A Hypothesis about Spiegel *et al.*'s 1989 paper on psychosocial intervention and breast cancer survival. *Psycho-Oncology*, **7**, 361-370.

[6] Spiegel, D., Bloom, J.R., Kraemer, H.C. and Gottheil, E. (1989) Effect of psychosocial treatment on survival of patients with metastatic breast cancer. *Lancet*, **2**, 888-891.

[7] Boesen, C. and Johansen, C. (2008) Impact of psychotherapy on cancer survival: Time to move on? *Current Opinion in Oncology*, **20(4)**, 372-377.

[8] Stephen, J.E., Rahn, M., Verhoef, M. and Leis, A. (2007) What is the state of the evidence on the mind-cancer survival question, and where do we go from here? A point of view. *Support Care Cancer*, **15(8)**, 923-930.

[9] Andersen, B.L., Yang, H.C., Farrar, W.B., Golden-Kreutz, D.M., Emery, C.F., Thornton, L.M., Young, D.C. and Carson, W.E., 3rd (2008) Psychologic intervention improves survival for breast cancer patients: A randomized clinical trial. *Cancer*, **113(12)**, 3450-3458.

[10] Kuchler, T., Bestmann, B., Rappat, S., Henne-Bruns, D. and Wood-Dauphinee, S. (2007) Impact of psychotherapeutic support for patients with gastrointestinal cancer undergoing surgery: 10-year survival results of a randomized trial. *Journal of Clinical Oncology*, **25**, 2702-2708.

[11] Grulke, N., Bailer, H., Hertenstein, B., Kachele, H., Arnold, R., Tschuschke, V. and Heimpel, H. (2005) Coping and survival in patients with leukemia undergoing allogeneic bone marrow transplantation—long-term follow-up of a prospective study. *Journal of Psychosomatic Research*, **59(5)**, 337-346.

[12] Palesh, O., Butler, L.D., Koopman, C., Giese-Davis, J., Carlson, R. and Spiegel, D. (2007) Stress history and breast cancer recurrence. *Journal of Psychosomatic Research*, **63(3)**, 233-239.

[13] Beresford, T.P., Alfers, J., Mangum, L., Clapp, L. and Martin, B. (2006) Cancer survival probability as a function of ego defense (adaptive) mechanisms versus depressive symptoms. *Psychosomatics*, **47**, 247-253.

[14] Jones, D., Goldblatt, P.O. and Leon, D.A. (1984) Bereavement and cancer: Some results using data on deaths of spouses from the OPCD longitudinal study. *British Medical Journal*, **284**, 461-464.

[15] Johansen, C., Schou, G., Soll-Johanning, H., Mellemgaard, A. and Lynge, E. (1998) Marital status and survival in colorectal cancer. *Ugeskr Laeger*, **160(5)**, 635-638.

[16] Faller, H., Bülzebruck, H., Drings, P. and Lang, H. (1999) Coping, distress, and survival among patients with lung cancer. *Archives of General Psychiatry*, **56**, 756-762.

[17] Riehl-Emde, A., Buddeberg, C., Muthny, F.A., Landolt-Ritter, C., Steiner, R. and Richter, D. (1989) Causal attribuation and coping with the disease in breast cancer patients. *Psychother Psychological Medicine*, **39**, 232-238.

[18] Akechi, T., Okamura, H., Okuyama, T., Furukawa, T.A., Nishiwaki, Y. and Uchitomi, Y. (2009) Psychosocial factors and survival after diagnosis of inoperable non-small cell lung cancer. *Psychooncology*, **18(1)**, 23-29.

[19] Johansen, C. and Olsen, J.H. (1997) Psychosocial stress, cancer incidence and mortality from non-malignant diseases. *British Journal of Cancer*, **75(1)**, 144-148.

[20] Leissner, J., Hohenfellner, R., Thüroff, J.W. and Wolf, H.K. (2000) Lymphadenektomie in patients with transitional cell carcinoma of the urinary bladder: Significance for staging and prognosis. *BMU Int*, **85**, 817-823.

[21] Hardt, J., Petrak, F., Filipas, D. and Egle, U.T. (2004) Adaptation to life after surgical removal of the bladder—an application of Graphical Markov Models for analysing longitudinal data. *Statistics in Medicine*, **23**, 649-666.

[22] Muthny, F.A. and Koch, U. (1998) Spezifität der Krankheitsverarbeitung bei Krebs. In: Koch, U. and Weis, J., Eds., *Krankheitsbewältigung bei Krebs und Möglichkeiten der Unterstützung*, Stuttgart, Schattauer, 49-58.

[23] R Development Core Team: R: A language and environment for statistical computing. Vienna, R Foundation for Statistical Computing, 2007.

[24] Golka, K., Goebell, P.J. and Rettenmeier, A.W. (2007) Ätiologie und Prävention des Harnblasenkarzinoms. Deutsches Ärzteblatt, **104**, 719-723.

[25] Wolfson, C., Wolfson, D.B., Ashgarian, M., M'lan, C.E., Ostby, T., Rockwood, K. and Hogan, D.B. (2001) A reevaluation of the duration of survival after the onset of dementia. *New England Journal of Medicine*, **344(15)**, 1111-1116.

Vol.2, No.5, 435-440 (2010)
doi:10.4236/health.2010.25065

Effect of apigenin on the reproductive system in male mice

Hui Li, Hong-Bo Li, Ming Zhang, Fang Yan, Zhong-Xian Zhang, Zhi-Lan Li*

School of Public Health, Lanzhou University, Lanzhou, China; *Corresponding Author: lizhl@lzu.edu.cn

Received 23 November 2009; revised 7 January 2010; accepted 8 January 2010.

ABSTRACT

This study aimed to characterize the effect of apigenin on the reproductive system in male mice. Adult male mice were treated with intraperitoneal injection of apigenin at the dose levels of 5, 10, 15, 20 and 25 mg/kg.bw, 0.05% DMSO and 0.9% normal saline daily for seven days. Then, testis and epididymis sperms in sperm motility, sperm morphology, the percentages of ploidy cells and seminiferous epithelium cells at the cell-circle phase, and the ratio of ploidy cells were evaluated. The results showed that sperm density significantly reduced in the 25 mg/kg group compared with the solvent control group. The abnormal sperms were mainly amorphous; non-hook sperms took the second largest group; and banana, double-tail and folded-tail sperms were rare. Abnormal sperms were mainly in the head sperm. Moreover, after intraperitoneal injection of 5 mg/kg apigenin, the percentage of 1C population increased, and the percentage of 4C declined, leading to a significant increase of the 1C:4C ratio, compared with the solvent and negative control groups. The percentage of seminiferous epithelium cells at the cell-circle phase of G0/G1 exhibited a significant increase in the 25 mg/kg group compared with the control groups. Taken together, that apigenin has adverse effects on the reproductive system in adult male mice is demonstrated.

Keywords: Apigenin; Male Mice; Intraperitoneal Injection; Sperm Motility; Sperm Morphology; Flow Cytometry

1. INTRODUCTION

In the early 1970s, several studies in the United States first suggested a possible decline in human sperm concentration [1]. Since then, there has been increased awareness of the possible effects of chemicals on male fertility [2,3]. It is of paramount importance to assess potential health risks associated with exposure to chemical or physical agents since these agents may interfere with the ability of individuals to produce normal progeny. Apigenin is a common flavone present in diet: it is not only in aromatic plants (camomilla, rosemary and parsley), but also in celery, apple, honey, fennel and wheat germ [4-6].

Although apigenin is less active than its homologous isoflavone, genistein, much attention has been paid to its endocrine properties and potential effects on fertility recently. As a ligand of estrogen receptor [7,8], in vitro apigenin has estrogenic activity on the growth of transfected cells that are estrogen-dependent and have additive effects on 17-estradiol [6]. It has been shown that apigenin reduces the endogenous level of estrogen receptors in mouse uterus [9], enhances the estrogenicity of low-dose estradiol in immature rats [10], and has a protective effect on skin tumorigenesis, a hormonal-dependent cancer [11]. Moreover, anti-fertility properties of apigenin have been observed: apigenin is an active constituent of Striga orobanchioides, a medicinal plant with contraceptive properties [12]. Many potential mechanisms have been proposed to explain these (anti-) estrogenic and (anti-) carcinogenic properties including interaction with estrogen receptors [6,13], modulation of biosynthesis and metabolism of steroidhormones [14,15], enhancement of gap-junction intercellular communication, and apoptosis induction [16,17].

While the effect of apigenin on the reproductive system has been studied, to our best knowledge, the adverse effects of apigenin on sperm motility, sperm morphology and is still not very clear. Here these effects in male mice were investigated.

2. MATERIALS AND METHODS

2.1. Test Materials

Apigenin was obtained from Shanxi Hui Ke Plant Ex-

This work was supported by the research foundation for the young and the middle-aged scientist in Gansu Province, China (Grant No. 099RJYA003).

ploitation Limited Company (China), whose purity was 98.00%. Dimethyl sulfoxide (DMSO) was purchased from Shanxi Hua Mei Bioengineering Company (China). All other chemicals were obtained from standard commercial sources and were with the highest available quality.

2.2. Animals

Eight-four healthy, adult male SPF mice of Kunming strain, whose body weights ranged from 32 to 35 g, approximately seven weeks old, were obtained from Experimental Animal Center, Gansu College of Traditional Chinese Medicine, Lanzhou, Gansu, China (Animal Certificate of Quality No: SCXK [Gan]: 2004-0006-0000 328). The use of these animals was approved by the Chinese Association for Laboratory Animal Sciences. They were housed in the GLP laboratory (Laboratory Certificate of Quality No: SCXK [Gan]: 2004-0006-000 0328-0000120), where the temperature of 25°C, the relative humidity of approximately 50%, and a photoperiod of 12 h light: 12 h dark were maintained. All animals were housed on sawdust beds in cages and given the standard diet and distilled water of ad libitum during the whole study.

2.3. Chemicals and Experimental Design

A stock solution of 1.88 mg/ml apigenin was prepared by dissolving 376 g apigenin in 1ml DMSO and 199 ml 0.9% normal saline (NS). The solvent was 0.05% DMSO, and different concentrations of apigenin were prepared after the dilution with 0.05% DMSO. According to their body weights, male mice were randomly assigned to seven groups (12 animals per group) including one solvent group, one negative control group, and five experimental groups. The solvent and negative control groups were given 0.05% DMSO and 0.9% NS by intraperitoneal injection, respectively. The five experimental groups were given apigenin at the dose levels of 5, 10, 15, 20 and 25 mg/kg.bw once a day for seven consecutive days. Solution concentrations were adjusted so that a 30 g mouse received a solution of 0.4 ml. The body weights were recorded since the start of dosing, then once every two days, until the day of necropsy. The actual dose volume was adjusted according to the recorded body weight. Male mice were killed after cervical dislocation on the day following the last injection. On the day of sacrifice, the body weight was recorded. Immediately after sacrifice, both testes and epididymes were excised, trimmed free fat, placed on a paper towel to remove any liquid, and then weighted separately.

2.4. Sperm Motion Analysis

Sperm motion was analyzed with the WLJY-9000 sperm quantity detection system (Beijing Wei Li New Century Science and Technology Development Limited Company, China).The left epididymes were excised and placed in a pre-warmed peridish (37°C) consisting of 1 ml NS (0.86% sodium chloride). The tissue was made three cuts in the mid-to-distal region with a scalpel blade to release spermatozoa into the medium, which was then placed in a 37°C thermostatic water-bath box for 30 min prior to the measurement of sperm motility. The suspension was stirred, and 10 μl was placed on a clean counter board, mounted with a special cover lip, and then observed with four to six microscope fields. With the sperm quantity detection system, the percentages of mobile sperms, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), mean moving angle (MAD), amplitude of lateral displacement (ALH), beat cross frequency (BCF), linearity (LIN), straightness (STR), wobble (WOB)and sperm density were calculated, respectively.

2.5. Sperm Morphology Analysis

The right epididymes were minced with an eye scissor in 3 ml NS (0.86% NaCl), repetitively beaten by pipette, kept for 5 min and then filtered with four-layer lens paper. The sperm morphology was assessed by smearing the sperm suspension on a pre-cleaned slide with a drop from the filtrate. Once air-dried, the samples were fixed with methanol for 15 min, stained with 2% eosin for 1h, rinsed thoroughly with distilled water and dried in air. For each sample, 1,000 sperms from different fields were observed under 40 × magnification with a light microscope (Olympus, Japan), and according to the description of Huang *et al.* [18], classified as normal, amorphous, non-hook, banana, double-tail and folded-tail.

2.6. Cell Cycle Analysis

The right testis was surgically removed, stripped of adhering fat and connective tissues, and weighted. The germ cells were released from seminiferous tubules in cold phosphate buffered saline (PBS) at pH 7.4 by mincing the testes with fine curved scissors. The cell suspension was aspirated with PBS and filtered in a 200 mesh nylon mesh in order to remove tissue debris. The suspension was centrifuged at 1,500 rpm for 5 min. The supernatant was discarded, and the pellet was re-suspended in 10 ml PBS, and then centrifuged at 800 rpm for 5 min. After rinsing three times as above, the pellet was fixed in 75% ethanol and stored at 4°C. After 24 h, the fixed germ cells were washed with PBS to remove ethanol, and then the suspension was centrifuged at 1,500 rpm for 5 min. The supernatant was discarded, and the pellet was stained with Propidium iodide (PI) in darkness for 30 min. The pellet was filtered in a 320 mesh nylon mesh to remove cell mass. The filtrates were detected with flow cytometry (Epics. COULTER XL, USA).

2.7. Statistical Analyses

Statistical analyses were performed with SPSS (version15.0). Body weights, organ indexes, sperm motility parameters, the percentages of cell subpopulation were analyzed by one-way ANOVA, and the obtained data were expressed as mean ± SD. For parameters with significant differences among groups, multiple comparison tests were carried out. The percentages of sperm morphology were analyzed by the $\chi 2$ test, and the data were expressed as the constituent ratio. The parameters values were compared at the 5% significance level.

3. RESULTS

3.1. Effect of Apigenin on Body and Reproductive Organ Weights

The body weights of mice in seven groups increased at different levels, but compared with the solvent and negative control groups, none of the experimental groups showed statistically significant differences in body weight, relative testes and epididymides weight (**Table 1**).

3.2. Effect of Apigenin on Sperm Motion and Density

The mouse sperm densities in five experimental groups reduced at different levels, and compared with the solvent control group, there was a statistically significance in the 25 mg/kg group (**Table 2**).

3.3. Effect of Apigenin on Sperm Morphology

The mean percentage of abnormal sperms ranged from 2.69% to 3.16% in the experimental groups. The five experimental groups did not significantly differ from each other or the control groups. Furthermore, abnormal sperms were mainly classified as amorphous; non-hook sperm took the second largest group; banana, double-tail and folded-tail sperms were rare, which were mainly concentrated in the head sperms.

3.4. Effect of Apigenin on Cell Cycle

After injection of 5 mg/kg apigenin, the percentage of 1C population increased significantly compared with the other groups; that of 4C decreased compared with the negative control group; and a significant increase of 1C:4C ratio was observed compared with the control groups.

Compared with the solvent and negative control groups, the percentage of seminiferous epithelium cells at the cell-circle phase of G0/G1 exhibited a significant increase in the 25 mg/kg group.

4. DISCUSSION

The study on apigenin investigated whether apigenin had any toxic effect on the reproductive system in adult male mice. Intraperitoneal injection of apigenin for seven days to mice led to no obvious change in their body weights. The relative weights of testis and epididymis showed no marked changes as well, when normalized with the whole body weights.

Sperm motion is important for the sperm functional capacity, and the assessment of sperm motion is very useful for detecting or evaluating male reproductive toxicity [19]. For this purpose, both percentage of mobile spermatozoa and characteristics of sperm movement might provide key information. More quantitative and qualitative evaluation of toxic effects on sperm motion (e.g., motility) has been possible with the computer-assisted sperm analysis (CASA) system. Besides the conventional parameters, the system also describes sperm kinematics movements [20-27]. In this study,the results showed that compared with the solvent control group, sperm density had a significantly reduction in the 25 mg/kg group was shown, which is consistent with the results of Pu *et al.* [28]. On the other side, others parameters did not show significant variation in any experimental group. Taken together, our results suggested that apigenin had some effects on sperm motion parameters in mice.

Sperm morphology is another important aspect in assessing sperm quality as well as a key index to evaluate reproductive toxicity and mutagenicity of exogenous chemicals [29]. The results showed that abnormal sperms were mainly concentrated in the head sperm, which is consistent with the observation that the changes of sperm morphology are mainly in the head.

In recent years, the flow cytometry analysis has grown rapidly, which allows the recognition of several cell types at various stages of spermagenesis [30,31]. In particular, after treatment with toxic agents, the flow cytometry analysis of testicular tissue can be used to detect variations in terms of the relative fractions of different cell subpopulations, thus providing crucial evidence on possible toxic effects [32]. In this study, the percentages of different germ cell types in male mice as a function of dose after the treatments with different doses of apigenin were reported. Based on the DNA content, three germ-cell peaks could be identified through flow cytometry: 1C (round spermatids), 2C (spermatogonia) and 4C (primary spermatocytes). After intraperitoneal injection of 5 mg/kg apigenin, the percentage of 1C population increased, the percentage of 4C decreased and so the 1C:4C ratio significantly increased, indicating that primary spermatocytes decreased but round spermatids increased. This observation suggested that apigenin stimulated the meiosis of spermatogonia, and the effect

of apigenin in male mice occurred at the last stage of spermagenia. Moreover, compared with the control groups, the sperm density and quality of this dose group did not show any significant change.

Compared with the solvent and negative control groups, the percentage of seminiferous epithelium cells at the cell-circle phase exhibited a significant increase in the 25 mg/kg group. This indicated that the dose of 25 mg/kg can slow the proliferation speed of germ cells, and spermatogonia were blocked in G0/G1. This inhibition of spermatogonia is consistent with the decreased sperm density in the group.

In conclusion, the results show that as a single agent, apigenin can produce adverse effects on the reproductive system in adult male mice at a dose of 25 mg/kg/day. Thus, more efforts are required to elucidate the mechanisms related the apigenin effects on sperm quality and spermatogenesis.

Table 1. Body and organ weights (mean ± s.d.).

	Apigenin (mg/kg/d)					0.05% DMS0	0.9% N.S
	5	10	15	20	25		
weight change (g)	3.86 ± 1.64	3.81 ± 2.62	4.53 ± 1.87	4.03 ± 1.25	4.15 ± 1.63	4.58 ± 1.37	4.42 ± 1.97
Relative organ weights (g/g.bw)							
Testes	5.73 ± 0.74	5.42 ± 0.93	5.26 ± 0.54	5.51 ± 0.74	5.51 ± 0.76	6.22 ± 0.93	5.64 ± 0.78
Epididymides	2.00 ± 0.25	1.81 ± 0.21	1.87 ± 0.31	1.79 ± 0.28	1.87 ± 0.30	1.76 ± 0.31	1.87 ± 0.23

Table 2. Sperm motility in male mice treated with apigenin (n = 12/group; mean ± s.d.).

	Apigenin (mg/kg/d)					0.05% DMS0	0.9% N.S
	5	10	15	20	25		
a-grade sperm	7.52 ± 5.97	6.08 ± 3.86	9.66 ± 5.86	9.42 ± 6.05	10.01 ± 4.73	9.88 ± 5.63	7.72 ± 3.26
b-grade sperm	14.19 ± 5.32	13.24 ± 6.77	10.48 ± 5.28	12.90 ± 5.78	16.44 ± 5.35	14.99 ± 4.90	14.22 ± 4.57
c-grade sperm	42.63 ± 15.77	46.36 ± 7.28	41.00 ± 11.60	36.85 ± 12.37	38.70 ± 17.02	40.93 ± 8.24	37.38 ± 8.07
d-grade sperm	35.66 ± 20.03	34.32 ± 13.01	38.85 ± 16.36	40.72 ± 10.07	34.85 ± 18.56	34.19 ± 13.21	40.72 ± 10.72
a + b sperm	20.71 ± 8.81	19.32 ± 8.81	19.37 ± 8.96	22.42 ± 9.11	26.45 ± 10.00	24.87 ± 8.90	21.9 ± 5.94
a + b + c sperm	64.34 ± 20.03	65.68 ± 13.01	61.10 ± 16.37	59.27 ± 10.07	65.15 ± 18.56	65.85 ± 13.21	59.28 ± 10.72
VCL(μm/s)	55.80 ± 10.11	51.94 ± 4.73	54.28 ± 9.01	55.21 ± 7.90	60.02 ± 6.68	55.52 ± 6.46	58.56 ± 8.51
VSL(μm/s)	17.19 ± 6.11	15.20 ± 4.60	18.14 ± 6.64	19.57 ± 5.62	20.22 ± 4.43	18.70 ± 5.31	18.61 ± 3.70
VAP(μm/s)	24.27 ± 6.51	21.23 ± 4.39	24.31 ± 7.06	26.14 ± 4.98	26.74 ± 4.61	24.72 ± 5.19	25.15 ± 3.58
MAD(deg)	68.87 ± 8.76	71.40 ± 5.79	68.65 ± 8.08	69.03 ± 5.83	69.52 ± 6.93	69.00 ± 6.28	69.53 ± 6.26
ALH(μm)	1.87 ± 1.02	1.81 ± 0.81	1.83 ± 0.84	2.12 ± 0.80	2.64 ± 1.10	2.23 ± 0.92	2.57 ± 0.68
BCF(Hz)	10.54 ± 1.82	10.59 ± 3.44	9.38 ± 2.81	10.30 ± 1.37	9.95 ± 1.57	10.04 ± 1.58	9.82 ± 0.69
LIN	28.93 ± 6.10	28.17 ± 6.03	29.94 ± 6.12	32.75 ± 7.85	31.08 ± 6.49	31.47 ± 4.37	29.44 ± 4.00
WOB	44.57 ± 3.54	42.89 ± 3.73	46.60 ± 4.12	48.30 ± 4.73	46.17 ± 4.68	46.57 ± 2.30	45.03 ± 3.89
STR	63.33 ± 8.85	63.66 ± 9.57	63.42 ± 7.75	63.15 ± 9.31	68.27 ± 10.57	66.68 ± 6.76	66.96 ± 5.34
Sperm density ($\times 10^6$/ml)	1.25 ± 0.82	1.15 ± 0.38	1.51 ± 0.67	1.48 ± 0.85	0.96 ± 0.48★	2.22 ± 1.50	1.92 ± 0.66

Note: ★significantly different from the solvent control group at $P < 0.05$.
a: fast progressive motility
b: slow or dull progressive motility
c: non-progressive motility
d: immobility
a + b: progressive motility
a + b + c: total sperm motility

Table 3. Sperm morphology (n = 12/group; mean ± s.d.).

	Apigenin (mg/kg/d)					0.05% DMS0	0.9% N.S
	5	10	15	20	25		
Total count	8000	10000	9000	7000	5000	9000	7000
Abnormal	215(2.69)	292(2.92)	264(2.93)	221(3.16)	156(3.12)	251(2.79)	165(2.36)
Amorphous	173(80.47)	232(79.45)	195(45.44)	185(83.37)	120(76.92)	205(81.67)	124(75.15)
Non-hook	36(16.74)	49(21.12)	66(25.00)	31(14.02)	34(21.79)	43(17.13)	35(21.21)
Banana	1(0.47)	1(3.13)	1(0.38)	3(1.36)	0(0.00)	0(0.00)	5(3.03)
Double-tail	5(2.33)	10(31.30)	2(0.76)	2(0.90)	1(0.64)	3(1.82)	1(0.61)
Folded-tail	0(0.00)	0(0.00)	0(0.00)	0(0.00)	1(0.64)	0(0.00)	0(0.00)

Table 4. Sperm motility parameters t (mean ± s.d.).

	Apigenin (mg/kg/d)					0.05% DMS0	0.9% N.S
	5	10	15	20	25		
percentages of ploidy cells							
1C	86.23 ± 2.60■	77.50 ± 4.66	75.90 ± 2.12	76.68 ± 2.37	80.20 ± 1.15	77.13 ± 4.49	76.43 ± 5.12
2C	7.72 ± 1.07	11.35 ± 2.32	13.53 ± 0.05	11.69 ± 2.24	11.25 ± 0.66	10.86 ± 4.00	12.32 ± 2.75
4C	4.085 ± 0.46★	7.281 ± 1.85	6.469 ± 2.44	5.270 ± 0.63	5.282 ± 1.56	6.048 ± 2.30	6.731 ± 1.78
AP	1.925 ± 1.66	1.060 ± 1.50	0.000 ± 0.00	3.833 ± 4.25	3.033 ± 1.91	2.125 ± 4.25	0.875 ± 1.75
percentages of seminiferous epithelium cells in cell circle phase							
G0/G1	65.30 ± 2.01	54.10 ± 6.19	56.35 ± 4.74	60.67 ± 5.75	67.60 ± 5.84◆	53.60 ± 6.22	55.93 ± 10.4
S-phase	0.00 ± 0.00	11.68 ± 8.24	17.20 ± 3.11	11.23 ± 10.15	1.33 ± 1.19	16.60 ± 6.99	14.13 ± 9.63
G2/M	34.70 ± 2.01	34.26 ± 2.24	26.50 ± 7.78	28.07 ± 7.17	31.07 ± 4.95	29.80 ± 2.69	29.93 ± 3.58
ratio of ploidy cells							
1C:2C	11.39 ± 1.97	7.14 ± 1.93	5.61 ± 0.18	6.73 ± 1.32	7.14 ± 2.95	8.61 ± 5.50	6.51 ± 1.84
1C:4C	21.36 ± 2.94▲	11.39 ± 3.72	12.70 ± 5.11	14.73 ± 2.36	16.09 ± 4.71	15.37 ± 9.38	12.40 ± 5.28
4C:2C	0.53 ± 0.05	0.63 ± 0.04	0.48 ± 0.18	0.46 ± 0.12	0.47 ± 0.11	0.56 ± 0.08	0.55 ± 0.14

Note: ■significantly different from others groups at $P < 0.05$.
★significantly different from the NS control group at $P < 0.05$.
▲significantly different from the NS and 0.05% DMSO control groups at $P < 0.05$.
◆significantly different from the NS and 0.05% DMSO control
1C: haploidcell (round spermatids)
2C: diploidcell (spermatogonia)
4C: Tetraploidcell, (primary spermatocytes)

5. ACKNOWLEDGEMENTS

This work was supported by the research foundation for the young and the middle-aged scientist in Gansu Province, China (Grant No. 099RJYA003). We thank lab members An Jing, Yu-Hui Dang, Chen Ya and Shu-Yu Liu for their assistance. We also thank Dr. Si-Wu Fu and Dr. Xing-Rong Liu for critical reading the manuscript.

REFERENCES

[1] Nelson, C.M.K. and Bunge, R.G. (1974) Semen analysis: Evidence for changing parameters of male fertility potential. *Fertility and Sterility*, **25(6)**, 503-507.

[2] Carlsen, E., Giwereman, A., Keiding, N. and Skakkebaek, N.E. (1992) Evidence fordecreasing quality of semen during past 50 years. *British Medical Journal*, **305(6854)**, 609-613.

[3] Sharpe, R.M. (1993) Declining sperm counts in men—is there an endocrine cause? *Journal of Endocrinology*, **136**, 357-360.

[4] Hertog, M.G., Hollman, P.C. and Katan, M.B. (1992) Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the

Netherlands. *Journal of Agricultural and Food Chemistry*, **40(12)**, 2379-2383.

[5] Havsteen, B.H. (2002) The biochemistry and medicinal significance of the flavonoids. *Pharmacology & Therapeutics*, **96(2-3)**, 67-202.

[6] Le Bail, J.C., Varnat, F., Nicolas, J.C. and Habrioux, G. (1998) Estrogenic and antiproliferative activities on MCF-7 human breast cancer cells by flavonoids. *Cancer Letters*, **130(1)**, 209-216.

[7] Breinholt, V.M., Offord, E.A., Brouwer, C., Nielsen, S.E., Brosen, K. and Friedberg, T. (2002) In vitro investigation of cytochrome P450-mediated metabolism of dietary flavonoids. *Food and Chemical Toxicology*, **40(5)**, 609-616.

[8] Havsteen, B.H. (2002) The biochemistry and medicinal significance of the flavonoids. *Pharmacology & Therapeutics*, **96(2-3)**, 67-202.

[9] Breinholt, V., Hossaini, A., Svendsen, G.W., Brouwer, C. and Nielsen, E. (2000) Estrogenic activity of flavonoids in mice. The importance of estrogen receptor distribution, metabolism and bioavailability. *Food and Chemical Toxicology*, **38(7)**, 555-564.

[10] Stroheker, T., Cabaton, N., Berges, R., Lamothe, V., Lhuguenot, J.C. and Chagnon, M.C. (2003) Influence of dietary soy isoflavones on the accessory sex organs of the Wistar rat. *Food and Chemical Toxicology*, **41(8)**, 1175-1183.

[11] Birt, D.F., Hendrich, S. and Wang, W. (2001) Dietary agents in cancer prevention: Flavonoids and isoflavonoids. *Pharmacology & Therapeutics*, **90(2-3)**, 157-177.

[12] Hiremath, S.P., Badami, S., Hunasagatta, S.K. and Patil, S.B. (2000) Antifertility and hormonal properties of flavones of Striga orobanchioides. *European Journal of Pharmacology*, **391(1-2)**, 193-197.

[13] Miksicek, R.J. (1995) Estrogenic flavonoids: Structural requirements for biological activity. *Proceedings of the Society for Experimental Biology and Medicine*, **208(1)**, 44-50.

[14] Dai, R., Jacobson, K.A., Robinson, R.C. and Friedman, F.K. (1997) Differential effects of flavonoids on testosterone-metabolizing cytochrome P450s. *Life Sciences*, **61**, L75-L80.

[15] Ibrahim, A.R. and Abul-Hajj, H.Y. (1990) Aromatase inhibition by flavonoids. *Journal of Steroid Biochemistry and Molecular Biology*, **37(2)**, 257-260.

[16] Suschetet, M., Siess, M.H., Le Bon, A.M. and Canivenc-Lavier, M.C. (1998) Anticarcinogenic properties of some flavonoids, In: Vercauteren, J., Che`se, C. and Triaud, J. Eds., *Polyphenols* 96, INRA Editions, Versailles, 165-204.

[17] Wang, I.K., Lin-Shiau, S.Y. and Lin, J.K. (1999) Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukae mia HL-60 cells. *European Journal of Cancer*, **35(10)**, 1517-1525.

[18] Huang, X. and Chen, X. (1985) Methodology of mutagenic, teratogenic and carcinogenic environmental chemicals, Zhejiang Science & Technology Press, Hangzhou, 64.

[19] Perreault, S.D. (1997) The mature spermatozoa as a target for reproductive toxicants. In: Oekelheide, K., Chapin, R.E., Hoyer, P.B. and Harris, C., Eds., *Comprehensive toxicology, Reproductive and Endocrine Toxicology*, **10**, 165-179.

[20] Seed, J., Chapin, R.E., Clegg, E.D., Dostal, L.A., Foote, R.H., Hurtt, M.E., *et al.* (1996) Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: A consensus report. *Reproductive Toxicology*, **10(3)**, 237-244.

[21] Slott, V.L., Suarez, J.D. and Perreault, S.D. (1991) Rat sperm motility analysis: Methodologic considerations. *Reproductive Toxicology*, **5(5)**, 449-458.

[22] Toth, G.P., Stober, J.A., George, E.L., Read, E.J. and Smith, M.K. (1991) Sources of variation in the computer-assisted motion analysis of rat epididymal sperm. *Reproductive Toxicology*, **5(6)** 487-495.

[23] Chapin, R.E., Filler, R.S., Gulati, D., Heindel, J.J., Katz, D.F., Mebus, C.A., *et al.* (1992) Methods for assessing rat sperm motility. *Reproductive Toxicology*, **6(3)**, 267-273.

[24] Slott, V.L., Suarez, J.D., Poss, P.M., Linder, R.E., Strader, L.F. and Perreault, S.D. (1993) Optimization of the Hamilton-Thorn computerized sperm motility analysis system for use with rat spermatozoa in toxicological studies. *Fundamental and Applied Toxicology*, **21(3)**, 298-307.

[25] Slott, V.L., Linder, R.E. and Dyer, C.J. (1994) Method of euthanasia does not affect sperm motility in the laboratory rat. *Reproductive Toxicology*, **8(4)**, 371-374.

[26] Wier, P.J. and Rumberger, D. (1995) Isolation of rat sperm from the vas deferens for sperm motion analysis. *Reproductive Toxicology*, **9(3)**, 327-330.

[27] Dostal, L.A., Faber, C.K. and Zandee, J. (1996) Sperm motion parameters in vas deferens and cauda epididymal rat sperm. *Reproductive Toxicology*, **10(3)**, 231-235.

[28] Pu, Y., Li, Z., Zhao, J., Wang, X., Dang, Y. and An, J. (2007) The experimental study on the effect of celery on sperm quality in mice. *Health Vocational Education*, **25**, 122-124.

[29] Wang, H., Meng, Z. and Chang, F. (2006) The toxic effects of in vivo derivatives of sulfur dioxide on sperms in male mice. *Journal of Application and Environmental Biology*, **12**, 363-366.

[30] Petim, J.M., Ratinaud, M.H., Cordelli, E., Spano, M. and Julien, R. (1995) Mouse testis cell sorting according to DNA and mitochondrial changes during spermatogenesis. *Cytometry*, **19(4)**, 304-312.

[31] Suter, L., Bechter, R., Koch, E. and Bohadilla, M. (1998) Three parameter flow cytometric analysis of rat spermatogenesis. *Cytometry*, **27(2)**, 161-168.

[32] Spano, M., Baroleschi, C., Cordelli, E., Leter, G., Tiveron, C. and Pacchierotti, F. (1996) Flow cytometric assessment of trophosophamide toxicity on mouse spermatogenesis. *Cytometry*, **24**, 174.

Vol.2, No.5, 441-447 (2010)
doi:10.4236/health.2010.25066

Blood lipids may have influence on the emotional well-being in young men

Edyta Kramek, Sylwia Jastrzebska, Renata Walczak-Jedrzejowska, Katarzyna Marchlewska, Elzbieta Oszukowska, Anna Guminska, Krzysztof Kula, Jolanta Slowikowska-Hilczer*

Department of Andrology and Reproductive Endocrinology, Medical University of Lodz, Lodz, Poland;
*Corresponding Author: jolanta.slowikowska-hilczer@umed.lodz.pl

Received 14 January 2010; revised 4 February 2010; accepted 6 February 2010.

ABSTRACT

Anamnestic data on general health and medical conditions were achieved from 136 men (20-49 yrs). Beck Depression Inventory II (BDI-II) questionnaire was used to assess depressive symptomatology. Body weight, height, waist and hip circumference, arterial blood pressure were measured. Serum levels of total cholesterol (TC), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), triglicerydes (TG), glucose, SHBG, total testosterone, DHEA-S and estradiol were determined. Calculated were body mass index (BMI), waist to hip ratio (WHR) and free testosterone index (FTI). In men aged 40-49 general health significantly got worse, BMI, WHR, blood pressure increased and mean serum levels of FTI, DHEA-S, estradiol decreased in comparison to younger men. Only in 40-49 age band BDI-II scoring was negatively related with FTI, however, in the whole group there were no significant correlations. Nevertheless, some symptoms of depression were negatively related with LDL-C or HDL-C and positively with TG. Similar relations were found among young men, but not in the middle-aged. Conclusions: Only blood lipids may have influence on emotional well-being in young healthy men. The decreased testosterone level becomes probably the main risk factor for the lower mood in middle-aged men. Atherosclerosis risk factors and general health worsen with the advancing age, but they have no significant effect on psychological situation.

Keywords: Man; Depression; Sex Steroids; Lipids; General Health

1. INTRODUCTION

There are evidences of the relationship between hormonal changes in menstrual cycle and changes in mood in women [1,2]. Recent long-term, prospective studies have demonstrated that the transition to menopause is associated with higher risk for new onset and recurrent depression [3]. In men, however, interrelationship between the emotional state and sex steroids serum levels are controversial. Low testosterone serum level in hypogonadal men is characterised by depressed mood and emotional instability [4,5]. Androgen deficiency in the aging male, also referred to as late-onset hypogonadism (LOH), is a clinical and biochemical syndrome associated with advancing age and deficiency in serum testosterone levels below the young healthy adult male reference range [6]. It includes, among other symptoms, depressed mood, decreased vitality and low sex drive [7,8]. Several studies showed the significant improvement in the emotional well-being after testosterone [9-11] or dehydroepiandrosterone sulphate (DHEA-S) administration in hypogonadal men [12,13]. Nevertheless, physiologic studies were usually not successful in demonstrating a significantly negative relation of androgens with lower mood, especially in young men [14,15].

Other evidences indicate that vascular disease may cause depression and thus cerebral atherosclerosis may be responsible for late-life depressed mood [16,17]. On the other side, several studies assessed the negative association between serum levels of total cholesterol or LDL-cholesterol and depressive symptoms, what denies the “vascular theory” [18,19].

The aim of our study was to assess if emotional well-being in young men demonstrates a relationship with sex hormones or atherosclerosis risk factors in comparison to middle-aged men.

2. METHODS

2.1. Subjects and Study Design

The study was performed after the approval of the Bioethical Committee of the Medical University in Lodz,

Poland. A group of 300 men was randomly recruited from the Lodz's city population register for participation in this study. The only criterion of the selection was age between 20-49 years. Subjects were invited by a letter to attend for a screening visit at the Department of Andrology and Reproductive Endocrinology. The overall response rate for participation was 46%. Men who agreed to participate in the study (136 subjects, mean age 35.5 ± 10 years) were divided into age bands: 20-29 (44 subjects), 30-39 (36 subjects) and 40-49 (56 subjects).

Subjects were asked to complete an interviewer- assisted questionnaire gathered information on sociodemographic, medical conditions and medications. General health was scored from 0, when very good, to 3, when very bad.

Although it is not possible to do it concisely, but we defined emotional well-being as the capacity to live a full and creative life, and the flexibility to deal with life's inevitable challenges. Overall it means the lack of depression symptoms [20]. To measure depressive symptomatology the Beck Depression Inventory II (BDI-II) questionnaire was used [21]. BDI-II contains 21 questions concerning: 1) sadness, 2) pessimism, 3) past failures, 4) loss of pleasure from things a man used to enjoy, 5) feelings of guilty, 6) feelings of punishment, 7) self-dislike, 8) self-criticism, 9) suicidal thoughts and wishes, 10) crying, 11) agitation, 12) loss of interest in other people or activities, 13) indecisiveness, 14) worthlessness, 15) loss of energy, 16) changes in sleeping pattern, 17) irritability, 18) changes in appetite, 19) concentration difficulty, 20) tiredness or fatigue, and 21) loss of interest in sex during past two weeks including a day of meeting. Each answer was scored on a scale value of 0 to 3. Cutoffs used: 0-13—lack or minimal depression, 14-19—mild depression, 20-28—moderate depression, 29-63—severe depression.

Body weight and height, waist and hip circumference and arterial blood pressure were measured using standard procedures. Calculated were body mass index (BMI = weight/height^2) and waist to hip ratio (WHR).

2.2. Hormone and Lipid Measurements

Single venous blood samples were taken in the morning hours (8.00-10.00) in a fasting state, 12 hours after the last meal. Serum was separated and stored at –80^{o}C until assayed at the end of the study, not longer than 6 months.

Serum determinations of total testosterone, DHEA-S, estradiol and sex hormone binding globulin (SHBG) were performed. All hormones were measured using chemiluminescence immunoassay (Immulite 1000, DPC, USA). Detection limits were for: total testosterone—0.5 nmol/L, DHEA-S—3 µg/dl, estradiol—20 pmol/L and SHBG—0.2 nmol/L. Free testosterone index (FTI) was calculated as following: total testosterone/SHBG × 100.

Serum levels of total cholesterol (TC, normal range: 3.0-5.2 mmol/L), LDL cholesterol (LDL-C, normal range: < 2.6 mmol/L), HDL cholesterol (HDL-C, normal range: > 1.0 mmol/L), triglicerydes (TG, normal range: < 1.7 mmol/L), and glucose (normal range: 3.3-5.5 mmol/L) were determined with the use of enzymatic methods (Cobas Integra 800, Roche Diagnostics, Poland).

2.3. Statistical Analysis

All statistical analyses were performed using Statistica for Windows PL software, version 8.0 (Statsoft, Cracow, Poland). Mean ± standard deviation (SD) and median values have been used to express group data. Nonparametric analysis (Mann-Whitney U-test) was applied for comparison between groups after verification of values distribution (ANOVA). Correlations were examined using Spearman's linear regression analysis. $P < 0.05$ was considered significant.

3. Results

Table 1 presents self-reported health and physical status. General health significantly got worse in men aged 40-49 in comparison to younger ones. Significantly increased BMI, WHR, systolic and diastolic blood pressure were found also in this age band. One or more morbidities were reported by 16 men. The most frequent

Table 1. Results of physical and self-reported health characteristics.

	Age band (years)		
	20-29 *n* = 44	30-39 *n* = 36	40-49 *n*= 56
General health (score)	1.1 ± 1.5	1.8 ± 1.5	2.0 ± 1.3[a]
BMI (kg/m^2)	24.7 ± 3.6	26.1 ± 3.7[c]	26.9 ± 3.9[a]
WHR	0.88 ± 0.1	0.92 ± 0.1	0.96 ± 0.1[a]
Systolic BP	131.5 ± 12.8	132.1 ± 13.5	142.9 ± 20.4[a]
Diastolic BP	76.9 ± 9.1	78.0 ± 9.6	88.3 ± 12.9[a]
Self-reported morbidities:			
Hypertension	1	1	4
Arrhythmia	0	0	1
CAD	0	0	1
GIU	0	0	8
Neurosis	0	0	1
Depression	0	0	1
BPH	0	0	1

[a]$p < 0.001$; [c]$p < 0.05$ vs. 20-29 age band; Mann-Whitney U-test; n – number of subjects; BMI – body mass index; WHR – waist to hip ratio; BP – blood pressure; CAD – coronary artery disease; GIU – gastrointestinal ulcer; BPH – benign prostate hyperplasia

were gastrointestinal ulcer (50%) and hypertension (37.5%). Nobody used antidepressants, psychotropic drugs, narcotics or lipid-lowering medications. None was denoted as alcoholic or drug addict.

Mean serum levels of DHEA-S, estradiol, as well as FTI, were significantly decreased, while SHBG increased, in men aged 40-49 in comparison to men aged 20-29 as shown in **Table 2**. In men 30-39 years old

Table 2. Results of hormonal determinations and serum levels of lipids in men between 20 and 49 years of age.

Hormones and lipids		Age band (years)		
		20-29 n = 44	30-39 n = 36	40-49 n = 56
TT (nmol/L)	x ± SD	18.5 ± 4.1	18.4 ± 6.4	18.3 ± 5.7
	median	17.9	18.7	18.0
	range	8.4-28.0	7.5-34.6	7.4-35.6
FTI	x ± SD	84.5 ± 30.4	71.8 ± 26.7[c]	45.5 ± 13.8[a]
	median	76.6	66.1	44.5
	range	27.1-168.9	31.9-160.2	23.1-83.9
DHEA-S (μg/dl)	x ± SD	287.3 ± 107.7	259.0 ± 102.3	246.5 ± 89.7[c]
	median	251.5	233.0	229.5
	range	107.0-572.0	129.0-572.0	88.4-456.9
Estradiol (pmol/L)	x ± SD	124.5 ± 41.1	100.3 ± 36.9[b]	90.0 ± 26.1[a]
	median	117.3	93.5	87.6
	range	45.5-208.8	44.1-178.1	45.3-164.4
SHBG (nmol/L)	x ± SD	24.0 ± 8.4	27.8 ± 12.8	43.3 ± 19.4[a]
	median	22.7	25.4	39.4
	range	8.9-49.9	12.5-69.5	15.4-106.0
TC (mmol/L)	x ± SD	4.2 ± 0.9	4.8 ± 1.0[b]	6.1 ± 0.9[a]
	median	4.1	4.6	6.2
	range	2.6-7.5	3.0-7.5	3.8-7.5
HDL-C (mmol/L)	x ± SD	1.5 ± 0.3	1.5 ± 0.4	1.6 ± 0.2[c]
	median	1.4	1.5	1.6
	range	0.9-2.3	0.9-2.4	1.2-2.6
LDL-C (mmol/L)	x ± SD	2.2 ± 0.8	2.7 ± 0.9[c]	3.5 ± 1.2[a]
	median	2.2	2.5	3.3
	range	1.0-5.0	1.2-5.3	1.2-6.1
TG (mmol/L)	x ± SD	1.1 ± 0.7	1.5 ± 1.2	1.9 ± 1.2[a]
	median	0.9	1.1	1.5
	range	0.4-4.4	0.3-5.7	0.8-5.8

[a]$p < 0.001$; [b]$p < 0.01$; [c]$p < 0.05$ vs. 20-29 age band, Mann-Whitney U-test; n – number of subjects, x - mean value, SD - standard deviation; TT – total testosterone; FTI – free testosterone index; DHEA-S – dehydroepiandrosterone sulphate; SHBG – sex hormone binding globuline; TC – total cholesterol; HDL-C – HDL cholesterol; LDL-C – LDL – cholesterol; TG – triglycerides

significantly decreased were only FTI and estradiol. The mean serum levels of total testosterone did not differ significantly between the age groups.

Serum levels of all lipids were significantly increased in the oldest group of men in comparison to 20-29 age band. In those aged 30-39 significantly increased were TC and LDL-C. The level of glucose was within the normal range in all of the subjects.

It was recognised that 12 men (8.8%) had mild depression (**Table 3**). One men from the youngest age band presented symptoms of severe depression. He had normal levels of all studied parameters (FTI-121.5). In 4 men aged 40-49 moderate depression was diagnosed, while in none of younger. All of them had low FTI (28.3-51.9) and increased TC (5.3-7.5 mmol/L). However, all of the subjects with more intensive depression reported adverse life events during last six months such as major financial problems, serious illness or injury to a close relatives, broken off a steady relationship and getting the sack from a job. These events might be reasons of lower mood.

Total scoring of BDI-II was not related with age, general health scoring, blood lipids and sex hormones serum levels. When correlations where performed for each age band separately it appeared that total scoring of BDI-II was negatively related with FTI, but only in 40-49 age band ($r = –0.26$, $p < 0.05$). Other correlations of total BDI-II where not found.

Among different symptoms of depression only difficulties in concentration were positively associated with the age ($r = 0.18$, $p < 0.05$). Among hormones a negative association was observed between FTI and loss of interest in sex ($r = –0.21$, $p < 0.05$). More associations were found between lipids and symptoms of depression: negative between LDL-C and loss of energy ($r = –0.22$, $p < 0.01$) or loss of pleasure from things a man used to enjoy ($r = –0.2$, $p < 0.05$), HDL-C and feelings of past failures ($r = –0.31$, $p < 0.01$) or suicidal thoughts or wishes ($r = –0.24$, $p < 0.05$). TG were positively correlated with feelings of past failures ($r = 0.29$, $p < 0.01$) and negatively with the self-assessment of general health ($r = –0.18$, $p < 0.05$). Serum levels of TC were not associated with the separate symptoms of depression. Similar relations were found among young men, but not in the middle-aged.

BMI, WHR and blood pressure were not correlated with the total scoring of BDI-II or single depression symptoms or the scoring of general health in the whole group, as well as in each age band.

Table 3. Results of the Beck Depression Inventory II (BDI-II) in men 20-49 years old.

Depression intensity	Age band (years)		
	20-29 *n* = 44 (%)	30-39 *n* = 36 (%)	40-49 *n* = 56 (%)
Lack or minimal	40 (90.9)	31(86.1)	48 (85.8)
Mild	3 (6.8)	5 (13.9)	4 (7.1)
Moderate	0	0	4 (7.1)
Severe	1 (2.3)	0	0

4. DISCUSSION

Although total testosterone serum levels were unchanged with the age in our study, FTI, which is a counterpart of biologically active testosterone, decreased significantly. Moreover, DHEA-S and estradiol decreased with the advancing age, while the level of SHBG increased. Such phenomena were described in several studies [22-24]. There is a general consensus that serum levels of testosterone decline with age. This decline begins at about the 30^{th} year of life and decreases progressively as men get older. The European Male Aging Study (EMAS) provides one of the largest data sets to investigate hormonal variations in aging men. It revealed that total testosterone decreased by only 0.4%/year, whereas free testosterone decreased by 1.3%/year [24]. Serum free testosterone concentrations decrease more than the total testosterone because of the increased levels of SHBG in older men. Since the circulating non-SHBG-bound testosterone (free and albumin-bound) is the biologically available form for activity at the target organs, measurements of this non-SHBG-bound testosterone better reflect the clinically important state of circulating testosterone than the total testosterone concentrations [25]. It is also known that serum concentrations of adrenal androgens such as dehydroepiandrosterone and its sulphate (DHEA and DHEA-S) and androstendione decrease with the increasing age [26,27]. The age trend of serum estrogen in men has been reported variously as declining [27,28] or steady [29].

Although significant decrease in serum levels of sex steroids were observed with the advancing age, in this study we have not found the direct relationship between emotional well-being and sex steroids in young men. Only in 40-49 age band total scoring of BDI-II was negatively related with FTI. It may indicate that after 40 testosterone serum level is so low that it may negatively influence the mood. Nevertheless, although most of the men with higher intensity of depression were above 40, the most severe depression occurred in the 25-year-old man, who had normal levels of sex steroids. The reason of lower mood was probably situational, because all of the depressed men reported recent adverse life events. The results of other studies on the influence of sex steroids on mood in men are controversial. In the Rancho Bernardo Study of 856 men aged 50-89, the BDI score was significantly and inversely associated with

bioavailable testosterone, independent of age, weight change and physical activity [30]. Similar associations were seen for dihydrotestosterone, while estradiol was not associated with depressed mood. The Massachusetts Male Aging Study (MMAS) of 1709 men aged 40-70 revealed no correlation between the Center for Epidemiologic Studies Depression Scale (CES-D) and serum testosterone level [31]. Rubin *et al.* [15] showed that age was negatively correlated with baseline serum testosterone in patients with endogenous depression, but not in controls. Similar results, revealing that among men with major depression testosterone secretion may be reduced, were achieved by others [32,33]. However, signs and symptoms of major depression may overlap with apparent psychiatric sequel of hypogonadism. Woodman and Williams [34] found that 39% of hypogonadal men treated in an endocrinology clinic were also treated with medication for a psychiatric illnesses. These data suggest that some depressed older men may have state-dependent low testosterone levels and that some of them may improve with androgen treatment.

Among sex steroids only FTI was associated with the lost of interest in sex as the separate parameter of depression. There are a lot of studies showing the relationship between libido and androgens [7,35]. EMAS revealed that men with the lowest levels of total and free testosterone reported lower overall sexual function (OSF) scores in comparison to men with the highest testosterone levels [36]. There is also an improvement in the sexual function after treatment with testosterone [37].

We investigated also the state of general health because it may, as the only factor, lead to significant deterioration of mood. The oldest men worse estimated their health in comparison to younger and they reported more morbidities. They had more atherosclerosis risk factors: higher BMI, WHR, blood pressure and increased serum levels of TC, LDL-C and TG. In hypogonadal men, as well as in aging men, increase in total adipose tissue mass and the redistribution of fat from peripheral subcutaneous depots to central, intra-abdominal depots is well-documented [38-40]. Decreased testosterone level and visceral adipose tissue accumulation in the aging male probably represents the most important factor for premature morbidity and mortality from cardiovascular disease [41,42]. However, we did not find the impairment of mood with the advancing age or associations of total BDI-II scores with blood lipids, anthropometric results and the general health scoring. Nevertheless, there were relations between some symptoms of depression and blood lipids. LDL-C correlated negatively with the loss of energy and the loss of pleasure from things a man used to enjoy. It was found previously by other authors that low LDL-C level may predispose to acute depression and the risk of suicidal behaviour [43]. HDL-C correlated negatively with feelings of past failures or suicidal thoughts. Nevertheless, we did not find associations between TC and symptoms of depression what was shown by other authors [44]. Maes *et al.* [45] suggested that major depression was accompanied by reduced formation of cholesterol esters and perhaps by impairment of reverse cholesterol transport. The latter is accompanied by lower serum HDL-C. Serum HDL-C was significantly lower in depressed men who had made suicidal attempts than in those without such suicidal behaviour. Maimanee and Al-Hazimi [46] suggested that the youth with low level of TC was more exposed to acute depression than the elders. It is known that brain membranes have a very high content of polyunsaturated fatty acids (PUFAs): omega 3 and 6. These two classes of fatty acids cannot be synthesised by the organism and have to be taken from alimentation. Phospholipids composed of PUFAs chains increase the membrane fluidity which is also determined by the phospholipids/free cholesterol ratio, as cholesterol increases membrane viscosity. Any dietary lack of essential PUFAs has consequences on cerebral development [44,47]. It has been suggested that the increased prevalence of depression in the European countries in the 20th century could be related to changes in alimentary patterns, in which consumption of omega 3 PUFAs constantly diminished [48]. These findings have challenged the public health programs aimed at promoting the decrease of cholesterol, and even suggested to suspend the administration of lipid lowering drugs [44].

5. CONCLUSIONS

Only low blood lipids may have influence on emotional well-being in young healthy men. The decreased testosterone level becomes probably the main risk factor for the lower mood in middle-aged men. Atherosclerosis risk factors and general health worsen with the advancing age, but they have no significant effect on psychological situation.

REFERENCES

[1] Bancroft, J. (1995) The menstrual cycle and the well being of women. *Social Science & Medicine*, **41(6)**, 785-791.

[2] Chrisler, J.C. and Caplan, P. (2002) The strange case of Dr. Jekyll and Ms.Hyde: How PMS became a cultural phenomenon and a psychiatric disorder. *Annual Review of Sex Research*, **13**, 274-306.

[3] Frey, B.N., Lord, C. and Soares, C.N. (2008) Depression during menopausal transition: A review of treatment strategies and pathophysiological correlates. *Menopause International*, **14(3)** 123-128.

[4] Zarrouf, F.A., Artz, S., Griffith, J., Sirbu, C. and Kommor, M. (2009) Testosterone and depression: Systematic review and meta-analysis. *Journal of Psychiatric Practice*,

15(4), 289-305.

[5] Joshi, D., van Schoor, N.M., de Ronde, W., Schaap, L.A., Comijs, H.C., Beekman, A.T. and Lips, P. (2009) Low free testosterone levels are associated with prevalence and incidence of depressive symptoms in older men. *Clinical Endocrinology* (*Oxford*) [ahead of print].

[6] Nieschlag, E., Swerdloff, R., Behre, H.M., Gooren, L.J., Kaufman, J.M., Legros, J.J., Lunenfeld, B., Morley, J.E., Schulman, C., Wang, C., Weidner, W. and Wu, F.C. (2006) Investigation, treatment, and monitoring of late-onset hypogonadism in male: ISA, ISSAM, and EAU recommendations. *Journal of Andrology*, **27(2)**, 135-137.

[7] Morales, A., Buvat, J., Gooren, L.J., Guay, A.T., Kaufman, J.M., Tan, H.M. and Torres, L.O. (2004) Endocrine aspects of sexual dysfunction in men. *Journal of Sexual Medicine*, **1(1)**, 69-81.

[8] Nease, D.E. and Malouin, J.M. (2003) Depression screening: A practical strategy. *Journal of Family Practice*, **52(2)**, 118-124.

[9] Burris, A.S., Banks, S.M., Carter, C.S., Davidson, J.M. and Sherins, R.J. (1992) A long-term prospective study of the physiologic and behavioral effects of hormone replacement in untreated hypogonadal men. *Journal of Andrology*, **13(4)**, 297-304.

[10] Morley, J.E., Perry, H.M. 3rd, Kaiser, F.E., Kraenzle, D., Jensen, J., Houston, K., Mattammal, M. and Perry, H.M. Jr. (1993) Effect of testosterone replacement therapy in older hypogonadal males: A retrospective study. *Journal of the American Geriatric Society*, **41(2)**, 149-152.

[11] Wang, C., Cunningham, G., Dobs, A., Iranmanesh, A., Matsumoto, A.M., Snyder, P., Weber, T., Berman, N., Hull, L. and Swerdloff, R.S. (2004) Long-term testosterone gel (Androgel) treatment maintains beneficial effects on sexual function and mood, lean and fat mass, and bone mineral density in hypogonadal men. *Journal of Clinical Endocrinology & Metabolism*, **89(5)**, 2085-2098.

[12] Libe, R., Barbetta, L., Dall'Asta, C., Salvaggio, F., Gala, C., Beck-Pecoz, P. and Ambrosi, B. (2004) Effects of dehydroepiandrosterone (DHEA) supplementation on hormonal, metabolic and behavioral status in patients with hypoadrenalism. *Journal of Endocrinological Investigation*, **27(8)**, 736-741.

[13] Von Muhlen, D., Laughlin, G.A., Kritz-Silverstein, D. and Barrett-Connor, E. (2007) The dehydroepiandrosterone and wellness (DAWN) study: Research design and methods. *Contemporary Clinical Trials*, **28(2)**, 153-168.

[14] Sachar, E.J., Halpern, F., Rosenfeld, R.S., Gallagher, T.F. and Hellman, L. (1973) Plasma and urinary testosterone levels in depressed men. *Archives of General Psychiatry*, **28(1)**, 15-18.

[15] Rubin, R.T., Poland, R.E. and Lesser, I.M. (1989) Neuroendocrine aspects of primary endogenous depression VIII. Pituitary-gonadal axis activity in male patients and matched control subjects. *Psychoneuroendocrinology*, **14(3)**, 217-229.

[16] Jones, B.N. and Reifler, B.V. (1994) Depression co-existing with dementia: Evaluation and treatment. *Medical Clinics of North America*, **78(4)**, 823-840.

[17] Thomas, A.J., Kalaria, R.N. and O'Brien, J.T. (2004) Depression and vascular disease: What is the relationship? *Journal of Affective Disorders*, **79(1)**, 81-95.

[18] Shin, J.Y., Suls, J. and Martin, R. (2008) Are cholesterol and depression inversely related? A meta-analysis of the association between two cardiac risk factors. *Annals of Behavioral Medicine*, **36(1)**, 33-43.

[19] Lehto, S.M., Hintikka, J., Niskanen, L., Tolmunen, T., Koivumaa-Honkanen, H., Honkalampi, K. and Vijnamaki, H. (2008) Low HDL cholesterol associates with major depression in a sample with a 7-year history of depressive symptoms. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, **32(6)**, 1557-1561.

[20] Sutton, P.W., Love, J.G., Bell, J., Christie, E., Mayrhofer, A., Millman, Y., Williams, H. and Yuill, C. (2005) The emotional well-being in young people: A review of the literature. School of Applied Social Studies, Robert Gordon University, Aberdeen, Scotland.

[21] Beck, A.T., Steer, R.A. and Brown, G.K. (1996) Manual for the Beck Depression Inventory-II. Psychological Corporation, San Antonio, TX, USA.

[22] Kaufman, J.M. and Vermeulen, A. (2005) The decline of androgen levels in elderly men and its clinical and therapeutic implications. *Endocrine Review*, **26(6)**, 833-876.

[23] Liu, P.Y., Beilin, J., Meier, C., Nguyen, T.V., Center, J.R., Leedman, P.J., Seibel, M.J., Eisman, J.A. and Handelsman, D.J. (2007) Age-related changes in serum testosterone and sex hormone binding globulin in Australian Men: longitudinal analyses of two geographically separate regional cohorts. *Journal of Clinical Endocrinology & Metabolism*, **92(9)**, 3599-3603.

[24] Wu, F.C., Tajar, A., Pye, S.R., Silman, A.J., Finn, J.D., O'Neil, T.W., Bartfai, G., Casanueva, F., Forti, G., Giwercman, A., Huhtaniemi, I.T., Kula, K., Punab, M., Boonen, S., Vanderschurren, D. and the EMAS study group (2008) Hypothalamic-pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: The male aging study. *Journal of Clinical Endocrinology & Metabolism*, **93**, 2737-274.

[25] Swerdloff, R.S. and Wang, C. (2002) Androgens and the aging male. In: Lunenfeld, B. and Gooren, L., Eds., *Textbook of men's health*. The Parthenon Publishing Group, 148-157.

[26] Nafziger, A.N., Bowlin, S.J., Jenkins, P.L. and Pearson, T.A. (1998) Longitudinal changes in dehydroepiandrosterone concentrations in men and women. *Journal of Laboratory & Clinical Medicine*, **131(4)**, 316-323.

[27] Feldman, H.A., Longcope, C., Derby, C., Johannes, C.B., Araujo, A.B., Coviello, A.D., Bremner, W.J. and McKinlay, J.B. (2002) Age trends in the level of serum testosterone and other hormones in middle-aged men: Longitudinal results from the Massachusetts Male Aging Study. *Journal of Clinical Endocrinology & Metabolism*, **87(2)**, 589-598.

[28] Ferrini, R.L. and Barrett-Connor, E. (1998) Sex hormones and age: A cross-sectional study of testosterone and estradiol and their bioavailable fractions in community-dwelling men. *American Journal of Epidemiology*, **147(8)**, 750-754.

[29] Barrett-Connor, E. (1990) A prospective, population-based study of androstendione, estrogens and prostate cancer. *Cancer Research*, **50**, 169-173.

[30] Barrett-Connor, E., Von Mühlen, D.G. and Kritz-Silverstein, D. (1999) Bioavailable testosterone and depressed mood in older men: The Rancho Bernardo Study.

Journal of Clinical Endocrinology & Metabolism, **84(2)**, 573-577.

[31] Araujo, A.B., Durante, R., Feldman, H.A., Goldstein, I. and McKinlay, J.B. (1998) The relationship between depressive symptoms and male erectile dysfunction: Cross-sectional results from the Massachusetts Male Aging Study. *Psychosomatic Medicine*, **60(4)**, 458-465.

[32] Seidman, S.N. and Walsh, B.T. (1999) Testosterone and depression in aging male. *American Journal of Geriatric Psychiatry*, **7(1)**, 18-33.

[33] Carnahan, R.M. and Perry, P.J. (2004) Depression in aging men: The role of testosterone. *Drugs & Aging*, **21(6)**, 361-376.

[34] Woodman, C.L. and Williams, W.R. (1996) Testosterone, mood, and psychotropic medication. American Psychiatric Association, New York.

[35] Travison, T.G., Morley, J.E., Araujo, A.B., O'Donnell, A.B. and McKinlay, J.B. (2006) The relationship between libido and testosterone levels in aging men. *Journal of Clinical Endocrinology & Metabolism*, **91(7)**, 2509-2513.

[36] O'Connor, D.B., Corona, G., Forti, G., Tajar, A., Lee, D.M., Finn, J.D., Bartfai, G., Boonen, S., Casanueva, F.F., Giwercman, A., Huhtaniemi, I.T., Kula, K., O'Neill, T.W., Pendleton, N., Punab, M., Silman, A.J., Vanderschueren, D., Wu, F.C.W. and the European Male Ageing Study group (2008) Assessment of sexual health in aging men in Europe: Development and validation of the European Male Ageing Study sexual function questionnaire. *Journal of Sexual Medicine*, **5(6)**, 1374-1385.

[37] Morley, J.E., Charlton, E., Patrick, P., Kaiser, F.E., Cadeau, P., McCready, D. and Perry, H.M. 3rd (2000) Validation of a screening questionnaire for androgen deficiency in aging males. *Metabolism*, **49(9)**, 1239-1242.

[38] Vermeulen, A., Goemaere, S. and Kaufman, J.M. (1999) Sex hormones, body composition and aging. *Aging Male*, **2(1)**, 8-15.

[39] Isidori, A.M., Giannetta, E., Gianfrilli, D., Bonofacio, V., Aversa, A., Isidori, A., Fabbri, A. and Lenzi, A. (2005) Effects of testosterone on body composition, bone metabolism and serum lipid profile in middle-aged men: A meta-analysis. *Clinical Endocrinology* (*Oxford*), **63(4)**, 381- 394.

[40] Slowikowska-Hilczer, J., Marchlewska, K., Walczak-Jedrzejowska, R., Oszukowska, E., Guminska, A., Kramek, E., Jastrzebska, S., Zawadzka, E., Kula, W., Habib, M., Trzuskowska, D., Jakubowski, L. and Kula, K. (2007) High risk of atherosclerosis in men aged 20-39 from Lodz agglomeration. *Polski Merkuriusz Lekarski*, **23(138)**, 417-425.

[41] Marin, P. (2002) Testosterone, aging and body composition. In: Lunenfeld, B. and Gooren, L., Eds., *Textbook of men's health.* The Parthenon Publishing Group, 227-234.

[42] Wu, F.C.W. and Von Eckardstein, A. (2003) Androgens and coronary artery disease. *Endocrine Review*, **24(2)**, 183-217.

[43] Rabe-Jablonska, J. and Poprawska, I. (2000) Levels of serum total cholesterol and LDL-cholesterol in patients with major depression in acute period and remission. *Medical Science Monitor*, **6(3)**, 539-547.

[44] Colin, A., Reggers, J., Castronovo, V. and Ansseau, M. (2003) Lipids, depression and suicide. *Encephale*, **29(1)**, 49-58.

[45] Maes, M., Smith, R., Christophe, A., Vadoolaeghe, E., Van Gastel, A., Neels, H., Demedts, P., Wauters, A. and Meltzer, H.Y. (1997) Lower serum high-density lipoprotein cholesterol (HDL-C) in major depression and in depressed men with serious suicidal attempts: Relationship with immune-inflammatory markers. *Acta Psychiatrica Scandinavica*, **95(3)**, 212-221.

[46] Maimanee, T.A. and Al-Hazmi, S. (2009) Relationship between blood cholesterol level and acute depression. *Journal of the Egyptian Society of Parasitology*, **39(2)**, 653-663.

[47] Owen, C., Rees, A.M. and Parker, G. (2008) The role of fatty acids in the development and treatment of mood disorders. *Current Opinion in Psychiatry*, **21(1)**, 19-24.

[48] Young, G. and Conquer, J. (2005) Omega-3 fatty acids and neuropsychiatric disorders. *Reproduction Nutrition Development*, **45(1)**, 1-28.

Vol.2, No.5, 448-453 (2010)
doi:10.4236/health.2010.25067

The effects of slight atmospheric pressure fluctuations on the occurrence of emergency transport due to suicidal injuries

Lyudmyla Aleksandrovna Didyk[1]*, Yuriy Pavlovich Gorgo[2], Joris Jan Josef Dirckx[3], Irina Aleksandrovna Semenova[4], Nataliya Petrovna Didyk[1], Dmytro Sergeevich Gorlov[5]

[1]Institute of Physics, National Academy of Sciences of Ukraine, Kyiv, Ukraine; *Corresponding Author: la_didyk@iop.kiev.ua
[2]Inter-University Medical Engineering Department, National Technical University of Ukraine "Kyiv Polytechnic Institute", Kyiv, Ukraine; yugorgo@ukr.net
[3]Biomedical Physics, University of Antwerp, Antwerp, Belgium; Joris.Dirckx@ua.ac.be
[4]Department of Pediatric, Social and Forensic Psychiatry, National Medical Academy of Post-graduate Education named after P.L. Shupyk, Kyiv, Ukraine; elly14@mail.ru
[5]Biological Department, Taras Shevchenko National University, Kyiv, Ukraine; tavricheskiy@yahoo.com

Received 15 January 2010; revised 1 February 2010; accepted 3 February 2010.

ABSTRACT

The objective of this study was to test the hypothesis that a relation exists between slight atmospheric pressure fluctuations (APF) in the far infrasound frequency range and daily number of emergency transport events due to suicidal injuries (EESU). The regression models to compare groups were used to assess the relation of EESU to the mean daily amplitude of APF (APF-A) and to the ratio of APF amplitude averaged over the daytime to the APF amplitude averaged over the nighttime (Rdn). To eliminate the confounding effects of basic meteorological parameters and annual trend in EESU, the non-parametric smoothing method was used in a stepwise manner. The low APF-A (95% CI = 1.06-1.16 Pa) compared to their common middle levels and the high (95% CI = 3.18-3.64 Pa), as well the low Rdn (CI = 0.83-0.92) and very high Rdn (CI = 3.05-3.77) compared to their more closed to common regular values (CI = 1.69-1.90) turned out to be more beneficial factors promoting the decrease in the incidence of EESU. We suppose that more attention needs to be paid to the meteorotropic effects of APF on certain kinds of psychopathology resulting in suicidal behaviour, and further investigations in different geographical and climatic conditions, especially in those with more intense atmospheric perturbations, are necessary.

Keywords: Atmospheric Pressure Fluctuations; Suicidal Injuries; Emergency Events

1. INTRODUCTION

The weather effects upon human mental state and behaviour have long been discussed in literature. There are considerable numbers of evidences on the severe negative weather after-effects on people with psychical disorders resulting in suicidal behaviour. The basic meteorological parameters (ambient temperature, humidity, atmospheric pressure and wind velocity), as well the certain physical weather condition (hours of sunlight, cloud cover and precipitation) were suggested as the external triggers for development of suicidal ideation and action [1-7].

However, very little attention has been paid to possible effects of other atmospheric parameters on human behaviour. It was suggested, that slight atmospheric pressure fluctuations (APF) in the far infrasound frequency range is important meteorotropic factor influencing on mental function and behaviour [8-10]. The APF penetrate into buildings [11,12], therefore they could influence on people indoors and, as well as outdoors.

Long ago Mezernitsky P.G. [13] pointed to the high sensitivity of the central nervous system to atmospheric pressure "micropulsations". Vladymirsky B.M. [9] suggested the psychotropic effects of natural atmospheric infrasound. In line with these suggestions Green J.E. and Dunn F. [8] reported the correlation between strong naturally occurring infrasonics and selected kinds of human behaviour.

The possible effects of natural APF on human mental activity and autonomic indices, as well the symptoms of central nervous systems and vestibular disorders were confirmed by the experimental investigations with artificial pressure oscillations in the far infrasound frequency

range [10,14].

Some authors believed that the increase in anxiety levels in people with mental disorders or incidences of suicides during episodes of strong wind could be at least partly due to meteorotropic effects of APF created by wind induced turbulence of airflows [10,15].

The objective of this study was to examine whether a relation exists between natural APF and suicidal behaviour. For this the daily number of emergency transport events due to suicidal injuries (EESU) was related to mean daily integral amplitude of APF (APF-A) in the far infrasound frequency range and to the ratio of APF integral amplitude averaged over the daytime to the APF integral amplitude averaged over the nighttime (Rdn). The study was performed in Kiev locality (Ukraine) with moderate climatic condition, where calm and relatively slight windy weather is prevailing.

2. MATERIALS AND METHODS

Daily numbers of emergency transport events on suicidal injuries (EESU) for the period from 1 July 2005 to 30 June 2006 were obtained from Kyiv Station of emergency services and medicine of catastrophes. During this time the outdoor continuous measurements of atmospheric pressure have been performed with a standard microbarometer. The value of atmospheric pressure was recorded every 0.5 s. A special computer program developed by us was used to calculate average integral amplitudes of APF in the range of their periods from 3 s to 120 s over each 1 h of the day. The range of APF periods was selected allowing for the results of previous investigations of biological effects of APF in the far infrasound frequency range [10,12].

The mean daily value of APF integral amplitude (APF-A) and the ratio of APF integral amplitude averaged over the daytime (from 8:00 to 20:00 hours) to APF integral amplitude averaged over the nighttime (from 20:00 to 8:00 hours) (Rdn) were used for the analysis allowing for the results of previous investigations of biological effects of APF in the far infrasound range of their periods [12].

Three-hourly meteorological data were obtained from Kyiv Geophysical Observatory for the same year period. Mean daily temperature (T), relative humidity (RH) and atmospheric pressure (AP) were considered as potential confounding variables for the relation of EESU to APF-A.

As APF are causally related to wind induced turbulence, the possible interrelation between effects of APF-A and wind velocity (WV) was examined.

The number of a day in the course of the year (Y) was included in the model to control the effects of time-varying environmental factors on the year dynamics of EESU. This variable has associations with annual changes in length of daylight, weather parameters and social factors influencing on suicidal rates.

The polynomial approximation was used to assess visually the functional shape of yearly dynamics of EESU, as well as EESU relations with APF-A and Rdn.

The regression models to compare groups [16] were used to assess the relation of EESU to APF-A and Rdn.

If significant difference in EESU was revealed between two categories of the values of independent variable the numbers of days in first and second categories were defined by changing the boundary between them until the most significant difference in EESU was obtained.

The negative or positive effect of the one category of values of independent variable versus the other category was determined correspondingly as a percentage increase or decrease in number of EESU.

To eliminate the effects of potential confounding variables, the non-parametric smoothing method (Loess technique) was used in a stepwise manner [17].

We controlled the day of the week effects by dummy variables. All public holidays were excluded from the data. A few days of EESU and atmospheric data were lost. Hence 345 days were used for the analysis.

The number of EESU and values of atmospheric parameters were not normally distributed. Therefore, non-parametric (Mann-Whitney U-test and Spearman's rank correlation test) estimations were used. Matlab 6.5 (Curve Fitting Toolbox), Statistica 6 and MS Excel were applied for statistical analysis.

3. RESULTS

3.1. Atmospheric Data

Table 1 provides data on APF-A and Rdn values. Maximal value for hourly amplitude of APF during the year was 22.5 Pa.

We found high correlation between APF-A and WV ($r = 0.72$, $p < 0.00001$), though this correlation was significantly reduced ($r = 0.23$, $p = 0.04$, $n = 77$) in the range of low WV values (< 1.5 m/s). Dependency of the correlation coefficient on the value of WV was consistent with the non-linear character of causal relationships between APF and WV [18]. According to this character, at high WV (above 2 m/s) the APF increased rapidly with increasing WV, as the wind-induced effects dominated. At lower WV value (less than 0.5-1.5 m/s) there was only a moderate increase in the APF, as they were less dependent on the wind.

3.2. EESU Data

The total number of EESU for 345 days was 1036.

The yearly dynamics of EESU revealed significant seasonality (**Figure 1**). The significant season-specific

Table 1. Data on daily values of APF and Rdn during the year.

	95% CI	Maximum	Minimum
APF-A (Pa)	2.64-3.05	11.07	0.7
Rdn	1.43-1.62	7.2	0.21

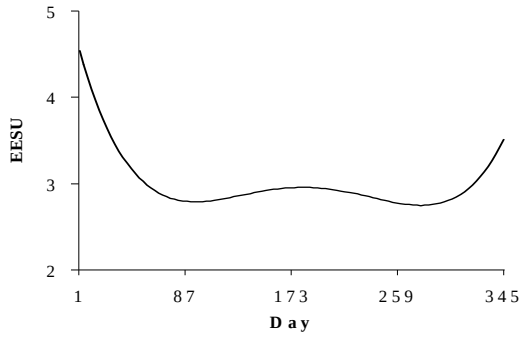


Figure 1. Polynomial model of yearly dynamics (from 1 July 2005 to 30 June 2006) of the number of EESU.

difference in the number of EESU was found between warm days (T, CI = 17.40°-19.18°) during summer-autumn season (from 1 July to 5 October 2005 and from 2 June to 30 June 2006, n = 122) and the other colder days (T, CI = 1.45°-3.75°) of the year (from 6 October 2005 to 1 June 2006, n = 223). The number of EESU proved to be greater (CI = 3.01-3.72) during warm summer-autumn period in comparison with the colder period of the year (CI = 2.60-3.06, p = 0.01).

3.3. The Relation between EESU and APF-A

The APF-A did not reveal significant relation with EESU when their raw data were considered (**Figure 2**). The functional form of the relation between EESU and APF-A was changed after adjustment for the Y and T variables. Significant difference in EESU was found only between two categories of APF-A values, which we considered as low and middle-high (**Table 2**). The number of EESU turned out to be significantly less on days with low APF-A than on days with middle-high APF-A. The category of low APF-A included about one fourth of all days of the year (n1 = 85). The other days (n2 = 260) with middle and high APF-AI compiled the second category.

Significant difference in the number of EESU between two categories of APF-A values, which was revealed after adjustment for Y and T, pointed to interfering effects of these two confounding variables on the relation between EESU and APF-A. It was mentioned above that greater numbers of EESU were observed during warm summer-autumn season compared to the other colder time of the year. Even more pronounced difference in EESU was found between two categories of T values. The number of EESU was significantly

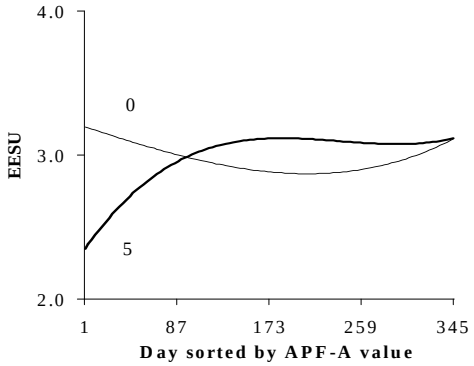


Figure 2. The number of EESU plotted against the day sorted by APF-A value in ascending order. Polynomial model: thin line (0)—raw data, thick line (5)—data adjusted for the four potential confounding variables (Y, T, AP, RH) and Rdn.

Table 2. 95% Confidence intervals for two categories of APF-A (low and middle-high) and corresponding number of EESU for data adjusted for two variables—Y and T (EESU/2) and five variables—Y, T, AP, RH and Rdn (EESU/5).

	Low APF-A 95% CI n1 = 85	Middle-high APF-A 95% CI n2 = 260	P Value
APF-A (Pa)	1.06-1.16	3.18-3.64	
EESU/2	2.34-3.03	2.90-3.31	0.046
EESU/5	2.25-2.90	2.95-3.29	0.0034

Note: p - significance of the difference in the number of EESU between two categories of APF-A values.

greater (p < 0.002) on days with high T (17.15°C (CI = 15.97-18.32), n2 = 128) compared to days with low T (2.8°C (CI = 1.66-4.03°C), n = 217). The ranges of T values on days with low and middle-high APF-A turned out to be significantly different also. The value of T was almost two times higher (13.35°C (CI = 11.23-15.46)) on days with low APF-A compared to days with middle-high APF-A (6.39°C (CI = 5.11-7.66)). Therefore, the decrease in number of EESU on days with low APF-A, which prevailed during warm season, could be interfered by simultaneous effects of the comfort warm T promoting an increase in number of EESU.

The difference in number of EESU between two categories of APF-A values remained significant after following stepwise adjustment for the AP and RH variables (p = 0.02). After additional adjustment for the Rdn the significant difference in EESU between low and middle-high APF-A became more pronounced (p = 0.0034, **Table 2**). The percentage decrease in the number of EESU on days with low APF-A versus the middle-high APF-A was –17.6% (–23.7 - –11.8).

This difference was also significant after additional adjustment for WV (after adjustment for the four confounding variables and WV p = 0.033) that suggested the

independent on WV effects of APF-A on EESU.

3.4. The Relation between EESU and Rdn

Figure 3 shows the functional shape of plots of EESU (raw data and data adjusted for the four variables and APF-A) vs the day sorted by Rdn value in ascending order. This shape suggested the three categorical model of the relation between EESU and Rdn. After adjustment for the four variables and APF-A significantly greater number of EESU proved to be at days with Rdn middle-high (n2 = 161) values comparing to days with Rdn low (n1 = 150) or very high (n3 = 34) values (**Table 3**). This classification of Rdn values was used taking into consideration the mean year magnitude of Rdn (**Table 1**). The percentage decrease in the number of EESU on days with low or very high Rdn versus the middle-high Rdn was correspondingly: –10.3% (–10.4 - –10.4) and –23.6% (–32.9 - –16.1).

The relation of EESU with Rdn remained significant after additional adjustment for WV (after adjustment for the four confounding variables and WV $p_{1,2} = 0.03$, $p_{2,3} = 0.01$).

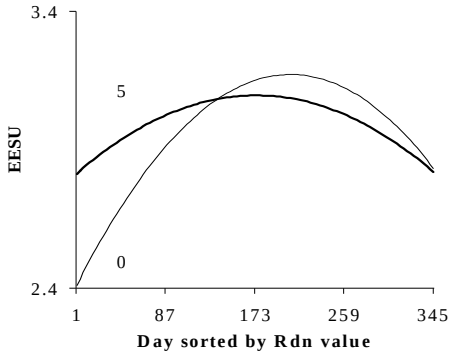


Figure 3. The number EESU plotted against the day sorted by Rdn value in ascending order. Polynomial model: thin line (0)—raw data, thick line (5)—data adjusted for the four potential confounding variables (Y, T, AP, RH) and APF-A.

Table 3. 95% Confidence intervals for three categories of Rdn (low, middle-high and very high) and corresponding number of EESU for raw data (EESU/0) and data adjusted for five variables - Y, T, AP, RH and APF-A (EESU/5).

	Low Rdn 95% CI n1 = 150	Middle-high Rdn 95% CI n2 = 161	Very high Rdn 95% CI n2 = 34
Rdn	0.83-0.92	1.69-1.80	3.05-3.77
EESU/0	2.47-3.02 $P_{1,2} = 0.02$	2.96-3.57	2.00-3.14 $P_{2,3} = 0.07$
EESU/5	2.67-3.11 $P_{1,2} < 0.05$	2.98-3.47	2.00-2.91 $P_{2,3} = 0.005$

Note: $P_{1,2}$; $P_{2,3}$ - significance of the difference in the number of EESU between days with correspondingly: low Rdn and middle-high Rdn; middle-high Rdn and very high Rdn.

4. DISCUSSIONS

4.1. The Relation between EESU and APF-A

To our knowledge, only one communication has been published on the relation between natural APF and human behaviour. Green J.E. and Dunn F. [8] found a correlation between the presences of strong infrasonic disturbances generated by natural atmospheric phenomena and automobile accidents, as well the schoolchildren behavioural demonstrations of being unwell.

We found that the number of EESU was greater at days with common middle and high APF-A compared to the days with low APF-A. These findings suggest the high sensitivity of people with suicidal psychopathology to APF levels, which is in line with the some authors' belief that weather sensitivity increases in people with psychical disorders [19-22]. Obviously the APF middles levels as well as the high ones were relevant external stimulus to provocation of suicidal intentions. Previously, the stimulating effects of artificial atmospheric pressure oscillations in the range of their natural levels on human normal purposeful behavioural activity were also revealed in the experimental study [10].

In contrast, the lack of activating influences of APF at days with their low levels seems to be the positive factor favouring to decrease in risk of suicidal actions, as well the incidence of EESU. The decrease in number of suicidal injuries at days with low APF could be also connected with increasing passiveness for the lack of APF in the environment, which was previously assumed [10]. From this point of view, the low levels of APF can particularly interfere with transition from suicidal ideation to suicidal action.

It follows from our results that the Y and T were important confounding variables for the relation between EESU and APF-A. These variables can be associated with certain slow time-varying environmental factors such as seasonal changes in weather, certain physical conditions and social aspects influencing on suicidal events. In considerable numbers of studies the daylight and sunlight duration, comfort ambient temperature, as well the warm months of the year (particularly summer and late spring) are considered to be an optimal condition for the suicide actions [3,4,7,23]. We found that simultaneous effects of these two variables and the APF-A on the number of EESU had opposite character. Due to interfering effects of Y and T the significant relation between EESU and APF-A was found only after adjustment for these two variables.

It is necessary to note that the present study demonstrates the significant effects of natural APF-A having lower values, than those in the experimental studies [10,14]. Apparently biological effectiveness of natural APF-A is depended on dose effects of their prolonged actions.

4.2. The Relation between EESU and Rdn

The previous study showed that daily dynamics of APF was characterized by pronounced regularity [12]. According to this regularity the mean hourly amplitude of APF increases from night to day followed by a decrease from day to night. Therefore, the possible association of daily dynamics of APF with circadian rhythms of human psychical activity could be suggested. From standpoint of behavioural continuum from sleeping to high level of vigilance the regular daily dynamics of APF could be considered as favourable factor enabling an organism to adopt to the needs of higher activity levels while awake.

We found that the number of EESU was significantly decreased at days with low (CI = 0.83-0.92) and very high (CI = 3.05-3.77) Rdn compared with middle-high Rdn (CI = 1.69-1.90) closed to mean year values of Rdn (CI = 1.43-1.62). These results indicated that the number of EESU was greater at days when the ratio of diurnal APF values to their preceding nocturnal values closed to common mean values. When nocturnal APF were higher or much less, than their diurnal values, the number of EESU was decreased. Therefore, it could be suggested that effectiveness of diurnal levels of APF in relation to EESU was depended on the foregoing nocturnal levels.

As to the activating effects of APF the decrease in their diurnal levels in comparison with the nocturnal levels indicated the decrease in effectiveness of APF as external provoking stimulus for the suicidal actions and as a consequence the decrease in the number of EESU during the daytime. The decrease in the number of EESU at days when diurnal APF were highest in comparison with the nocturnal ones could be due to "paradoxical" or antagonistic reaction with opposite character. According to the opinion of Wein A.M. *et al*. [24], such character of reaction could be evoked by irritant when the initial level of activation was abruptly altered.

4.3. The Additional Findings and Possible Implication of the Results

The previous study showed interrelated effects of high APF and WV on the number of emergency transport events due to circulatory system diseases [12]. Possible reason for the independent on WV significant decrease in EESU on days with low APF-A revealed in this study could be the non-linear relation between these two atmospheric variables [18]. According to this non-linearity, the causal relation between APF and WV is well pronounced only during strong wind, as the low WV is insufficient contributor to the APF generation. Apparently, due to the weak association of APF with low WV the relation between EESU and APF-A remained significant after adjustment for the WV.

The additional findings obtained in this study did not support also the conception of a spring peak of suicide incidence [23-25]. According to our data the number of EESU (without the reference to the sex difference) was higher during warmer time of the year in Kyiv. This time was summer and early autumn. Though, Parker G. and Walter S. [26] reported an early summer peak for both genders and early autumn peak in women.

As the future implication of this study results we suggest that the meteorological forecast of APF levels would be helpful for hospital emergency services in order to be able to prepare for potential increases in workload due to increase in traumatic accidents. The evidence, received in this study may be useful for the behavioural self-control in individuals with propensity toward suicidal ideation, as well for the relevant medical professionals.

5. CONCLUSIONS

The results of this study corroborate the suggestion that mean daily amplitudes of APF and ratio of diurnal levels of APF to their nocturnal levels are related to the risk for injuries due to psychopathological suicidal behaviour.

In this preliminary study we only found threshold effects of APF. The essential limitation of our study was the moderate climatic conditions of Kyiv locality with still or slightly windy weather prevailing. Perhaps, the functional shapes of the relation of EESU with APF are more complex than those revealed in the present study. To clear up this question it will be necessary to extend the observation over a longer period and different geographical and climatic areas, especially those where strong atmospheric pressure perturbation are frequent. Because there may be further uncontrolled factors our findings require additional corroboration.

6. ACKNOWLEDGEMENTS

This study was supported by the NATO Program Security Through Science, Collaborative Linkage Grant number 98376, and by funds from the University of Antwerp (BOF-NOI).

REFERENCES

[1] Mills, C.A. (1934) Suicides and homicides in their relation to weather changes. *The American Journal of Psychiatry*, **91(3)**, 669-677.

[2] Anderson, A.C. (1982) Environmental factors and aggressive behaviour. *Journal of Clinical Psychiatry*, **43**, 280-283.

[3] Souêtre, E., Wehr, T.A., Douillet, P. and Darcourt, G. (1990) Influence of environmental factors on suicidal behavior. *Psychiatric research*, **32(3)**, 253-263.

[4] Salib, E. and Gray, N. (1997) Weather conditions and fatal self-harm in North Cheshire 1989-1993. *British Journal of Psychiatry*, **171**, 473-477.

[5] Deisenhammer, E.A., Kemmler, G. and Parson, P. (2003) Association of meteorological factors with suicide. *Acta Psychiatrica Scandinavica*, **108(6)**, 455-459.

[6] Deisenhammer, E.A. (2003) Weather and suicide: The present state of knowledge on the association of meteorological factors with suicidal behaviour. *Acta Psychiatrica Scandinavica*, **108(6),** 402-409.
[7] Page, L.A., Hajat, S. and Kovats, R.S. (2007) Relationship between daily suicide counts and temperature in England and Wales. *British Journal of Psychiatry*, **191(2)**, 106-112.
[8] Green, J.E. and Dunn, F. (1968) Correlation of naturally occurring infrasonics and selected human behaviour. *Journal of the Acoustical Society of America*, **44(5)**, 1456-1457.
[9] Vladymirsky, B.M. (1982) The atmospheric infrasound as a possible physical agent transferring solar activity influence to the biosphere. *Problemy Kosmicheskoi Biologii*, in Russian, **43**, 174-179.
[10] Delyukov, A. and Didyk, L. (1999) The effects of extra-low-frequency atmospheric pressure oscillations on human mental activity. *International Journal of Biometeorology*, **43(1)**, 31-37.
[11] Richner, H. and Graber, W. (1978) The ability of non-classical meteorological parameters to penetrate into buildings. *International Journal of Biometeorology*, **22 (2)**, 242-248.
[12] Didyk, L.A., Gorgo, Y.P., Dirckx, J.J.J., Bogdanov, V.B., Buytaert, J.A.N., Lysenko, V.A., Didyk, N.P., Vershygora, A.V. and Erygina, V.T. (2008) Atmospheric pressure fluctuations in the far infrasound range and emergency transport events coded as circulatory system diseases. *International Journal of Biometeorology*, **52(7)**, 553-561.
[13] Mezernitsky, P.G. (1934) Medical meteorology. Ginásio Integrado Magdalena Kahn group, Yalta-Crimea, in Russian.
[14] Kompanets, V.S. (1968) The effects of repeated bi-directional changes in barometric pressure on man. *Voenno-Medicinsky Zhurnal*, **6**, 61-63.
[15] Bedard, A.J. and Georges, T.M. (2000) Atmospheric Infrasound. *Physics Today*, 53(**3)**, 32-37.
[16] McNamee, R. (2005) Regression modelling and other methods to control confounding. *Occupational and Environmental Medicine*, **62(7)**, 500-506.
[17] Storlie, C.B. and Helton, J.C. (2005) Multiple predictor smoothing methods for sensitivity analysis. *Proceedings of the 37th Winter Simulation Conference*, Orlando, 231-239.
[18] Woodward, R., Israelsson, H., Bondar, I., McLaughlin, K., Bowman, J.R. and Bass, H. (2005) Understanding wind-generated infrasound noise. *Proceedings of the 27th Seismic Research Review: Ground-Based Nuclear Explosion Monitoring Technologies*, Rancho Mirage, 866-875.
[19] Persinger, M.A. (1987) Mental processes and disorders: A neurobehavioral perspective in human biometeorology. *Experientia*, **43(1)**, 39-48.
[20] Barnston, A.G. (1988) The effect of weather on mood, productivity, and frequency of emotional crisis in temperate continental climate. *International Journal of Biometeorology*, **32(2)**, 134-143.
[21] Verhoef, M.J., Rose, M.S. and Ramcharan, S. (1995) The relationship between chinook conditions and women's physical and mental well-being. *International Journal of Biometeorology*, **38(3)**, 148-151.
[22] Rose, M.S., Verhoef, M.J. and Ramcharan, S. (1995) The relationship between chinook conditions and women's illness-related behaviours. *International Journal of Biometeorology*, **38(3)**, 156-160.
[23] Petridou, E., Papadopoulos, F., Frangakis, C., Skalkidou, A. and Trichopoules, D. (2002) A role of sunshine in the triggering of suicide. *Epidemiology*, **13(1)**, 106-109.
[24] Wein, A.M., Solovieva, A.D. and Kolosova, O.A. (1990) Autonomic-vascular distonia. *Medicine*, Moscow, in Russian.
[25] Chew, K.S. and McCleary, R. (1995) The spring peak in suicides: A cross-national analysis. *Social Science & Medicine*, **40(2)**, 223-230.
[26] Parker, G. and Walter, S. (1982) Seasonal variation in depressive disorders and suicidal deaths in South Wales. *British Journal of Psychiatry*, **140**, 626-632.

Vol.2, No.5, 454-457 (2010)
doi:10.4236/health.2010.25068

Association of the plasminogen activator inhibitor-1(PAI-1) gene 4G/5G promoter polymorphism in Buerger's disease (Tromboangiitis obliterans)

Sinasi Manduz[1], Nurkay Katrancioglu[1], Oguz Karahan[1], Oztürk Ozdemir[2]*

[1]Department of Cardiovascular Surgery, Faculty of Medicine, Cumhuriyet University, Sivas, Turkey
[2]Department of Medical Genetics, Faculty of Medicine, Cumhuriyet University, Sivas and University of COMU, Faculty of Medicine, Canakkale, Turkey; *Corresponding Author: ozdemir615@yahoo.com

Received 3 January 2010; revised 26 January 2010; accepted 27 January 2010.

ABSTRACT

Thromboangiitis obliterans (TAO) is an unusual tobacco-associated vasculopathy that is a non-atherosclerotic inflammatory disorder of unknown etiology that affects small and medium-sized vessels of the extremities. The single guanosine nucleotide deletion/insertion polymorphism (4G/5G) at -675 bp in promoter region of the PAI-1 gene is the major genetic determinant of PAI-1 expression. Plasma PAI-1 level is higher in people with the homozygous 4G genotype than in those with the 5G/5G genotype and renders higher transcription activity. The aim of this study was to determine the status and the role of PAI-1 gene 4G/5G promoter polymorphism in patients with Buerger's disease (Thromboangiitis obliterans—TAO). The current case-control study included 30 consecutive patients with Buerger's disease (mean age 42.9 ± 14.3 years, 28 men and 2 women), and 30 healthy volunteers (mean age 40.9 ± 4.79 years, 27 men and 3 women) between January 2006 and September 2009. Patients and control cases were genotyped for the 4G/5G polymorphism using the multiplex PCR based stripassay reverse hybridisation technique. It was found that heterozygote PAI-1 gene polymorphisms ($p < 0.05$) was significantly more frequent in patients with TAO in the current results. There was a significant difference in genotype distribution between the two groups ($P < 0.001$). The 4G allele occurred more frequently in the patient group of heavy smokers ($P = 0.05$). The current study shows the higher prevalence of of 4G allele in TAO patients in Sivas population means gene may predispose to TAO.

Keywords: Thromboangiitis Obliterans; Genetic Polymorphisms; PAI-1

1. INTRODUCTION

In 1908 Buerger suggested the name thromboangiitis obliterans (TAO) for a disease of the peripheral arteries which he had studied in eleven lower limbs amputated for "presenile spontaneous gangrene" [1]. TAO (Buerger's syndrome) is an unusual tobacco-associated vasculopathy that is a nonatherosclerotic inflammatory disorder of unknown etiology that affects small and medium-sized vessels of the extremities [1-3]. Etiopathogenesis of disease could not be evenly revealed. Researchers think that tobacco is rolled for starting and progressing of disease and by the way genetic factors, hipercoagulability, endothelium function and immune mechanisms etc. are suspicious factors that enrolled such as tobacco [1,2,4,5]. For example HLA-9 and HLA-B5 was excessively documented in patients. This may sign that genetic factors likely play a role [5].

Highly cellular thrombus occludes much of the lumen and lymphocytes, giant cells, fibrous hyperplasia, intimae thickness have seen around vaso-vasorums in histological investigation of vessel that affected by TAO [5]. A highly cellular thrombus occludes much of the lumen. A multinucleate giant cell and microabscess are present within the thrombus [5].

Both genetic and thrombotic events, reminds that prothrombotic gene deletions might act a part in this disorder. Prothrombotic genes detected significantly in some studies about TAO [6]. Plasminogen is one of mediators of fibrinolysis that synthesized in the liver and then circulates in plasma and other extracellular fluids. This respect presents in all tissues [7]. Urokinase-type plasminogen activator (uPA) and the tissue-type PA (tPA) are two regulative enzymes that directed the modulation of plasminogen to plasmin. The enzymatic activity of uPA can be inhibited plasminogen activator inhibitor

(PAI) -1 and PAI-2 [8]. PAI-1 is a major inhibitor of the fibrinolytic system. The PAI-1 gene polymorphism is known as a deletion/insertion of G base (5G/4G) in codon -675 and the major genetic determinant of PAI-1 expression. The 4G allele renders higher transcription activity than the PAI-1 promoter with the 5G allele in stimulated MCs [9]. The polymorphic PAI-1 gene is a member of plasminogen cascade with an inhibitory role in plasmin activation by response to cytokines, hormones and many growth factors. Isordila *et al.*, claimed that the 4G allele is an independent risk factor for acute myocardial infarction in young patients, as are smoking, hypertension and a family history of inherited cardiovascular disease [9]. It was aimed to find out the possible role of polymorphic 5G/4G alleles of PAI-1 gene in TAO in the current results.

2. MATERIAL AND METHOD

Thirty patients diagnosed with TAO at 1998 to 2009 in our clinic were included the study. Total genomic DNA from peripheral blood samples were used for each patient and control for 4G/5G polymorphism in PAI-1 gene. Control group was composed of healthy individuals who had no vascular complaints.

Clinical diagnosis of TAO was made according to criteria of Shionoya and defined as fallows; a history of smoking, disease on set before the age of 50 years, infrapopliteal arterial occlusions, upperlimb involvement or phlebitis migrans, and the absence of atherosclerotic risk factors other than smoking. In Doppler ultrasound of cases, the decreased arterial flow pattern was also revealed. Because of this arteriography was applied all cases. In arteriography, arterial occlusion and corkscrew collaterals revealed typically.

Mutation Analysis: Two ml of peripheral blood-EDTA samples were used for total genomic DNA isolation (Invitek, Berlin, Germany). Multiplex PCR-based reverse hybridisation stripAssay technique (Vienna Lab, Labordiagnostika GmbH, and Vienna, Austria) was used for mutation analysis (ProfiBlot T48, Tecan, Switzerland). Genotyping of PAI-1 gene was made and compared to the control group individuals.

Statistical Analysis: The SPSS software (ver; 14.0) was used for evaluation of data, χ^2-test (chi-square) was used to compare the significance of the differences between groups. The test of importance between the mean of two groups was used for accordance of two groups and margin of error was approved to as 0.05. Furthermore appropriateness of groups was evaluated with importance test of difference of between two groups.

3. RESULTS

The informed consents of the patients were also obtained. 30 TAO patients (28 males, 2 females and mean ages was 42.9 ± 14.3 years) and 30 healthy controls (27 males, 3 females and mean ages was 40.9 ± 4.79 years) were included in the current study. Two groups were similar regarding age and sex distribution [(t = 0.69); P = 0.487; p > 0.05]. Twenty nine (97%) cases had a history of smoking in TAO group and 22 (73%) cases had a history of smoking in control group. Detection of etiopathogenesis is important for meticulous care of these patients. Homozygous 4G/4G profile was 7% for TAO and 4% for control individuals. The homozygous wide type 5G/5G genotype was 10 % in patients with TAO and 53% in healthy controls (**Table 1**). Heterozygote mutation of 4G/5G profile was detected in 25 (83.3%) patients with TAO and 13 (43.3%) control subjects. The current results indicate that the 4G/5G gene polymorphism of PAI-1 is significantly associated with TAO while 4G/4G type may probably be an important hereditary risk factor as well. We found that heterozygote PAI gene polymorphisms ($p < 0.05$) were significantly more frequent in patients with TAO. We detected PAI-1 5G/4G polymorphism was significantly higher in TAO group ($p = 0.001$) (**Table 1** and **Figure 1**). Most possibly the 4G/5G gene polymorphism for PAI-1 may be an acceptable risk factor of TAO.

Table 1. The percantages of polymorphic alleles (5G/4G) for PAI-1 gene in the current TAO and control groups.

GROUP	ALTERNATIVE GENOTYPES FOR PAI-1 GENE			
	4G/4G	**4G/5G**	**5G/5G**	**Total (n-%)**
TAO (n-%)	2–7	25–83	3–10	30–100
Control (n-%)	1–4	13–43	16–53	30–100
***P**	**0.05**	0.001	0.001	

*p < 0.05 is significant

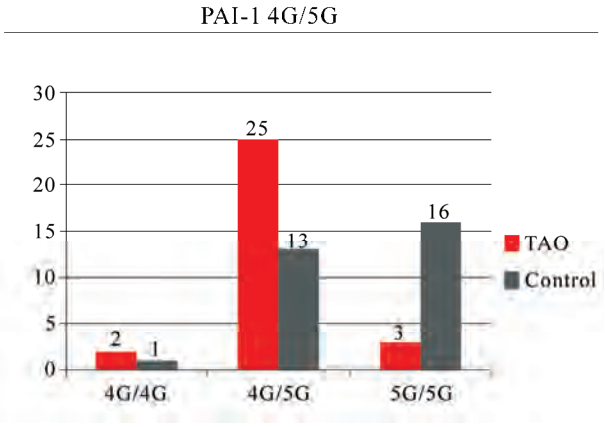


Figure 1. Shows the barr diagram of alternative 5G/4G alleles of PAI-1 gene in TAO and control groups.

4. DISCUSSIONS

Some association has been suggested between Buerger's disease (TAO) and other syndromes but there is still no consensus about diagnostic criteria. TAO is a nonatherosclerotic inflammatory disorder of unknown etiology that affects small and medium-sized vessels of the extremities [1-3]. It is usually present at less than 40 years old in male sex. However, last studies show that this disease can be appears in smoking females [5]. In our study, there were 28 (93%) males, 2 (7%) females' cases in TAO group and the mean age was 42.9 ± 14.3 years. On the other hand, control group was consisted of 27 (90%) males, 3 (10%) females. Additionally 29 (97%) subjects had smoking history in TAO group and 22 (73%) in control group. TAO is diagnosed and treated with difficulty because of elusive etiology (10]. There is no sufficient knowledge about the other risk factors and predisposing conditions in the literature [11,12]. It was documented that there was a strongly relationship between TAO and HLA-9 and HLA-B5. This situation remind to genetical predisposition can be rolled in background of this disorder [5]. The effected vessel was filled with the thrombus formation in microscopical scanning [5]. Prothrombotic risk factors were observed in this disease because of genetical suspicions and thrombotic findings [6]. The 4G/5G alleles in PAI-1 and MTHFR C677T gene polymorphisms may help to identify the couples at risk for recurrent pregnancy loss [13]. Abboud *et al*. showed that the risk of myocardial infarction (MI) was notably high in 4G and -844A carriers with elevated plasma PAI-1 and were associated with reduced tPA levels [14]. PAI-1 concentration increases because of the 4G deletion of 675th promoter region of PAI gene. Therefore, fibrinolytic activity is reduces and predisposing thrombotic events are increases [15]. It was demonstrated that all alone or combined effect of this gene deletion was impacted deep venous thrombosis (DVT), in studies [16]. Choudhury and friends detected that the free PAI-1 levels can be significant venous occlusion in TAO patients [17]. This gene mutation accused in Turkish DVT patients [18]. A meta-analyze signs to 4G/4G genotype was 20% folds increased of myocardial infarction risk that have included 9 studies [19]. Another meta-analyze points to each 4G allele was slightly increased of myocardial infarction risk that have included 37 studies [20]. Recently another meta-analyze that included 17 studies points that there was average relation ship between 4G allele and venous thromboemboli in subjects which have no genetical and acquired prothrombotic risk factors [21]. In our study we detected PAI-1 4G/5G deletion in 25 (83%) subjects in TAO group (**Figure 1**). Although there were 13 (43%) subjects detected with PAI-1 4G/5G deletion in control group. Evaluation of the difference between two groups was statistically significant (p = 0.001) (**Table 1**). Analysis of etiopathogenesis of TAO disease has importance for loss of extremity and labor force by reason of this disease and these results should be verified by larger population studies. In view of previous and current results, there is a clear need to redefine the diagnostic algorithm and the criteria for diagnosing TAO (Buerger's disease).

In conclusion, our study revealed the presence of suspicious relationship between TAO and PAI-1 gene polymorphism. However this is a pilot study including limited number of subjects and it must be supported with large cohort studies. In addition, it is believed that the current results could be helpful for analysis of etiopathogenesis of TAO in the future studies. Additionally, analyses of etiopathogenesis of the TAO disease have importance for prevention of potential losses of extremity and labor force caused by this disease.

REFERENCES

[1] Buerger, L. (1952) Thrombo-angiitis obliterans: A study of the vascular lesions leading to presenile spontaneous gangrene. *American Journal of Medicine*, **13(5)**, 526-532.

[2] Brodmann, M., Renner, W., Stark, G., Winkler, M., Pabst, E., Hofmann, C. and Pilger, E. (2000) Prothrombotic risk factors in patients with thrombangitis obliterans. *Thrombosis Research*, **99(5)**, 483-486.

[3] Cooper, L.T., Tse, T.S., Mikhail, M.A., Mcbane, R.D., Stanson, A.W. and Ballman, K.V. (2004) Long-term survival and amputation risk in thromboangiitis obliterans (Buerger's disease). *Journal of the American College of Cardiology*, **44(12)**, 2410-2411.

[4] Taşoğlu, I., Hanedan, O. and Ulus, A.T. (2008) Thromboangiitis obliterans (buerger hastalığı). *Turkiye Klinikleri Journal of General Surgery Special Topics*, **1(3)**, 15-25.

[5] Szuba, A. and Cooke, J.P. (1998) Thromboangiitis obliterans. An update on Buerger's disease. *Western Journal of Medicine*, **168(4)**, 255-260.

[6] Özen, F., Manduz, Ş., Katrancıoğlu, N., Karahan, O., Köksal, B. and Özdemir, Ö. (2009) Tromboanjiitis obliterans hastalarında protrombotik gen polimorfizminin rolü turkiye klinikleri. *Journal of Cardiovascular Science*, **21(2)**, 160-164.

[7] Isserhoff, R.R. and Rifkin, D.B. (1983) Plasminogen is present in the basal layer of the epidermis. *Journal of Investigative Dermatology*, **80**, 297-299.

[8] Herouy, Y., Trefzer, D., Hellstern, M.O., Stark, G.B., Vanscheıdt, W., Schoè, P.F.E. and Norgauer J. (2000) Plasminogen activation in venous leg ulcers. *British Journal of Dermatology*, **143(5)**, 930-936.

[9] Isordia-Salas, I., Leaños-Miranda, A., Sainz, I.M., Reyes-Maldonado, E. and Borrayo-Sánchez, G. (2009) Association of the plasminogen activator inhibitor-1 gene 4G/5G polymorphism with ST elevation acute myocardial infarction in young patients. *Revista Española de Cardiología*, **62(4)**, 365-372.

[10] Foley, S. and Gibbs, H. (2008) Muir painful digital infarction in a male smoker treated as Buerger's disease. *Australasian Journal of Dermatology*, **49(2)**, 94-97.

[11] Stammler, F., Diehm, C., Hsu, E., Stockinger, K. and Amendt, K. (1996) The prevalence of hyperhomocysteinemia in thrombangitis obliterans. Does homocsteine play a role pathogenetically? *Deutsche medizinische Wochenschrift*, **121(46)**, 1417-1423.

[12] Chenl, Z., Takahashi, M., Naruse, T., Nakajima, T., Chen, Y.W., Inoue, Y., Ishikawa, I., Iwail, T. and Kimura, A. (2007) Synergistic contribution of CD14 and HLA loci in the susceptibility to Buerger disease. *Human Genetics*, **122(3-4)**, 367-372.

[13] Yenicesu, G.I., Cetin, M., Ozdemir, O., Cetin, A., Ozen, F., Yenicesu, C., Yildiz, C. and Kocak, N. (2009) A prospective case-control study analyzes 12 thrombophilic gene mutations in turkish couples with recurrent pregnancy loss. *American Journal of Reproductive Immunology*, **63(2)**, 126-136.

[14] Abboud, N., Ghazouani, L., Saidi, S., Ben-Hadj-Khalifa, S., Addad, F., Almawi, W.Y. and Mahjoub, T. (2010) Association of PAI-1 4G/5G and -844G/A gene polymorphisms and changes in PAI-1/tissue plasminogen activator levels in myocardial infarction: A case-control study. *Genetic Testing and Molecular Biomarkers*, **14(1)**, 23-27.

[15] Francis, C.W. (2002) Plasminogen activator inhibitor-1 levels and polymorphisms. *Archives of Pathology & Laboratory Medicine*, **126(11)**, 1401-1404.

[16] Akar, N., *et al*. (2000) Effect of plasminogen activator inhibitor-1 4G/5G polymorphism in turkish deep vein thrombotic patients with and without FV1691 G-A. *Thrombosis Research*, **97**, 227-230.

[17] Choudhury, N.A., Pietraszek, M.H., Hachiya, T., Baba, S., Sakaguchi, S., Takada, Y. and Takada, A. (1992) Plasminogen activators and plasminogen activator inhibitor 1 before and after venous occlusion of the upper limb in thromboangiitis obliterans (Buerger's disease). *Thrombosis Research*, **66(4)**, 321-329.

[18] Eroglu, A., Ulu, A. and Akar, N. (2006) Plasminogen activator inhibitor-1 gene 4G/5G polymorphism in cancer patients with and without thrombosis. *Journal of Thrombosis and Thrombolysis*, **22(2)**, 111-112.

[19] Boekholdt, S.M., Bijsterveld, N.R., Moons, A.H., Levi, M., Büller, H.R. and Peters, R.J. (2001) Genetic variation in coagulation and fibrinolytic proteins and their relation with acute myocardial infarction: A systematic review. *Circulation*, **104(25)**, 3063-3068.

[20] Ye, Z., Liu, E.H., Higgins, J.P., Keavney, B.D., Lowe, G.D., Collins, R. and Danesh J. (2006) Seven haemostatic gene polymorphisms in coronary disease: Meta-analysis of 66, 155 cases and 91,307 controls. *Lancet*, **367(9511)**, 651-658.

[21] Tsantes, A.E., Nikolopoulos, G.K., Bagos, P.G., Rapti, E., Mantzios, G., Kapsimali, V. and Travlou, A. (2007) Association between the plasminogen activator inhibitor-1 4G/5G polymorphism and venous thrombosis. A meta-analysis. *Journal of Thrombosis and Haemostasis*, **97(6)**, 907-913.

Vol.2, No.5, 458-464 (2010)
doi:10.4236/health.2010.25069

Effect of aerobic training on airflow obstruction, vo2 max, EIB in stable asthmatic children

Ganesan Kathiresan[1]*, Asokan[2]

[1]Department of Physiotherapy, Masterskill University College, Cheras, Malaysia; *Corresponding Author: gans_therapist@yahoo.co.in
[2]Paediatrics Department, Government children Hospital, Chennai, India

Received 7 January 2010; revised 22 January 2010; accepted 23 January 2010.

ABSTRACT

Children with bronchial asthma, primarily those with a clinically more severe disease, tend to have a sedentary lifestyle and therefore be inclined to have lower aerobic fitness than their healthy non-asthmatic peers. Aerobic training has a number of well known beneficial effects in both normal and asthmatic children. However, the impact of training on the clinical management of the underlying bronchial asthma remains controversial, particularly in the most severe patients. Clinical evaluation, spirometric tests, symptom limited maximum exercise testing, and exercise challenge tests were performed in a group of children with stable moderate to severe asthma. Forty two patients (24 boys) aged 8-16 were evaluated twice: before and after supervised aerobic training (group 1, n = 26) and two months apart (untrained group 2, n = 16). In results, Spirometric and maximal exercise variables in the initial evaluation were significantly reduced in group 1 ($p < 0.05$) but medication and clinical scores and the occurrence of exercise induced bronchospasm (EIB) did not differ between the two groups. Aerobic improvement with training (maximal oxygen uptake and/or anaerobic threshold increment > 10% and 100 ml) was inversely related to the baseline level of fitness and was independent of disease severity. Although the clinical score and the occurrence of EIB did not change after training, aerobic improvement was associated with a significant reduction in the medication score and the daily use of both inhaled and oral steroids ($p < 0.05$). In conclusion, results show that the less fit asthmatic children were able to normalize their aerobic fitness with a supervised training programme without clinical complications. Interestingly, I found a significant association between aerobic improvement and reduction in use of both inhaled and oral steroids.

Keywords: Asthma; Children; Exercise Training; Maximal Oxygen Uptake; Anaerobic Threshold; Physical Fitness

1. INTRODUCTION

Researchers have investigated a variety of rehabilitative modes of training in an attempt to ascertain the appropriate mode of exercise, dose, work load, number of repetitions and order and number of exercises in order to bring about favourable improvements in asthmatic symptomology. However, the research regarding the effects of exercise training on asthmatics is sparse and there has continually been a self-inflicted restriction of physical and sporting activities in asthmatics. This is so despite clinicians having advised that exercise can take place when asthmatics use beta-agonists prior to exercise, avoid conditions that are likely to produce exercise- induced asthma and participate in swimming which is deemed less asthmogenic than other forms of exercise [1,2]. Asthmatics can safely and successfully exercise with the correct interventions [3] as seen when 41 medals were won (albeit controversially due to the stimulant effects of asthma medication) at the 1984 Olympic Games by American athletes with a history of asthma or exercise-induced asthma (EIA) in high-respiratory events such as cycling and swimming [4].

Regular physical activity of adequate intensity and duration involving large muscle groups has been proved to have a number of potential beneficial effects on general health, including improvement in aerobic capacity, body composition, flexibility, muscular strength, and psychosocial measures [5]. This improvement might be particularly true for patients who suffer from chronic diseases such as congestive heart failure, chronic obstructive pulmonary disease (COPD), and bronchial asthma. However, patients with chronic respiratory dis-

eases tend to show less tolerance to exercise due to pulmonary limitation, self-restriction of activities, or lack of physical activity secondary to medical advice or family influence [6]. Thus, children with bronchial asthma, primarily those with a clinically more severe disease, tend to have a sedentary lifestyle and therefore be inclined to have lower aerobic fitness than their healthy non-asthmatic peers [6-10].

However, most of the published studies have failed to show a clinical advantage of the improvement in fitness with training [6,8,9,11]. Ludwick *et al.* found that normalisation of cardiopulmonary endurance in 65 severe asthmatic children was not related to improvement in pulmonary function [8]. Robinson *et al.* showed that significant aerobic improvement with training was not associated with changes in use of medication, symptoms, or bronchial responsiveness to histamine [9]. Cochrane and Clark reported that fitness level was not associated with several clinical and functional characteristics of the disease [1]. Recently, Thio *et al.* found that normalisation of cardiovascular fitness in asthmatic children was not related to a lower prevalence of exercise induced bronchospasm (EIB) [11]. These results contrast with the traditional notion that aerobic improvement may have a number of theoretical advantages for asthma control [6]. The objective of this study was to analyse the association between possible changes in aerobic capacity after training and the clinical and functional markers of the severity of bronchial asthma in a group of children with moderate to severe disease.

2. METHODOLOGY

The Research works started with an Education programme for all the patients and their parents) who were included for the study (Both Control and Experimental groups). Education sessions lasted for approximately 45 minutes and included topics like What Asthma? The Aetiological factors, Nutritional aspects and Psychological problems associated with respiratory disability, Other complications, Treatments available, Bronchodilator therapy and Inhaler devices, Nebulizer therapy, long- term oxygen therapy, Risk factor modification, Relaxation, Physiotherapy intervention which included Aerobic training programme. After the Education programme, written informed consent was obtained from the patients. Forty two (42 nos.) asthmatic children were randomly divided in to two groups—Experimental groups (Group 1) 26 children (15 boys and 11 girls) and Control group (Group 2)—16 children had no training (9 boys and 7 girls). Forty two (42 nos.) asthmatic children of mean (SD) age 12.4 (1.8) years (range 8-16) were studied. The mean (SD) body mass and height were 40.4 (9.8) kg and 146.8 (10.3) cm, respectively. Although they were physically active, none was following a regular exercise training programme. Medical follow up was performed by the physician. The children were in a stable phase of the disease, with no exacerbation during the 15 days before the start of the tests. The subjects were referred from an asthma management centre and were consecutively enrolled in an aerobic training programme.

2.1. Study Protocol

A clinical and medication history was obtained from all subjects, followed by physical examination, spirometric tests before and after bronchodilator, and a progressive incremental cardiopulmonary exercise test on a cycle ergometer. On another day the patients performed an exercise challenge test. This protocol was performed twice, before and after training by patients in group 1 and two months apart by those in group 2.

2.2. Outcome Measurements Clinical Evaluation

According to the clinical parameters of the International Consensus Report on Diagnosis and Management of Asthma [12], severe asthma in children is defined as frequent bronchospasm and nocturnal asthmatic symptoms almost daily, peak expiratory flow (PEF) values < 60% predicted at baseline despite optimal therapy, daily use of inhaled anti-inflammatory agents at high doses (beclomethasone > 800 μg/day), and frequent use of systemic corticosteroids (> 3 times/year). Moderate asthma is defined as symptoms requiring inhaled â2 agonists almost daily and nocturnal asthmatic symptoms more than twice a month but not daily, PEF values of 60-80% predicted at baseline but normal after bronchodilator, and daily use of an anti-inflammatory agent at low or moderate doses (beclomethasone < 800 μg/day). All patients were on an inhaled corticosteroid (beclomethasone 500-1500 μg/day) and 33 had a past history of frequent courses of oral corticosteroids (> 3 times/year); nine patients were on systemic steroids at the time of the study. According to the guidelines for the evaluation of impairment/disability in patients with asthma [13], 33 patients had a medication score of > 3 requiring treatment for adequate clinical control: bronchodilator on demand, inhaled beclomethasone in a daily dose of > 800 μg, or more than three courses of oral steroids per year.

2.3. Pulmonary Function Testing (Spirometric Tests)

Patients were asked to refrain from short acting bronchodilators for at least six hours before testing. Spirometric tests were performed in all subjects before and 10 minutes after the inhalation of 200 μg salbutamol via a pressurized metered dose inhaler connected to a spacer. The equipment used was a CPF-S (Medical Graphics Corporation, USA) with flow measurement carried out

with a pneumotachograph Fleisch No. 3. Technical procedures, acceptability and reproducibility criteria were those recommended by the American Thoracic Society [14]. Predicted normal values for all spirometric variables were those of Knudson *et al.* [15]. A positive response to bronchodilator was defined as a 12% and 200 ml increase in the forced expiratory volume in one second (FEV1) [14].

2.4. Cardiopulmonary Exercise Testing

Exercise tests were performed using a digital computer based exercise system (MGC-CPX System; Medical Graphics Corporation (MGC, USA) with breath by breath analysis of metabolic, ventilatory, and cardiovascular variables. The maximal symptom limited exercise test was carried out on a calibrated electromagnetically braked cycle ergometer (CPE 2000; Medical Graphics Corporation), modified with child pedal cranks. The selected work rate was continuously increased in a linear ramp pattern (15 W/min for height < 150 cm; 20 W/min for height > 150 cm) [16] so that the duration of the incremental exercise test was more than eight and less than 12 minutes. The following data were recorded as a moving average of eight breaths: oxygen uptake ($\tilde{V}O_2$, ml/min STPD); carbon dioxide production ($\tilde{V}CO_2$, ml/min STPD); respiratory exchange ratio (R); minute ventilation ($\tilde{V}E$, l/min BTPS), respiratory rate (f, bpm); ventilatory equivalent for oxygen and carbon dioxide ($\tilde{V}E/V\sim O_2$ and $\tilde{V}E/\tilde{V}CO_2$); end tidal partial pressures of oxygen and carbon dioxide ($PETO_2$ and $PETCO_2$, mm Hg); heart rate (HR, bpm), and oxygen pulse ($\tilde{V}O_2$/HR, ml/beat).The predicted $\tilde{V}O_2$max was calculated according to the equations of Cooper *et al.* for children [17], taking into account the close demographic and anthropometric similarity of the subjects. The lower limit of normality for $\tilde{V}O_2$max was defined according to the values proposed by these authors (lower 95% confidence limit; estimated –1.64 × SD): girls < 11 years = 25 ml/minckg; girls > 11 years = 27 ml/minckg; boys < 13 years = 32 ml/minckg; boys > 13 years = 37 ml/minckg. The $\tilde{V}O_2$ at the anaerobic threshold ($\tilde{V}O_2AT$) was measured by the gas exchange method, visually checking the inflection point of $\tilde{V}CO_2$ with regard to $\tilde{V}O_2$ (modified V slope) [18], and by the ventilator method in which $\tilde{V}E/\tilde{V}O_2$ and $PETO_2$ increased while $\tilde{V}E/\tilde{V}CO_2$ and $PETCO_2$ remained stable. The lower limit of normality for $\tilde{V}O_2AT$ was defined as 40% of the $\tilde{V}O_2$max predicted since, of the 109 children evaluated by Cooper *et al.*, only one presented with a value below this cut off point [17].

2.5. Exercise Challenge Test

The test for detection of EIB was carried out with a mechanically braked Monark® cycle ergometer. Methylxanthines and â2 adrenoceptor agonists were withheld 12 hours prior to the test. Before the exercise the patient performed a forced expiratory manoeuvre (Vitatrace® spirometer) with assessment of basal values of forced vital capacity (FVC) and FEV1. The test was carried out only in patients with normal spirometric values at rest—that is, FEV1 above the lower 95% confidence limit (n = 35) [15]. The tests were performed at a room temperature of 18-26°C, barometric pressure 680-703 mm Hg, and relative humidity 55-60%.After one minute of light exercise on the cycle ergometer the work load was quickly increased until the heart rate corresponded to 80% of predicted (220-age), and then maintained for six minutes [19]. Spirometric evaluations were performed successively at five, 10, and 20 minutes after the exercise. EIB was defined as being present if FEV1 showed a reduction equal to or greater than 10% of its pre-exercise values [16].

2.6. Aerobic Exercise Training

The two month indoor aerobic training programme on a cycle ergometer was performed by 26 asthmatic children (group 1). Medical supervision was provided during all training sessions. The sessions were performed three times a week (total 24). Inhaled â2 adrenergic bronchodilators were administered 10 minutes before training. The target heart rate during the training sessions was individualised. This initially corresponded to the anaerobic threshold and was increased every two weeks to an intensity which the subject was able to endure for 30 minutes. A typical session consisted of 10-15 minutes of callisthenics and stretching warm up exercises, 30 minutes of continuous aerobic activity on a cycle ergometer, and a five minute period of cooling down.

2.7. Statistical Analysis

Mean (SD) values are reported. Paired and non-paired *t* tests were used to compare within group and between group evaluations, respectively; the ÷ 2 (Fisher exact) test to evaluate the association between changes in clinical and functional outcomes and response to training; and Pearson product moment correlation to assess linear association between variables. The level of statistical significance was always set at $p < 0.05$.

3. RESULTS

3.1. Pre Evaluation

In the basal evaluation mean (SD) values of the main spirometric variables were significantly lower in patients in group 1 than in those in group 2 (**Table 1**). However, as anticipated by clinical stability, only two patients in group 1 had post-bronchodilator FEV1 values below the lower 95% confidence limit. Furthermore, in patients in group 1 the main parameters of maximum aerobic performance ($\tilde{V}O_2$max, $\tilde{V}O_2AT$, O_2 pulse max) were sig-

nificantly reduced when compared with those of group 2. On the other hand, the clinical and medication scores and prevalence of a positive EIB test were not significantly different between the two groups.

3.2. Post Evaluation

The supervised aerobic training was well tolerated and completed by all children in group 1. Only one child experienced a mild episode of EIB during a training session, but it was promptly reversed with an inhaled bronchodilator. Although significant only for the O_2 pulse max ($p < 0.05$), we found a trend towards an increase in the maximal exercise variables in the trained group. On the other hand, a worsening trend was identifiable in the untrained children, although this reached statistical significance only for $\tilde{V}O_2AT$. There were, however, no significant changes in the clinical outcomes in the two groups (**Table 1**). As the response to exercise training has consistently been reported to be inversely related to the baseline values [1,20,22], we analysed the relationship between the degree of improvement (post–pre/pre) and the initial level of fitness.

As shown in **Figure 1**, there was a strongly negative relationship between these variables—that is, the less fit the subject the higher the aerobic gain with training. A positive response to training ($\tilde{V}O_2max$ and/or $\tilde{V}O_2AT$

Table 1. Clinical and functional characteristics of the asthmatic children at Pre and Post evaluations.

Variables		Group 1 (n = 26)	Group 2 (n = 16)
FEV1 pre-BD (% pred)	Pre Evaluation	78.3 (16.4)	99.1 (18.8)*
	Post Evaluation	80.2 (15.5)	97.6 (17.4)*
FEV1 post-BD (% pred)	Pre Evaluation	92.1 (18.7)	109.0 (19.3)*
	Post Evaluation	91.4 (17.1)	106.5 (15.3)*
$\tilde{V}O_2max$ (% pred)	Pre Evaluation	83.4 (10.5)	99.0 (19.4)*
	Post Evaluation	87.8 (11.1)	91.1 (18.0)
$\tilde{V}O_2max$ (l/min)	Pre Evaluation	1444 (215)	1659 (287)*
	Post Evaluation	1523 (202)	1585 (273)
$\tilde{V}O_2AT/V\tilde{}O_2max$ (%)	Pre Evaluation	46.2 (7.8)	59.5 (12.6)*
	Post Evaluation	48.3 (9.1)	47.2 (13.4)†
O_2 pulse (% pred)	Pre Evaluation	91.2 (14.1)	114.1 (23.3)*
	Post Evaluation	106.5 (17.1)†	106.3 (30.1)
Severe asthma [9]	Pre Evaluation	22 (84%)	11 (69%)
	Post Evaluation	18 (69%)	13 (81%)
Medication score >3 [10]	Pre Evaluation	22 (84%)	11 (69%)
	Post Evaluation	15 (57%)	14 (87%)
EIB positive [16]	Pre Evaluation	13 (50%)	8 (57%)
	Post Evaluation	12 (46%)	10 (71%)

Values are mean (SD) for continuous variables and frequency (approximated%) for the others.
FEV1 = forced expiratory volume in one second; FVC = forced vital capacity;
BD = bronchodilator; $\tilde{V}O_2$ = oxygen consumption;
AT = anaerobic threshold; O_2 pulse = $\tilde{V}O_2$/heart rate; max = relative to maximum exercise.
*$p < 0.05$ (group 2 > group 1; non-paired *t* test).
†$p < 0.05$ (final evaluation vs initial evaluation; paired *t* test).

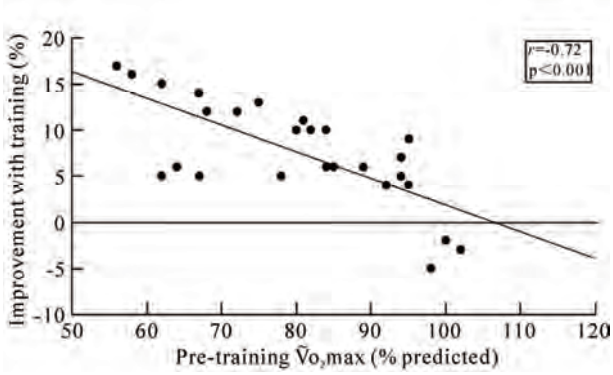


Figure 1. Relationship between baseline maximal aerobic fitness and degree of improvement after training in 26 children with moderate to severe asthma.

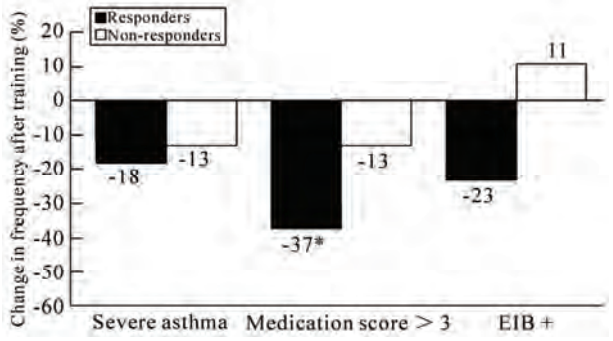


Figure 2. Association between changes in clinical indicators of asthma severity and positive (responders) or negative (non-responders) response to aerobic training. *p < 0.05 (Fisher's exact test).

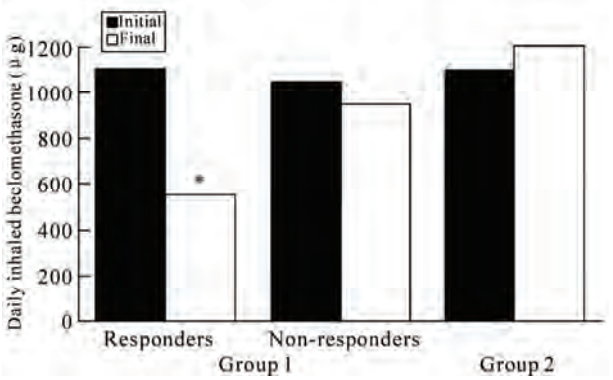


Figure 3. Mean values of daily inhaled beclomethasone in the initial and final evaluations. Group 1 = trained children with (responders) and without (non-responders) aerobic improvement after training; group 2 = untrained children.*p < 0.05 (paired t test).

post-training – pre-training/pre-training × 100 more than 10% and 100 ml) was found in 11 subjects (42%); All of these training "responders" presented an abnormally reduced $\tilde{V}O_2max$ and $\tilde{V}O_2AT$ at the initial evaluation. Although clinical and functional variables at baseline did not differ between "responders" and "non responders", after training the "responders" had a lower prevalence of subjects with a medication score of > 3 ($p < 0.05$, **Figure 2**). In addition, a significant reduction was seen in the mean dose of daily inhaled steroids in the "responders" group (from 1125 (225) to 575 (150) μg/day, $p < 0.05$, **Figure 3**). Finally, in five (of nine) children oral steroids were able to be withdrawn; interestingly, all of them showed aerobic improvement with training.

4. DISCUSSIONS

We have evaluated the effect of a supervised aerobic training programme on the cardio respiratory fitness and clinical indicators of control in a group of children with moderate to severe but stable asthma. The degree of response to training and the positive effect on the clinical management were strongly influenced by the level of fitness in the initial evaluation; beneficial effects were shown only in the less fit patients. Our results suggest that exercise therapy for the most untrained children can have a role, at least in the short term, in reducing the minimal edication needed for control of moderate to severe asthma. Previous studies in normal and asthmatic patients have shown that the initial grade of fitness and motivation is an important predictor of aerobic improvement after training [1,20,22]. Cochrane and Clark, for example, found that the relative gain in $\tilde{V}O_2max$ after training in asthmatic subjects was negatively related to symptom score on the training day and the baseline level of fitness, and positively to motivation [1]. Thus, the improvement after training in muscular capillarisation, oxidative capacity, muscular strength, and cardio circulatory adjustments is likely to occur in motivated subjects with worse baseline aerobic conditions. However, it should be recognised that the so called "regression to the mean" effect cannot be ruled out by this finding; the random longitudinal variation tends to increase the lower values of a given distribution or, alternatively, the higher values tend to randomly decrease with time. Nevertheless, as previously noted, this higher potential for improvement in the most unfit individuals is a well known phenomenon and it seems highly improbable that such a statistical artefact would be consistently related to another measurable biological effect such as the reduction in inhaled steroids (**Figures 2** and **3**). In this context, there is now growing evidence to show that the systemic effects of inhaled beclomethasone in children are dose dependent [23,24] and medication usage seems to be a particularly useful index of overall asthma control [12,13]. Although the respiratory system is usually considered to be largely insensitive to training effects per se

(with the possible exception of muscle ventilatory strength and endurance), a cause-effect relationship could explain this association. Thus, one can speculate that a possibly lower occurrence of EIB after training would induce a lower chronic release of inflammatory mediators and therefore reduce the need for inhaled steroids. However, we did not find a significant reduction in the prevalence of a positive EIB test with training, regardless of whether or not there was a positive response (**Figure 2**). Another more plausible hypothesis is that the improvement was related to a higher degree of acceptance and level of self-care in the least fit patients who usually have negative attitudes toward their disease and exertion [25,26]. Thus, Strunk *et al.* [25] showed that the wide variability in aerobic performance in a group of 90 children with moderate and severe asthma was mainly related to the degree of social and disease adjustment. Engström *et al.* [26] in a group of 10 severely asthmatic children submitted to physical training showed that only psychological modifications correlated significantly with aerobic improvement. Thus, individual variations in acceptance and knowledge of the disease seem to influence the usual level of physical activity in asthmatic children, and therefore their degree of fitness. In this context, exercise training may induce a more decided posture in relation to the disease, with consequences in the minimum medication required for clinical control.

Results are consistent with those of Thio *et al.* [11] who were not able to find a lower prevalence of EIB after dynamic exercise training, although in a previous cross sectional study we found an association between a reduction in $\tilde{V}O_2AT$ and a higher prevalence of EIB in asthmatic children [27]. While one can predict a reduction in EIB with aerobic improvement (secondary to training induced lower submaximal ventilation) [28], in our study this enhancement alone was probably not sufficient to reduce the EIB, at least when assessed in a formal challenge test.

A particularly notable finding was the relative inefficacy of the training programme in improving the maximal aerobic parameters in almost 60% of the children. However, one should recognise that maximal incremental testing is not representative of the daily pattern of exercise activities in the paediatric group (which is better characterised by short bursts of activity); new submaximal protocols have been suggested to be more suitable for evaluating training responses in children [29]. In addition, the degree of fitness in the initial evaluation was above that expected for asthmatic children (**Table 1**) and the low pre-intervention prevalence of unfit children could have induced a type II error. This finding is consistent, however, with the suggestion that secular trends do not reduce the average aerobic fitness of westernised children [30]. Our results suggest that this is also the case for asthmatic children, at least those with the studied profile. In conclusion, our results show that the less fit asthmatic children were able to normalize their aerobic fitness with a supervised training programme without clinical complications. Their ability to improve aerobic capacity was not related to clinical and spirometric severity before training. Interestingly, we found a significant association between aerobic improvement and reduction in use of both inhaled and oral steroids.

Further research using larger samples is needed to confirm these findings and to assess the duration of the training induced beneficial effects in treatment requirements, the effectiveness of long term aerobic exercise, the response to submaximal protocols and, finally, to determine which profile of childhood asthma is likely to respond to exercise therapy.

REFERENCES

[1] Cochrane, L.M. and Clark, C.J. (1990) Benefits and problems of a physical training programme for asthmatic patients. *Thorax*, **45(5)**, 345-351.

[2] Weisgerber, M., Guill, M., Weisgerber, J. and Butler, H. (2003) Benefits of swimming in asthma: Effect of a session of swimming lessons on symptoms and PFTS with review of the literature. *Journal of Asthma*, **40(5)**, 453-464.

[3] Nagel, F. Asthma, sport, and exercise. http://www.asthma.co.za/articles/

[4] Haas, F., Pasierski, S., Levine, N., Bishop, M., Axen, K., Pineda, H. and Haas, A. (1987) Effect of aerobic training on forced expiratory airflow in exercising asthmatic humans. *Journal of Applied Physiology*, **63(3)**, 1230-1235.

[5] Lea and Febiger (1991) Guidelines for graded exercise testing and training. 4th Edition, *American College of Sports Medicine*, Philadelphia.

[6] McFadden, E.R. (1984) Exercise performance in the asthmatic. *American Review of Respiratory Disease*, **129**, 584-587.

[7] Garfinkel, S.K., Kesten, S., Chapman, K.R. and Rebuck, A.S. (1992) Physiologic and non-physiologic determinants of aerobic fitness in mild to moderate asthma. *American Review of Respiratory Disease*, **145**, 741-745.

[8] Ludwick, S.K., Jones, J.K., Jones, T.X., Fukuhara, J.T. and Strunk, R.C. (1986) Normalization of cardiopulmonary endurance in severely asthmatic children after bicycle ergometry therapy. *Journal of Pediatrics*, **109(3)**, 446-451.

[9] Robinson, D.M., Egglestone, D.M., Hill, P.M., Rea, H.H., Richards, G.N. and Robinson, S.M. (1992) Effects of a physical conditioning programme in asthmatic patients. *New Zealand Medical Journal*, **10(937)**, 253-256.

[10] Strunk, R.C., Rubin, D., Kelly, L., Sherman, B. and Fukuhara, J. (1988) Determination of fitness in children with asthma: Use of standardized tests for functional endurance, body fat composition, flexibility, and abdominal strengh. *American Journal of Diseases of Children*, **142(9)**, 940-944.

[11] Thio, B.J., Nagelkerke, A.F., Ketel, A.G., van Keeken, B.L. and Dankert-Roelse, J.E. (1996) Exercise-induced

asthma and cardiovascular fitness in asthmatic children. *Thorax*, **51(2)**, 207-209.

[12] National Heart Lung and Blood Institute (1992) International consensus report on diagnosis and management of asthma, Publication No. 92-3091.

[13] American Thoracic Society (1993) Guidelines for the evaluation of impairment/disability in patients with asthma. *American Review of Respiratory Disease*, **147(4)**, 1056-1061.

[14] American Thoracic Society (1991) Lung function testing: Selection of reference values and interpretative strategies. *American Review of Respiratory Disease*, **144(5)**, 1202-1218.

[15] Knudson, R.J., Lebowitz, M.D., Holberg, C.J. and Burrows, B. (1983) Changes in the normal maximal expiratory flow-volume curve with growth and aging. *American Review of Respiratory Disease*, **127(6)**, 725-734.

[16] Godfrey, S., Davies, C.T.M., Wozniak, E. and Barnes, C.A. (1971) Cardiorespiratory responses to exercise in normal children. *Clinical Science*, **40**, 419-431.

[17] Cooper, D.M., Weiler-Ravell, D., Whipp, B.J. and Wasserman, K. (1984) Aerobic parameters of exercise as a function of body size during growth in children. *Journal of Applied Physiology*, **56(3)**, 628-634.

[18] Beaver, W.L., Wasserman, K. and Whipp, B.J. (1986) A new method for detecting the anaerobic threshold by gas exchange. *Journal of Applied Physiology*, **60(6)**, 2020-2027.

[19] Eggleston, P.A., Rosenthal, R.R., Anderson, S.A., Anderton, R. and Bierman, C.W. (1979) Guidelines for the methodology of exercise challenge testing of asthmatics. *Journal of Allergy and Clinical Immunology*, **64**, 642-645.

[20] Fink, G., Kaye, C., Blau, H. and Spitzer, S.A. (1993) Assessment of exercise capacity in asthmatic children with various degrees of activity. *Pediatric Pulmonology*, **15(1)**, 41-43.

[21] McArdle, W.D., Katch, F.I. and Katch, F.L. (1996) Training for anaerobic and aerobic power. 4th Edition, *Exercise physiology*, Baltimore: Williams & Wilkins, 393-415.

[22] Brodal, P., Ingjer, F. and Hermansen, L. (1977) Capillary supply of skeletal muscle fibers in untrained and endurance-trained men. *American Journal of Physiology*, **232**, H705-H712.

[23] Hanania, N.A., Chapman, K.R. and Kesten, S. (1995) Adverse effects of inhaled corticosteroids. *American Journal of Medicine*, **98(2)**, 196-208.

[24] Yiallourds, P.K., Milner, A.D. and Conway, E. (1997) Adrenal function and high dose inhaled corticosteroids for asthma. *Archives of Disease in Childhood*, **76(5)**, 405-410.

[25] Strunk, R.C., Mrazek, D.A., Fukuhara, J.T., Masterson, J., Ludwick, S.K. and LaBrecque, J.F. (1989) Cardio vascular fitness in children with asthma correlates with psychologic functioning of the child. *Pediatrics*, **84(3)**, 460-464.

[26] Engstrom, I., Falstrom, K. and Karlborg, E. (1991) Psychological and respiratory physiological effects of a physical exercise programme on boys with severe asthma. *Acta Paediatr Scand*, **80**, 1058-1062.

[27] Nery, L.E., Silva, A.C., Neder, J.A., Cabral, A.L. and Fernandes, A.L. (1994) Exercise tolerance and anaerobic threshold (AT) in children with moderate to severe bronchial asthma. *American Journal of Respiratory and Critical Care Medicine*, **149**, A786.

[28] Bungaard, A., Ingemann-Hansen, T., Schimdt, A. and Halkjaer-Kristensen, J. (1981) The importance of ventilation in exercise-induced asthma. *Allergy*, **36(6)**, 385-389.

[29] Cooper, D.M. (1995) Rethinking exercise testing in children: A challenge. *American Journal of Respiratory and Critical Care Medicine*, **152**, 1154-1157.

[30] Santuz, P., Baraldi, E., Filippone, M. and Zacchello, F. (1997) Exercise performance in children with asthma: Is it different from that of healthy controls? *European Respiratory Journal*, **10(6)**, 1254-1260.

Vol.2, No.5, 465-476 (2010)
doi:10.4236/health.2010.25070

Health

Health measurement

Paul Andrew Bourne

Department of Community Health and Psychiatry, The University of the West Indies at Mona, Kingston, Jamaica;
paulbourne1@yahoo.com

Received 21 December 2009; revised 14 January 2010; accepted 17 January 2010.

ABSTRACT

Jamaicans are not atypical in how they conceptualize health and/or how they address patient care as the antithesis of diseases or dysfunctions (*i.e.* health conditions). In the 1900s and earlier, Western Societies were using the biomedical model in the measurement and treatment of health, health attitudes and the utilization of health services. This approach emphasizes sickness, dysfunction, and the identification of symptomology or medical disorders to evaluate health and health care. Such an approach places significance on the end (*i.e.* genetic and physical conditions), instead of the multiplicity of factors that are likely to result in the existing state, or issues outside of the space of dysfunctions. Notwithstanding the limitations of the biomedical approach, it is still practiced by many Caribbean societies, and this is fundamentally the case in Jamaica. The current paper is an examination of health measurement, and provides at the same time a rationale for the need to have a more representative model as opposed to the one-dimensional approach of using pathogens in measuring health. Owing to the importance of health in development, patient care and its significance for other areas in society, this paper seeks to broaden more than just the construct, as it goes to the core of modern societies in helping them to understand the constitution of health and how patient care should be treated. Thus, it provides a platform for the adoption of the biopsychosocial model, which integrates biological, social, cultural, psychological and environmental conditions in the assessment of health and the outcome of research, by using observational survey data.

Keywords: Health; Biomedical Model; Biopsychosocial Model; Determinants; Antithesis of Diseases; Health Measurement; Jamaica

1. INTRODUCTION

The construct of health is more than a concept. It is a "leading characteristic of the members of a population..." [1] and, ergo, it plays a direct role in the images of health and health care. Among the plethora of reasons for the importance of health are not merely the images created by the construct, but also its contribution to the production of different tenets of human existence—illness, morbidity, comorbidity, disability, mortality, life expectancy, wellbeing, and so on, as well as the guide that it affords for health interactions and interventions. In addition to the aforementioned issues, it is of germane significance in aiding us to understand many of the things that we see. The definition of this single term "health" is important, as a precise use of the construct fashions and connects other important applications such as growth and development, productivity, health care and people's expectations of health care professionals. One scholar, in helping us to understand the meaning of a construct, says that "without a well-defined construct, it is difficult to write good terms and to derive hypotheses for validation purposes" [2]. Embedded in Spector's argument is the "theoretical abstraction" of the construct, and how we may use it for outcome research. In this paper, the author will review the existing literature and identify particular measures of health, examining how these differ from the WHO's conceptual definition of health [3]. At the same time, within the limitations of the biomedical model, the study will evaluate the usefulness of the biopsychosocial model in health and how the image of health influences the health care of people.

1.1. Image of Health

Health, however, is more than a "theoretical abstraction". There is an "objective reality" to this construct. It explains life, and life is an objective reality. Furthermore, health is a valuable tool that "drives" health policies and influences the determinants of health care. Then there is the issue of health care and how this is planned for, as well as the role that health plays in the development of a society. Health, wellbeing and poverty are well documented in developmental economics by scholars such as

Amartya Sen, Paul Streeten and Martin Ravallion as having critical roles in understanding human development (or the lack of it). The fascination with health and wellbeing in developmental studies is primarily because of the direct association between development and health.

Jamaica is not atypical in how its people conceptualize health and/or how they address patient care. In the 1900s and earlier, western societies used the biomedical approach in the measurement and treatment of health [5]. The biomedical approach emphasizes sickness, dysfunction, pathogens, and disability and medical disorders in the construction of health. This approach places importance on the outcome (or the end) instead of the multidimensional conditions that are likely to result in the existing state. Notwithstanding the limitations of the biomedical approach, it is still practiced by many Caribbean societies, and this is fundamentally the case in Jamaica. This is atypical in many Western nations, as contemporary demographers still use the antithesis of illness and disability to write about health [6-8]. Rowland wrote that "Measures of population health are of general interest to demographers, sociologists, geographers and epidemiologists. Interdisciplinary concerns here include comparing national progress through the epidemiologic transition, and identifying social and spatial variations within countries in patterns of disease and mortality [5].

The United States has left many Caribbean societies behind in how they conceptualize health and treat health care. As early as the commencement of the 20th century [4], the United States shifted their focus from negative wellbeing (*i.e.* antithesis of diseases) to positive wellbeing. The antithesis of diseases assumes a bipolar opposite between health and diseases. Embedded in this bipolar thinking is that for one to be healthy, he/she must not be experiencing any symptomology of dysfunctions. Hence, the health of people is measured by mortality or morbidity statistics. Health, however, is more than just the antithesis of diseases to positive psychology, inclusive of socio-cultural conditions and the environment. Positive wellbeing encapsulates the biomedical model in addition to psychological, socio-cultural and environmental conditions. The name that Engel gave to this new approach is the biopsychosocial model. The current paper is a discourse on the limitation of the biomedical model, which will provide a rationale for the need to have a more representative model as against this one-dimensional approach to the measurement of health.

Traditionally, health was conceptualised as the "antithesis of diseases" [4]. Using the antithesis of diseases, this construct utilizes a minimization approach or a negative perspective, adopted by western societies, which saw health as the absence of dysfunctions, morbidity conditions or comorbidity. "This definition of health has been largely the result of the domination of the biomedical sciences by a mechanistic conception of man. Man is viewed by physicians primarily as a physiochemical system" [9]. With this thinking, health professionals' evaluation of patient care and diagnostic treatments is based primarily on the identification of any symptomology of dysfunctions. Hence the standard that is used in the evaluation of health is the established norm of any deviation from diseases. Rather than conceptualizing health and stating its determinants, this approach uses the identification of symptomology to measure health. Therefore, life expectancy is used here as a measure of health. This assumes that once an individual is alive, it is because there are no dysfunctions to cause death. Embedded in this association is the influence of dysfunctions on health, but there are no other determinants of health except the various symptomologies of diseases.

Outside of diseases, there are other determinants of health. Based on the biopsychosocial model that George Engel [10,11] developed, he proposed an approach to the treatment of the health care of psychiatric patients that included biological, social and psychological conditions. Such a conceptual framework, unlike the biomedical sciences, introduces and identifies factors that are responsible for the health, and by extension the wellbeing, of a population. One scholar cites that "the states of health and disease are the expressions of the success or failure experienced by the organism in its efforts to respond adaptively to environmental changes" [12]. Again, when health is defined as the antithesis of diseases its determinant is solely biological, but this is clearly one -dimensional, and many scholars have shown that health is, in fact, multidimensional, and composed of biopsychosocial and environmental conditions.

Another aspect to health is the positive association between the determinants of health and health care policies. Health care policy makers use the determinants of health as the benchmark that directs their planning. Therefore, when health policies are too narrow, the health determinants which fashion a population's health care will take a minimal approach, as this is based on the image of health. One scholar puts it succinctly, "…health policies affect health through their effects on health determinants" [13], which speaks to the importance of "good" hypotheses in the schema of things. It should be noted that the hypotheses allow us to derive the possible determinants of health, which would be used to evaluate the effectiveness of the health policy, and so show how they affect health (see **Figure 1**).

The goal of the policy is to decrease the incidence of chronic diseases, high risk sexual behaviour/violence and injury through the adaptation of appropriate behaviours by the population and particularly young children, adolescents and young adults [14].

The general conceptualization of health in Jamaica is the "antithesis of diseases". This explains why many people emphasize health care for morbidity conditions,

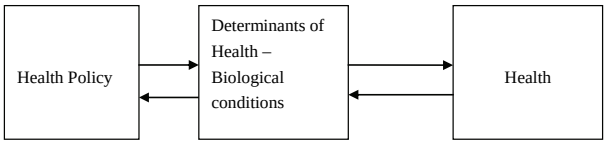


Figure 1. The relation between health policy and health, and the roles of health determinants.

genetics, or physical functioning (*i.e.* their biology). Another indicator of the usage of this perspective can be seen in how data are collected on health in Jamaica and/or in the wider Caribbean. Such a situation highlights the minimization or substantially negative approach in the construct of health. Despite the title of the Ministry of Health's "National Policy for the Promotion of Healthy Lifestyle in Jamaica", throughout the paper the MOH [14] emphasizes mortality, diseases, dysfunctions and reproductive health, which highlights Jamaicans' perspective on health. This is also evident in the Planning Institute of Jamaica which is responsible for policy, along with the Statistical Institute of Jamaica, collecting information on health by way of 1) preventative (*i.e.* behaviour modification), curative (surgical procedures, visits to health practitioners), restorative (physical rehabilitation), and palliative (*i.e.* pain management) measures, and ownership of health insurance. Thus, the hypotheses that arise from the collected data are in keeping with the narrowed definition for which the data was initially gathered by the research design exercise. The hypothesis of the presence of pathogens such as poor air being the cause of diseases, or classification of ill-health, is ancient, within the context that health has been expanding from mere physical functioning for some time. This hypothesis assumes that a person who does not have an ailment (or disease condition) is healthy, which is categorically false, as health psychologists have shown that psychological conditions do influence wellbeing [4]. This perspective dates back to Galen in Ancient Rome (*i.e.* 130 CE-200 CE). A point is even more forcefully made in a study by two economists, which found a strong direct relationship between happiness and wellbeing [15]. Other researchers found an association between "positive and/or negative" mood(s) and wellbeing [16]. This paper is in two parts, designed: 1) to provide detailed evidence that will support the rationale for an expanded concept which looks at health and wellbeing; and 2) to illustrate the purpose and significance of the expanded model that Engel termed the biopsychosocial model. This paper however is not arguing for a biopsychosocial hybrid model, which would include biological, economic, social, cultural, psychological and ecological conditions.

2. PHYSICAL FUNCTIONING

Caring for patients suffering from ill-health has a long history, which dates back to the Agrarian societies. During those earlier periods, man in his quest to address health conditions did so primarily from the standpoint of physical functionality. Based on the annals of time, the literature showed that people would treat biological dysfunctions and sometimes the "spirit" in their pursuit of making man healthier. This approach dates back as far as ancient Rome (*i.e.* 130 CE-200 CE). Despite the WHO offering us a better way in the pursuit of happiness and wellness, man continues to return to the biomedical model of health. One of the reasons for the continued acceptance of the use of the biomedical model is the dominance of technology in this process. As technology is still primarily intended to address physical dysfunctions and the absence of pathogens, many studies conducted in early societies have not only linked the concept of health to medical conditions and by extension health care, but have served as another important indicator in determining lifespan.

In 1884, an Englishman named Francis Galton who was both a mathematician and medical doctor researched the "physical and mental functioning" of some 9,000 people between the ages of 5 and 80 years [17]. Galton wanted to measure the human life span in relation to the physical and mental functioning of people, so he sponsored a health exhibition that would allow him to collect data for analysis. Health was traditionally defined as the "antithesis of diseases", which explains the predominance of physical functioning in policy making and health care, and justifies Galton's wanting data on the physical functioning of people.

The 20th century has brought with it massive changes in the typologies of dysfunctions, where deaths have shifted from infectious diseases such as tuberculosis, pneumonia, yellow fever, Black Death (*i.e.* Bubonic Plague), smallpox and "diphtheria" to illnesses such as cancer, heart disease and diabetes [14]. Although diseases have shifted from infectious to degenerate, chronic non-communicable illnesses and science, medicine and technology have expanded since then, and the image of health in contemporary Jamaica still lags behind many developed nations. Morrison [18] titled an article "Diabetes and Hypertension: Twin Trouble" in which he establishes that diabetes mellitus and hypertension have now become problems in Jamaicans and in the wider Caribbean. This situation was equally corroborated by Callender [19] and Steingo [20] at the 6th International Diabetes and Hypertension Conference, which was held in Jamaica in March 2000. They found that there is a positive association between diabetic and hypertensive patients—50% of individuals with diabetes had a history of hypertension [19,20]. Prior to those scholars' work, Eldemire [21] found that 34.8% of new cases of diabetes and 39.6% of hypertension were associated with senior citizens (*i.e.* ages 60 and over). Accompanying this period of the "age of degenerative and man-made ill-

nesses" are life expectancies that now exceed 50 years.

Before the establishment of the American Gerontology Association in the 1930s and their many scientific studies on the ageing process [17], many studies were done based on the biomedical model, *i.e.* physical functioning or illness and/or disease-causing organisms [4]. Many official publications used either reported illnesses or the prevalence of seeking medical care for measuring sicknesses. Some scholars have still not moved to the post biomedical predictors of health status. The dominance of this approach is so strong and present within the twenty-first century, that many doctors are still treating illnesses and sicknesses without an understanding of the psychosocial and economic conditions of their patients. To illustrate this more vividly, the researcher will quote a sentiment expressed by a medical doctor in The Caribbean Food and Nutrition Institute's Quarterly [22]. A public health nutritionist, Dr. Kornelia Buzina [23], says, "When used appropriately, drugs may be the single most important intervention in the care of an older patient … and may even endanger the health of an older patient …" This proposition highlights the paradox in biomedical sciences as well as showing the need to expand the image of health beyond this negative approach to it.

Within the context of the WHO's definition and growing numbers of studies that have concluded that health should be a multidimensional construct, in 2007 a group of medical practitioners used physical functionality and dysfunctions to treat an elderly patient who was suffering from a particular health condition [24]. The researchers put forward an examination of a 74-year old man who with "...a long history of ischaemic heart disease, presented with increasingly prolonged episodes of altered consciousness" [24]. The physicians cite the argument that "many elderly patients may have more than one cause for this symptom" [24], which summarizes their perspective and reliance on understanding medical disorders in the dispensing of patient care. Throughout the study, the scholars and medical practitioners did not seek to evaluate the psychological, social, and environmental conditions and their possible influence on the current state of dysfunction of the elderly patient. Despite the seeming complexity of the result of the detailed inquiry into the neurological conditions of the patient, and the keen medical examination of the patient, his medical condition continued for years unabated. This emphasises the dominance of the biomedical model, and it goes beyond this single study, as a review of publications in the West Indian Medical Journal—a medical journal in Jamaica—from 1960-2009 revealed a few studies that have gone beyond the use of the biomedical approach to the examination of patient care.

In seeking to treat the 74-year old patient, the medical practitioners examined and re-evaluated various medical problems. Thus, owing to the thinking of this group of researchers, they used "multiple medications" in the treatment of the patient's condition. It was clear from the perspective of the scholars that what guided their intervention were the biomedical sciences (*i.e.* physical functionality or dysfunctions). In this case, health is the "antithesis of diseases". It is the narrow definition of health —negative health (*i.e.* biomedical approach) —which explains the image of health and health care for those scholars and researchers. Apart from the reasons for the use of diagnosed conditions, life expectancy and other physical issues are utilized in examining health, because of the precision in using them to evaluate health as against other approaches that are more holistic and broader in scope.

2.1. Health Measurement

The narrow definition of health is the "antithesis of diseases" which Longest [13] says is the "…absence of infection or the shrinking of a tumour" which can be called dysfunctions (see [1,4]. As we mentioned earlier, the "antithesis of diseases" idea dates back to Galen in Ancient Rome. It was widespread in the 1900s, and so medical professionals used this operational definition in patient care. Another fact during this time was that technology was fashioned in this regard, addressing solely physical dysfunctions. This definitional limitation may be a rationale for the World Health Organization, nearing the mid-1900s, declaring that health is the "state of complete physical, mental, and social wellbeing, and not merely the absence of diseases or infirmity" [3]. It should be noted that this conceptual definition which is in the Preamble to the constitution of the WHO which was signed in July 1946 and became functional in 1948, according to one scholar, from the Centre of Population and Development studies at Harvard University, is a mouthful of sweeping generalizations. According to Bok [25], the definition offered by the WHO is too broad and difficult to measure, and at best it is a phantom. Other intelligentsia point to the WHO's definition as a difficulty for policy formulation, because its scope is "too broad" [26]. The question is "Is the conceptual definition formulated by WHO so broad that those policies faced difficulty in formation", and by extension should we regress to a pre-1946 conceptualization of health because a construct is difficult to operationalize today? Undoubtedly, health extends beyond diseases and is tied to cultural and psychological elements, personal responsibility, lifestyle, environmental and economic influences as well as quality nutrition [27-41]. Those conditions are termed determinants of health [26].

The WHO's perspective must have stimulated Dr. George Engel to pursue a modification of the narrow approach to the health and health care debate. Dr. Engel was a psychiatrist who formulated the construct called the biopyschosocial model in the 1950s. He believed that

when a patient comes to a doctor, for example for a mental disorder, the problem is a symptom not only of actual sickness (biomedical), but also of social and psychological conditions [10,11]. He therefore campaigned for years for physicians to use the biopsychosocial model for the treatment of patients' complaints, as there is an interrelationship between the mind, the body and the environment. He believed so deeply in the model, convinced that it would help in understanding sickness and providing healing, that he introduced it into the curriculum of Rochester medical school [42,43]. Medical psychology and psychopathology was the course that Engel introduced into the curriculum for first year medical students at the University of Rochester. This approach to the study and practice of medicine was a paradigm shift from the biomedical model that was popular in the 1980s and 1990s.

The Planning Institute of Jamaica and the Statistical Institute of Jamaica employ the biomedical model in capturing the health status and/or wellbeing of the populace. This approach was obsolete by the late 20th century, as in 1939 Cowdy, E.V. a cytologist in the United States; expanded on how ageing and health status should be studied in the future. Cowdy broadened the biomedical model in the measurement of the health status of older adults by including social, psychological and psychiatric information in his study entitled the "Problem of Ageing" [17]. The Ministry of Health [MOH] [14], however, has published a document in which it shows that health interfaces with biomedical, social and environmental conditions. One of the reasons put forward by the MOH to help in understanding why they arrived at the aforementioned position, was the rationale behind the explanation for the changes in the typology of diseases – that is, from infectious and communicable diseases to chronic conditions. The institution cites that this is substantially because of the lifestyle practices of Jamaicans. One of the ironies within the document was in the "main components of the policy for the promotion of a healthy lifestyle in Jamaica", which cites that the goal of the policy was to reduce the incidence of communicable and infectious diseases, which speaks to society's subconscious emphasis on the biomedical model in conceptualizing health and its treatment. Embedded within the MOH's 2004 publication are repetition and the focus on seeking to reduce physiological conditions that affect the individual. The MOH admits, however, that health interfaces with body and environment, which is an expansion of the biomedical model, but all indications in their document point to the biomedical science approach in the application of the policy. The institution recognized that psychological factors (for example, self-esteem, and resilience) play a role in influencing health, so much so that it included these within its "goal of the strategic approach", but they were not supported in the "broad objectives of the strategic approach".

Critical to all of this is the acceptance that the definition of health is fundamental to the construction of those hypotheses that are used to formulate health policies. According to Longest [13], the conceptualization of health is indeed critical to all the things that rely on its definition. Longest writes:

The way in which health is conceptualized or defined in any society is important because it reflects the society's values regarding health and how far the society might be willing to go in aiding and supporting the pursuit of health among its members [13].

In Jamaica health policies are still driven by physical functioning, which is an obsolete approach to addressing health and by extension wellbeing. This limited approach to health and wellbeing means that little consideration is given to other factors such as lifestyle, psychological state, the environment, crime and violence, among others. This of course implies that Jamaica's health policy is limited in its orientation, as it is largely driven by hypotheses that support physical functioning.

2.2. Biopsychosocial Approach

Dr. Buzina admits that wellbeing is fundamentally a biomedical process [23]. This conceptual framework derives from the Newtonian approach of basic science as the only mechanism that could garner information, and empiricism being the only apparatus to establish truth or fact. It is still a practice and social construction that numerous scholars and medical practitioners [24] continue to advocate despite new findings. Simply put, many scholarships still put forward a perspective that the absence of physical dysfunction is synonymous with wellbeing (or health, or wellness). Such a viewpoint appears to hold some dominance in contemporary societies, and this is a widespread image held in Jamaica. Then there are issues such as the death of an elderly person's life-long partner; a senior citizen taking care of his/her son/daughter who has HIV/AIDS; an aged person not being able to afford his/her material needs; someone older than 64 years who has been a victim of crime and violence and continues to be a victim; seniors who reside in volatile areas who live with a fear of the worst happening, the inactive aged, and generally those who have retired with no social support, are equally sharing the same health status as the elderly who have not been on medication because they are not suffering from biomedical conditions to the extent that they need to be given drugs.

Two medical doctors writing in Kaplan and Saddock's Synopsis of Psychiatry noted that physicians are frequently caught in theorizing that normality is a state of health [44]. They argued that doctors' definition of normality correlates with a traditional model (biomedical) that emphasizes observable signs and symptoms. Using

psychoanalytic theories, Saddock and Saddock [44] remarked that the absence of symptoms as a single factor is not sufficient for a comprehensive outlook on normality. They stated, "Accordingly, most psychoanalysts view a capacity for work and enjoyment as indicating normality…" [44]. Among the challenges associated with this method (biomedical model), is its emphasis only on curative care. Such an approach discounts the importance of lifestyle and preventative care. In that, health is measured based on experiences with illnesses and/or ailments, with limited recognition being placed on approaches that militate against sickness and/or diseases. The biomedical approach is somewhat biased against an understanding of multi-dimensional man, which is not in keeping with the holistic conceptualization of health as offered by the WHO.

2.3. Biopsychosocial Approach

In the 1950s, George Engel, a physician, teamed with John Romano, a young psychiatrist, to develop a biopsychosocial model for inclusion in the curriculum of the University of Cincinnati College of Medicine, which measured the health status of people. It is referred to as Engel's biopsychosocial model. Engel's biopsychosocial model [10,11,43], recognized that psychological and social factors coexisted along with biological factors. It was a general theory of illness and healing, a synergy between medicine, psychiatry and the behavioural sciences [42]. Therefore, from Engel's model, wellbeing must include factors such as motivation, depression (or the lack thereof), biological conditions (such as illnesses and diseases), social systems, cultural, environmental and familial influences on the appearance and occurrence of illness.

Some scholars may argue that this paper appears to believe that only quantitative studies may provide answers to the examination of the determinants of health. This is absolutely not so, and we use a qualitative study to show people's perception of what contributes to a particular medical condition. In a qualitative study that uses in-depth interviews with some 17 Malaysian men aged between 40 and 75 years old, some scholars examined the perception of these men in relation to erectile dysfunction (ED) —the sample was a convenient one of men who were suffering from ED and who were willing to speak about their condition. When the interviewers asked the participants about the possible causes of ED, many of them outlined biomedical conditions such as diabetes, hypertension, medications, past injuries, ageing and then came lifestyle practices (*i.e.* smoking) and psychosocial factors [45]. Embedded in this perception is the respondents' emphasis on pathophysiological conditions in health measurement and intervention. Although the sampled respondents do believe that psychosocial factors play a role in health status, it should be noted here that they did not itemize those conditions. This speaks to the conceptualization of health that these respondents have come to accept, and the fact that they believe that health is not limited to biomedical sciences. Using their definition of health, the study shows how culture plays a pivotal role in determining how men will seek health care irrespective of the nature of their condition.

According to a number of demographers [46,47], health has been conceptualized as "functioning ability". These pundits categorized "functioning ability" as 1) being able to provide both personal care and independent living but having some difficulty in performing these tasks or in getting about outside the home, 2) having no functioning difficulties, 3) being unable to independently provide personal care, and finally 4) being able to provide personal care but not able to manage life in the home independently" [46].

3. EXPANSION OF THE BIOMEDICAL MODEL

Studies reveal that positive moods and emotions are associated with wellbeing [48] as the individual is able to think, feel and act in ways that foster resource building and involvement with particular goal materialization [49]. This situation is later internalized, causing the individual to be self-confident, from which follow a series of positive attitudes that guide further actions [50]. Positive mood is not limited to active responses by individuals, but a study showed that "counting one's blessings," "committing acts of kindness", recognizing and using signature strengths, "remembering oneself at one's best", and "working on personal goals" all positively influence wellbeing [50,51]. Happiness is not a mood that does not change with time or situation; hence, happy people can experience negative moods [52].

Human emotions are the coalescence of not only positive conditions but also negative factors [53]. Hence, depression, anxiety, neuroticism and pessimism are seen as a measure of the negative psychological conditions that affect subjective wellbeing [54-56]. From Evans and colleague [54], Harris *et al.* [55] and Kashdon's monographs [56], negative psychological conditions affect subjective wellbeing in a negative manner (*i.e.* guilt, fear, anger, disgust); and the positive factors influence self-reported wellbeing in a direct way—this was corroborated in a study conducted by Fromson [57]; and by other scholars [53,58,59]. Acton and Zodda [60] aptly summarized the negative affective of subjective wellbeing in the sentence that reads "expressed emotion is detrimental to the patient's recovery; it has a high correlation with relapse to many psychiatric disorders."

From the theologians' perspective, spirituality and religiosity are critical components in the lifespan of people. They believe that man (including woman) cannot be

whole without religion. With this fundamental concept, theologians theorize that man cannot be happy, or feel comfortable without a balance between spirit and body [62]. In order to achieve a state of personal happiness, or self-reported subjective wellbeing, some pundits put forward a construct that people are fashioned in the image of God, which requires some religiosity before man can be happy or less stressed. Religion is, therefore, association with wellbeing [63-65] as well as low mortality [66]. Religion is seen as the opiate of the people from Karl Marx' perspective, but theologians, on the other hand, hypothesize that religion is a coping mechanism against unhappiness and stress. According to Kart [67], religious guidelines aid wellbeing through restrictive behavioural habits which are health risks, such as smoking, drinking alcohol, and even diet.

The discourse of religiosity and spirituality influencing wellbeing is well-documented [68,69]. Researchers have sought to concretize this issue by studying the influence of religiosity on quality of life, and they have found that a positive association exists between those two phenomena [70]. They found that the relationship was even stronger for men than for women, and that this association was influenced by denominational affiliation. Graham *et al.*'s [71] study found that blood pressure for highly religious male heads of households in Evans County was low. The findings of this research did not dissipate when controlled for age, obesity, cigarette smoking, and socioeconomic status. A study of the Mormons in Utah revealed that cancer rates were lower (by 80%) for those who adhered to Church doctrine [72,73] than those with weaker adherence.

In a study of 147 volunteer Australian males between 18 and 83 years old, Jurkovic and Walker [65] found a high stress level in non-religious as compared to religious men. The researchers in constructing a contextual literature quoted many studies that have made a link between non-spirituality and "dryness", which results in suicide. Even though Jurkovic and Walker's research was primarily on spiritual wellbeing, it provides a platform that can be used in understanding the linkages between the psychological status of people and their general wellbeing. In a study which looked at young adult women, the researchers found that spirituality affects the physical wellbeing of a populace [69]. Embedded within that study is the positive influence of spirituality and religion on the health status of women. Edmondson *et al.*'s work constituted of 42 female college students of which 78.8 percent were Caucasian, 13.5 percent African-American, 5.8 percent Asian and 92 percent were non-smokers.

Health psychologists concurred with theologians and Christians that religion influences psychological wellbeing [74,75]. Taylor [74] argued that religious people are more likely to cope with stressors than non-religious individuals, which explains the former's better health status. She put forward the position that this may be done through avoidance or vigilant strategies. This response is an aversive coping mechanism in addressing serious monologue or confrontational and traumatic events. Coping strategies, therefore, are psychological tools used by individuals to problem-solve issues, without which they are likely to construct stressors and threaten their own health status. Taylor [74] said that "some religious beliefs also lead to better health practices", producing lower mortality rates from all cancers in Orthodox Christians.

4. EVIDENCE OF USE FOR BIOPSYCHOSOCIAL MODEL

Even though policy makers are cognizant of the importance of healthy lifestyle practices and their influence on wellbeing [76], we continue to sideline them in understanding health status, and using this concept in the formulating of hypotheses that will drive a broader policy focus of health care for the populace. This is evident in our neglect to expand studies for policy purposes that collect data on health using the biopsychological model, meaning that policy formulators are emphasizing physical vulnerability or dysfunction to measure health status. Is there a study that has sought to use a maximization definition of health that will be able to better evaluate and plan for the wellbeing of Jamaicans?

A study conducted in Barbados reveals that there is a statistical causal relationship between socioeconomic conditions and health status. The findings revealed that 5.2% of the variation in reported health status was explained by the traditional determinants of health (disease indicators–See **Table 1**). Furthermore, when this was controlled for current experiences, the percentage fell to 3.2% (falling by 2%). When the current set of socioeconomic conditions were used they accounted for some 4.1% of the variations in health status, while 7.1% were due to lifestyle practices, compared to 33.5% that were as a result of current diseases [34]. It holds that the importance placed by medical practitioners on the current illnesses—as an indicator of health status—is not unfounded as people place more value on biomedical conditions as being responsible for their current health status. Despite this fact, it is obvious from the data—using 33.5%—that there are other indicators that explain some 67.5% of the reason why health status should be as it is. Furthermore, with an odds ratio of 0.55 for number of illnesses, there is a clear suggestion that the more people reporting illnesses, the lower will be their health status [34]; and this was equally so for more disease symptoms—odds ratio was 0.71).

Figure 1 above is a depiction of the use of the biopsychosocial model in the study of health status. This

Table 1. Potential determinants of self-reported health status, study of historical and current predictors of self-reported health status in elderly persons, Barbados, 1999-2000.

Predictor group	Individual predictor in each predictor group
Historical socioeconomic indictors	Education, occupation, childhood economic situation, childhood nutrition, childhood health, number of childhood diseases
Current socioeconomic indicators	Income, financial means, household crowding, living alone, currently married, number of people in household, number of siblings living outside household, number of other family and friends living outside household
Current lifestyle risk factors	Body mass index, waist circumference, categories of disease risk, nutrition, smoking, exercise
Disease indicators	Number of illnesses,[a] number of symptoms,[b] geriatric depression scale score, number of nights in hospitals in 4-month period, number of medical contacts in 4-month period

[a]Illnesses included hypertension, diabetes, cancer, chronic lung disease, coronary heart disease, cerebrovascular accident, and arthritis
[b]Symptoms included chest pain, shortness of breath, back pain, severe fatigue or tiredness, joint problems, persistent swelling in the feet or ankles, persistent dizziness, persistent headaches, persistent wheezing, cough or phlegm, persistent nausea or vomiting and persistent thirst or excessive sweating.
Source: Hambleton *et al*. [34]

research was conducted in Barbados between 1999 and 2000, in which health status was predicted by a composite function of five general typologies of variables. The model shows that health status is not primarily limited to biomedical conditions—such as diseases and ailments—as has been the custom of many scholars. While different indicators as used by these researchers may not be possible in this paper because of the limitation of the secondary dataset—for example "current lifestyle risk factors", "childhood nutrition", "childhood diseases", "environmental factors", to name a few—despite the data's shortcomings, the study emphasizes the use of a multidimensional approach in the study of wellbeing.

Bourne [27], using secondary data, encapsulates George Engel's conceptual idea of a multidimensional model which incorporates biological, social, psychological, environmental and social conditions in examining wellbeing. Wellbeing is operationally defined as material resources, illness and total expenditure of households. The sample is drawn from a nationally representative survey of 25,018 Jamaicans, some 9.3% of the sample being elderly. From a sample of 2,320 elderly Jamaicans (ages 65 + years), Bourne [27] found that 10 of the 14 predisposing variables explain 36.8% of the variance in wellbeing. Of the 10 statistically significant variables, the five most important ones, in descending order, are 1) area of residence ($\beta = 0.227$); 2) cost of medical care ($\beta = 0.184$); 3) psychological conditions [total positive affective conditions] ($\beta = 0.138$); 4) ownership of property ($\beta = 0.135$); and 5) crime ($\beta = 0.111$). Among the other factors, which are the 5 least important conditions, are negative affective conditions, marital status, educational level, average occupancy per room, age of residents, and the environment. Thus, whether or not we use Grossman's model [77], Hambleton *et al.*'s model [34] or Bourne's models [27-33] it is clear from them that wellbeing extends beyond biological conditions to include psychological, environmental, and social conditions.

Another study was conducted by Bourne [30] of some 3,009 elderly Jamaicans (60 years and older), with an average age of 71 years and 10 months ± 8 years and 6 months, of which 67% (n = 2,010) resided in rural areas, 21% (n = 634) dwelled in Other Towns and 12% (n = 365) lived in the Kingston Metropolitan Area. The mean General Wellbeing of elderly Jamaicans was low (3.9 out of 14 ± 2.3). Bourne's model [30] identified 10 explanatory variables which explain 40.1% (adjusted R-squared) of the variance in general wellbeing. In this study he deconstructed the general model into 1) economic wellbeing and 2) physical wellbeing (proxy by health conditions). Using the same set of explanatory variables, the latter model explains 3.2% of the variability in wellbeing (proxy by health conditions) compared to 41.3% for the former model (*i.e.* economic wellbeing using material economic resources). General Wellbeing was operational as material resources and functional limitation (or health conditions). Material economic resources constitute ownership of durable goods (such as motor vehicles, stereo, washing machines, et cetera); income (proxy by income quintile); and financial support (e.g. social security and other pensions). Hence, it follows that the biopsychosocial model is a better proxy for wellbeing; and that functional limitation is still not a good proxy for wellbeing as used by Hambleton *et al.* Grossman and even Smith and Kington [78].

Globally, regionally and especially domestically, the most popular space in research concerning wellbeing is the biomedical approach; its popularity is fuelled by the combination of the traditional operational definition of health (good physical health) and the dominance of the medical sciences in this field of enquiry. The number of studies on mortality, structural alterations and functional declines in body systems, genetic alterations induced by exogenous and endogenous factors, prevalence and incidence of diseases, and certain diseases as determinants of health, clearly justifies establishing leniency towards medical science in the study of health and health care. Engel [10,11] accredited the biomedical model that governs health care to the practice of pundits over the last 300 years. This model assumes that psychosocial processes are independent of the disease process. Engel argued for the bio-psychosocial model that it includes biological, psychological, and social factors, which is a close match to the multi-dimensional aspect of man.

With this as the base, it can be construed from Engel's thrust behind the biopsychosocial model that the previous model is a reductionistic model. Engel's biopsychosocial model in analyzing health emphasizes both health and illness, and maintains that health and illnesses are caused by a multiplicity of factors. Engel's theorizing, therefore, is better fitted for the definition of health coined by the World Health Organization.

In Jamaica, only a miniscule number of studies have sought to analyze the effect of the death of a family member or close friend, violence, joblessness, psychological disorders and sexual abuse, on wellbeing, or social change on health, area of residence on quality of life and the perception of ageing and its influence on health conditions. Morrison [18] alluded to a transitory shift from infectious communicable diseases to chronic non-communicable diseases as a rationale for the longevity of the Anglophone Caribbean populace. This was equally endorsed by Peña [79], the PAHO/WHO representative in Jamaica. They argued that this was not the only reason for the changing life expectancy. Morrison summarized this adequately, when he said that:

Aiding this transition is not only the increased longevity being enjoyed by our islanders but also the changing lifestyle associated with improved socioeconomic conditions [18].

With the post-1994 widened definition of health as put forward by the WHO, people are becoming increasingly cognizant of the fact that socio-cultural factors such as geographical location, income, household size and so on, as well as several psychological factors, explain wellbeing; hence the new definition of health has coalesced biomedical variables and socio-cultural and psychological variables in the new discourse on wellbeing.

Stressors may arise from within the individual or outside his/her environment. One such external stressor that may affect the individual is the death of loved ones. Response to the mortality of close family members may be more traumatic, depending on expectancy or non-expectancy. Bereavement influences the incidence of mortality. This may result in exhaustion of the individual's "adaptive reserve". The person's body wears down and becomes highly vulnerable to morbidity and even death. Rice put forward a study that contradicted an association between bereavement and mortality. He wrote that "Fathers who lost sons in war had lower mortality rates than those who lost son in accidents" [75]. Despite that study, Rice quoted other studies [80] that showed the impact of stress on human physiology. He argued that it is suppression during and after bereavement that creates the stressors, which become potent devices for mortality and morbidity. Lusyne, Page and Lievens' [81] study finds that there is an association between bereavement and mortality. However, this is more likely to occur in the short-run (*i.e.* during the first 6 months after the death of the spouse). As there are a number of confounding situations which in the long-run could offset the likelihood of mortality, such as remarriage, social support from other family members, grandchildren and so on, bereavement may not necessarily be a constant in one's life. Nevertheless, Lusyne, Page and Lievens affirm with other studies that the loss of a long-time partner may result in the death of the living spouse. The explanations given for this eventuality are 1) role theory as the surviving partner may find the role played by the other partner too stressful and so 2) may not be able to adapt to the new role alone; this is more a male phenomenon [81].

The Planning Institute of Jamaica and Statistical Institute of Jamaica collect data on ill-health, and questions are asked based on visits to health practitioners, healers and pharmacies, injuries, ailments, ownership of health insurance, duration of the disease or illness, cost of treatment for ailments and injuries, and mental disability. Those questions are clearly derivatives from the biomedical model, as they seek to address physical functioning without equally emphasizing culture, lifestyle behaviour, depression, stress, fatigue, trust for others, perception of one's position in current society and the likelihood of one's place in the future, religiosity, time periods, HIV/AIDS of family members or the individual and how it is likely to influence the his/her health and wellbeing, social involvement in various institutions, and issues on positive affective conditions.

5. CONCLUSIONS

In sum, any definition of the construct of health must be multidimensional in nature. Such a definition must include 1) personal and environmental conditions; 2) social factors; 3) psychological conditions; 4) diagnosed illness; and 5) self-determination of wellbeing. If health is solely based on illnesses (*i.e.* biomedical model), we would have failed in our bid to operationally define a construct that is comprehensive enough to encapsulate all the tenets that would capture man in his complex milieu. Health is not simply a construct. It plays a critical role in the formulation of policy for health care, and in the development of the society. Thus, if we emphasize only the biomedical approach to the study of health, its underpinnings could only be symptomology. This approach fails to capture issues outside of the mechanistic structure of man's conception of biomedical sciences. Concurringly if health care professionals were to use as their premise dysfunctions to indicate health, which is the deviation from the norm, this image of health would affect policy formulation and intervention programmes which are geared towards this narrow conceptualization. But this approach lacks are clear characteristics outside of illnesses that will encapsulate wellness, wellbeing, and healthy life expectancy in a multidimensional hu-

man. Thus, the biomedical model relies on illness identification to capture health and this fashions the health care system, which also limits health coverage outside of this negative view of health. This is undoubtedly suboptimal, and does not account for health. The health services in the Caribbean, and in particular Jamaica, are best described as medical services, as they are still fundamentally structured around the biomedical model which is embedded as the image of health, and not psychosocial, economic and ecological wellbeing. Although the WHO as early as the 1940s provides a definition of health that is comprehensive and complex, some scholars believe that it is elusive and by extension immeasurable. There are merits to the argument of those academics, but the emphasis should not be the difficulty of how operationalizing the construct labels it "elusive". Instead the goal should have been for researchers and academics alike to formulate a working definition of the conceptual framework created by the WHO. Thus, when Grossman in the 1970s moved away from the difficulty posed by the WHO's conceptual framework, he developed an econometric framework that laid the foundation for the measure of this seemingly "elusive" construct. Other scholars have built on the initial theoretical model introduced by Grossman, and Bourne in particular has added psychological and environmental conditions to the already established factors of the health model. The constitution of the World Health Organization (WHO) states that "Health is a state of complete physical, mental and social well-being and not merely the absence of diseases or infirmity" [3]. Hence, any use of morbidity statistics, dysfunctions, sickness, diseases or ill-health to conceptualize health is limited, and by extension is a negative approach to the treatment of this construct. Health, health care, and patient care are critical components in development, as unhealthy people will not be able to offer to the society their maximum, neither will they be able to comparatively contribute the same to productivity and production as their healthy counterparts. Therefore, the conceptualization of health is not merely a concept but a working product that affects all aspects of society.

REFERENCES

[1] Lamb, V. and Siegel, J.S. (2005) Health demography. 2nd Edition, *The Methods and Materials of Demography*, Elsevier Academic Press, San Diego, 341-363.

[2] Spector, P.E. (1992) Summated rating scale construction. An introduction. Sage Publication, London.

[3] World Health Organization (1948) *Preamble to the Constitution of the World Health Organization as Adopted by the International Health Conference*, New York, June 19-22, 1946; signed on July 22, 1946 by the representatives of 61 States (Official Records of the World Health Organization, **2**, 100) and entered into force on April 7, 1948. "Constitution of the World Health Organization, 1948." In Basic Documents, 5th Edition,. Geneva, Switzerland.

[4] Brannon, L. and Feist, J. (2007) Health psychology. 6th Edition, *An Introduction to Behavior and Health*, Wadsworth, Los Angeles.

[5] Rowland, D.T. (2003) Demographic Methods and Concepts. Oxford University Press, New York.

[6] Seigel, J.S. and Swanson, D.A. (ed.) (2004) The Methods and Materials of Demography. 2nd Edition, Elsevier Academic Press, San Diego.

[7] Spiegelman, M. (1980) Introduction to demography. 6th Edition, Harvard University Press, Boston.

[8] Shryock, H.S., Siegel J.S., *et al.* (1976) The Methods and Materials of Demography, Academic Press, San Diego.

[9] Smith, J.A. (1983) The idea of health: Implications for the nursing professional. Teachers College, New York.

[10] Engel, G.L. (1977) The need for a new medical model: A challenge for biomedicine. *Science*, **196(4286)**, 129-136.

[11] Engel, G.L. (1980) The clinical application of the biopsychosocial model. *American Journal of Psychiatry*, **137**, 535-544.

[12] Dubos, R. (1965) Man adopting. Yale University Press, New Haven.

[13] Longest, B.B. (2002) Health policymaking in the United States. 3rd Edition, Foundation of the American College Healthcare, Chicago.

[14] Ministry of Health. (2004) National policy for the promotion of health lifestyle in Jamaica. MOH, Kingston.

[15] Stutzer, A. and Frey, B.S. (2003) Reported subjective well-being: A challenge for economic theory and economic policy. http://www.crema-research.ch/papers/2003-07.pdf

[16] McConville, C., Simpson, E.E.A., Rae, G., Polito, A., Andriollo-Sanchez, Z., Meunier, N., Stewart-Knox, O., Connor, J.M., Boussel, A.M., Cuzzolaro, M. and Coudray, C. (2005) Positive and negative mood in the elderly: The Zenith study. *European Journal of Clinical Nutrition*, **59**, 22.

[17] Erber, J. (2005) Aging and older adulthood. *Thomson Learning*, Wadsworth, New York.

[18] Morrison, E. (2000) Diabetes and hypertension: Twin trouble. *Cajanus*, **33**, 61-63.

[19] Callender, J. (2000) Lifestyle management in the hypertensive diabetic. *Cajanus*, **33**, 67-70.

[20] Steingo, B. (2000). Neurological consequences of diabetes and hypertension. *Cajanus*, **33**, 71-83.

[21] Eldemire, D. (1995) A situational analysis of the Jamaican elderly. Planning Institute of Jamaica, Kingston.

[22] Caribbean Food and Nutrition Institute (1999) Health of the elderly. *Cajanus*, **32**, 217-240.

[23] Buzina, K. (1999) Drug therapy in the elderly. *Cajanus*, **32**, 194-200.

[24] Ali, A., Christian, D. and Chung, E. (2007) Funny turns in an elderly man. *West Indian Medical Journal*, **56**, 376-379.

[25] Bok, S. (2004) Rethinking the WHO definition of health. Working Paper Series, 14. http://www. golbalhealth.harvard.edu/hcpds/wpweb/Bokwp14073.pdf

[26] Evans, R.G. and Stoddart, G.L. (1990) Producing health, consuming health care. *Social Science and Medicine*, **31**, 1347-1363.

[27] Bourne, P. (2007) Using the biopsychosocial model to evaluate the wellbeing of the Jamaica elderly. *West India Medical Journal*, **56(3)**, 39-40.
[28] Bourne, P.A. (2009) Determinants of quality of life of youths in an English speaking Caribbean nation. *North American Journal of Medical Sciences*, **1**, 365-371.
[29] Bourne, P.A. (2008) Health determinants: Using secondary data to model predictors of well-being of Jamaicans. *West Indian Medical Journal*, **57**, 476-481.
[30] Bourne, P. (2007) Determinants of well-being of the Jamaican elderly. Unpublished Master of Science Thesis, The University of the West Indies, Mona, Jamaica.
[31] Bourne, P.A. (2008) Medical Sociology: Modelling Well-being for elderly People in Jamaica. *West Indian Medical Journal*, **57**, 596-604.
[32] Bourne, P.A. (2009) Social determinants of self-evaluated good health status of rural men in Jamaica. *Rural and Remote Health*, **9**, 1280.
[33] Bourne, P.A. (2009) A theoretical framework of good health status of Jamaicans: using econometric analysis to model good health status over the life course. *North American Journal of Medical Sciences*, **1**, 86-95.
[34] Hambleton, I.R., Clarke, K., Broome, H.L., Fraser, H.S., Brathwaite, F. and Hennis, A.J. (2005) Historical and current correlates of self-reported health status among elderly persons in Barbados. *Revista Panamericana de Salud Púrblica*, **17**, 342-352.
[35] WHO (2008) The social determinants of health. http://www.who.int/social_determinants/en/
[36] Walkinson, R.G. and Marmot, W. (2003) Determinants of health. 2nd Edition, *The Solid Facts*, World Health Organization, Copenhagen.
[37] Kelly, M., Morgan, A., Bonnefog, J., Beth, J. and Bergmer, V. (2007) The social determinants of health: Developing evidence base for political action, WHO Final Report to the Commission.
[38] Khetarpal, A. and Kocar, G. (2007) Health and well-being of rural women. *The Internet Journal of Nutrition and Wellness*, **3**, 1.
[39] Graham, H. (2004) Social determinants and their unequal distribution clarifying policy understanding. *The Mil-Bank Quarterly*, **82**, 101-124.
[40] Pettigrew, M., Whitehead, M., McIntyre, S.J., Graham, H. and Egan, M. (2004) Evidence for public health policy on inequalities: The reality according to policymakers. *Journal of Epidemiology and Community Health*, **5**, 811-816.
[41] Anthony, B.J. (1999) Nutritional assessment of the elderly. *Cajanus*, **32**, 201-216.
[42] Dowling, A.S. (2005) Images in psychiatry: George Engel, 1913-1999. http://ajp.psychiatryonline.org/cgi/reprint/162/11/2039
[43] Brown, T.M. (2000). The growth of George Engel's biopsychosocial model. http://human-nature.com/free-associations/engel1.html
[44] Saddock, B.J. and Saddock, V.A. (2003) Kaplan and Saddock's synopsis of psychiatry: Behavioral sciences/ clinical psychiatry. 9th Edition, Lippincott Williams and Wilkins, Philadephia.
[45] Low, W.Y., Ng, C.J., Choo, W.Y. and Tan, H.M. (2006) How do men perceive erectile dysfunction and its treatment? A qualitative study on opinions of men. *The Age-ing Male*, **9**,175-180.
[46] Crimmins, E.M., Hayward, M.D. and Saito, Y. (1994) Changing mortality and morbidity rates and the health status and life expectancy of the older population. *Demography*, **31**, 159-175.
[47] Portrait, F., Lindeboom, M. and Deeg, D. (2001) Life expectancies in specific health states: Results from a joint model of health status and mortality of older persons. *Demography*, **38**, 525-536.
[48] Leung, B.W., Moneta, G.B. and McBride-Chang, C. (2005) Think positively and feel positively: Optimism and life satisfaction in late life. *International Journal of Aging and human development*, **61**, 335-365.
[49] Lyubomirsky, S., King, L. and Diener, E. (2005) The benefits of frequent positive affect: Does happiness lead to success? *Psychological Bulletin*, **6**, 803-855.
[50] Sheldon, K.M. and Lyubomirsky, S. (2006) How to increase and sustain positive emotion: The effects of expressing gratitude and visualizing best possible selves. *Journal of Positive Psychology*, **1**, 73-82.
[51] Abbe, A., Tkach, C. and Lyubomirsky, S. (2003) The art of living by dispositionally happy people. *Journal of Happiness Studies*, **4**, 385-404.
[52] Diener, E. and Seligman, M.E.P. (2002) Very happy people. *Psychological Science*, **13**, 81-84.
[53] Watson, D., Wiese, D., Vaidya, J. and Tellegen, A. (1999) The two general activation systems of affect: Structural findings, evolutionary considerations and psychobiological evidence. *Journal of Personality and Social Psychology*, **76**, 820-838.
[54] Evans, R.G. (1994) Introduction. In: Evans, R.G., Barer, M.L. and Marmor, T.R., Eds., *Why are some people healthy and others not? The determinants of health of populations*. *Aldine de Gruyter*, New York.
[55] Harris, P.R. and Lightsey, J.O.R. (2005) Constructive thinking as a mediator of the relationship between extraversion, neuroticism and subjective well-being. *European Journal of Personality*, **19**, 409-426.
[56] Kashdan, T.B. (2004) The assessment of subjective wellbeing (issues raised by the Oxford Happiness Questionnaire). *Personality and Individual Differences*, **36**, 1225-1232.
[57] Fromson, P.M. (2006) Self-discrepancies and negative affect: The moderating roles of private and public self-consciousness. *Social behaviour and Personality*, **34(4)**, 333-350.
[58] McCullough, M.E., Bellah, C.G., Kilpatrick, S.D. and Johnson, J.L. (2001) Vengefulness: Relationships with forgiveness, rumination, well-being and the big five. P*ersonality and Social Psychology Bulletin*, **27**, 601-610.
[59] Watson, D., Clark, L.A. and Tellegen, A. (1988) Development and validation of brief measures of positive and negative affect: The PANAS scale. *Journal of Personality and Social Psychology*, **54**, 1063-1070.
[60] Watson, D., Clark, L.A. and Tellegen, A. (1988) Positive and negative affectivity and their relation to anxiety and depressive disorders. *Journal of Abnormal Psychology*, **97**, 346-353.
[61] Acton, G.S. and Zodda, J.J. (2005) Classification of psychopathology: Goals and methods in an empirical approach. *Theory of Psychology*, **15**, 373-399.
[62] Whang, K.M. (2006) Wellbeing syndrome in Korea: A

view from the perspective of biblical counseling. *Evangelical Review of Theology*, **30**, 152-161.
[63] Krause, N. (2006) Religious doubt and psychological well-being: A longitudinal investigation. *Review of Religious Research*, **47**, 287-302.
[64] Moody, H.R. (2006) Is religion good for your health? *The Gerontologist*, **14**, 147-149.
[65] Jurkovic, D. and Walker, G.A. (2006) Examining masculine gender-role conflict and stress in relation to religious orientation and spiritual well-being in Australian men. *Journal of Men's Studies*, **14(1)**, 27-46.
[66] House, J.S., Robbins, C. and Metzner, J.L. (1982) The association of social relationships and activities with mortality: Prospective evidence from the Tecumseh Community health study. *American Journal of Epidemiology*, **116**, 123-140.
[67] Kart, C.S. (1990) The realities of aging: An introduction to gerontology. 3rd Edition, Allyn and Bacon, Boston.
[68] Frazier, C., Mintz, L.B. and Mobley, M. (2005) A multidimensional look at religious involvement and psychological well-being among urban elderly African Americans. *Journal of Counseling Psychology*, **52**, 583-590.
[69] Edmondson, K.A., Lawler, K.A., Jobe, R.L., Younger, J.W., Piferi, R.L. and Jones, W.H. (2005) Spirituality predicts health and cardiovascular responses to stress in young adult women. *Journal of Religion and Health*, **44**, 161-171.
[70] Franzini, L. and Fernandez-Esquer, M.E. (2004) Socioeconomic, cultural and personal influences on health outcomes in low income Mexican-origin individuals in Texas. *Social Sciences and Medicine*, **59**, 1629-1646.
[71] Graham, T.W., Kaplan, B.H., Cornoni-Huntley, J.C., James, S.A., Becker, C., Hames, C.G. and Heyden, S. (1978) Frequency of church attendance and blood pressure elevation. *Journal of Behavioral Medicine*, **1,** 37-43.
[72] Gardner, J.W. and Lyon, J.L. (1982) Cancer in Utah Mormon men by lay priesthood level. *American Journal of Epidemiology*, **116**, 243-257.
[73] Gardner, J.W. and Lyon, J.L. (1982) Cancer in Utah Mormon women by church activity level. *American Journal of Epidemiology*, **116**, 258-265.
[74] Taylor, S. (1999) Health psychology. 4th Edition, McGraw-Hill, Chicago.
[75] Rice, P.L. (1998) Health psychology. Brooks/Cole Publishing Company, Los Angeles.
[76] Jamaica Social Policy Evaluation (2003) *Annual Progress Report on National Social Policy Goals* 2003. Cabinet Office, Kingston.
[77] Grossman, M. (1972) The demand for health: A theoretical and empirical investigation. *National Bureau of Economic Research*, New York.
[78] Smith, J.P. and Kington, R. (1997) Demographic and economic correlates of health in old age. *Demography*, **34**, 159-170.
[79] Peña, M. (2000) Opening remarks and greetings from the Pan American health organization. *Cajanus*, **33**, 64-70.
[80] Jemmott, J.B. and Locke, S.E. (1984) Psychosocial factors, immunologic mediation and human susceptibility to infectious diseases: How much do we know? *Psychological Bulletin*, **95**, 78-108. In: Rice, P.L., Ed., *Health Psychology*, 1998. Brooks/Cole Publishing Company, Los Angeles.
[81] Lusyne, P., Page, H. and Lievens, J. (2001) Mortality following conjugal bereavement, Belgium 1991-1996: The unexpected effect of education. *Population Studies*, **55**, 281-289.

Vol.2, No.5, 477-483 (2010)
doi:10.4236/health.2010.25071

Responses of the perfused liver of neonatal type 2 diabetic rats to gluconeogenic and ammoniogenic substrates

Mirian Carvalho-Martini, Fumie Suzuki-Kemmelmeier, Denise Silva de Oliveira, Jurandir Fernando Comar, Adelar Bracht*

Department of Biochemistry, University of Maringá, Maringá, Brazil; *Corresponding Author: adebracht@uol.com.br

Received 23 December 2009; revised 6 February 2010; accepted 8 February 2010.

ABSTRACT

The responses of livers from rats with type 2 diabetes to alanine (gluconeogenesis and ammonia detoxification) and other gluconeogenic substrates were investigated. The experimental system was the isolated perfused rat liver. Neonatal type 2 diabetes was induced with streptozotocin. Ammoniogenesis from endogenous substrates was 610% higher in livers from diabetic rats when compared to the control condition. Alanine (2.5 mM) ammoniogenesis was 285% higher in livers of diabetic rats. Gluconeogenesis from the following substrates was smaller in the liver of diabetic rats: Alanine (–43.5%), lactate (–28.3%) and glycerol (–30.5%). Pyruvate gluconeogenesis was normal. The high rate of ammoniogenesis explains the moderate hyperammonemia of type 2 diabetic rats. The enzymatic machinery of the gluconeogenic pathway of type 2 diabetic rats seems to be adapted to low rates of glucose removal by extrahepatic tissues. A significant contribution of gluconeogenesis to the fasting hyperglycemia can be expected only by short-term up-regulation mechanisms.

Keywords: Type 2 Diabetes; Gluconeogenesis; Ammoniogenesis; Alanine; Lactate

1. INTRODUCTION

Increased hepatic glucose production is characteristic of type 1 diabetes mellitus [1,2]. Particularly in the fasted state, elevated hepatic gluconeogenesis seems to be the main cause for the hyperglycemic condition in type 1 diabetes [3]. If the hyperglycemic condition in type 2 diabetes is also at least partly dependent on enhanced gluconeogenesis is not clear. There are reports claiming that this dependence is more accentuated in severely hyperglycemic patients and that it tends to diminish or even vanish in moderately hyperglycemic patients [4]. There are also studies in which no enhanced gluconeogenesis was found in type 2 diabetic patients [5]. It is generally believed that the enhanced gluconeogenesis is caused by an increased efficiency of the gluconeogenic pathway in combination with an augmented mobilization of glucose precursors to the liver [6,7].

Although gluconeogenesis can be measured in vivo using appropriate tracer techniques [4,8,9] it is of interest to reproduce the increased hepatic gluconeogenesis in isolated cell systems because this allows conclusions about its mechanisms. In the isolated perfused rat liver for example, the gluconeogenic activity reflects the enzymatic capacities. These, in turn, reflect the medium- and long-term effects of the circulating hormones on the expression of enzymes and other factors. For type 1 diabetes mellitus, increased gluconeogenesis in isolated hepatocytes or the isolated perfused liver from a variety of substrates has been found [10-15]. When alanine was the substrate, enhanced gluconeogenesis was found in the fasted state, combined with increased rates of urea production and increased rates of alanine incorporation into proteins [11]. These observations with alanine are consistent with the increased rates of mobilization of this amino acid in type 1 diabetes [6]. All these data reveal that short-term regulation operates most probably as a secondary mechanism for the enhanced gluconeogenesis in type 1 diabetes, the medium and long-term expression of key enzymes playing the decisive role [16].

Experiments in which gluconeogenesis was measured in the liver of rats with type 2 diabetes have not been done until now. They are of interest, however, because they will provide the same information about the enzymatic machinery that is already available for type 1 diabetes. The hypothesis that can be formulated is that en-

This work was sponsored by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Programa Nacional de Núcleos de Excelência (PRONEX, Fundação Araucária-CNPq).

hanced gluconeogenesis should be detectable in the isolated rat liver of type 2 diabetic rats in the same way as it was detected in livers from type 1 diabetic animals. Enhanced gluconeogenesis in the isolated organ would be reflecting mainly medium- and long-term effects of the circulating hormones on enzyme expression. With this hypothesis in mind, in the present work experiments were conducted with livers of type 2 diabetic rats. The neonatal streptozotocin-induced rat model of type 2 diabetes mellitus was used. It has been praised as a good model for type 2 diabetes in humans because it presents several of its characteristics [17]. In addition to gluconeogenesis, nitrogen metabolism from alanine was also measured. Evaluation of nitrogen metabolism is of interest because, in addition to hyperglycemia, type 2 diabetic rats also present moderate hyperammonemia [18]. For comparative purposes other gluconeogenic precursors, such as lactate and pyruvate, were also investigated.

2. MATERIALS AND METHODS

The liver perfusion apparatus was built in the workshops of the University of Maringá. Enzymes and coenzymes used in the assay procedures and streptozotocin were purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals were from the best available grade.

Neonatal type 2 diabetes mellitus was induced as previously described [19,20]. Male newborn (2 days old) Wistar rats were injected intraperitoneally with streptozotocin (160 mg/kg) dissolved in citrate buffer. Control rats were injected with citrate buffer. Seven weeks later, diabetes was confirmed by blood glucose levels (8-10 mM), glucose appearance in urine and 24 hours urinary volume (generally 500% above normal). After seven weeks the mean weights of the control and diabetic rats were 220 ± 2.8 and 203.2 ± 5.2 g, respectively. All animal experiments were done according to the universally accepted standards for animal experimentation.

Rats were fed *ad libitum* with a standard laboratory diet (Purina®), but food was withdrawn 24 hours prior to the perfusion experiments. For the surgical procedure, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Hemoglobin-free, non-recirculating perfusion was undertaken according to the technique described elsewhere [21,22]. After cannulation of the portal and cava veins the liver was positioned in a plexiglass chamber. The hepatic artery was closed (monovascular perfusion) and the bile duct was left open. The flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France) and was adjusted to between 30 and 35 ml min^{-1}, depending on the liver weight. The perfusion fluid was Krebs-Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37°C. The composition of the Krebs-Henseleit-bicarbonate buffer is: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM Na_2SO_4, 1.18 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and 2.5 mM $CaCl_2$. L-Alanine (2.5 mM), lactate (2.5 mM), pyruvate (1 mM) or glycerol (2 mM) were dissolved in the perfusion fluid. Samples of the effluent perfusion fluid were collected at 4 minute intervals and analyzed for their metabolite content. The following compounds were assayed by means of standard enzymatic procedures [23]: lactate, pyruvate, glucose, urea, ammonia, glutamine and glutamate. The oxygen concentration in the outflowing perfusate was monitored polarographically employing a teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate [22].

Rats after a 24-hours' fast were used because this minimizes interference of endogenous glycogen [24]. As shown by previous work the fasting glycogen levels of control and type 2 diabetic rats are very low when compared to the fed state [18].

Basal rates (absence of substrates) as well as increments caused by substrates (L-alanine, lactate, pyruvate or glycerol) were evaluated. The latter were calculated by subtracting the basal rates (before substrate infusion) from the steady-state rates found at the end of the substrate infusion period. The error parameters presented in graphs and tables are standard errors of the means. Differences between pairs of means were analyzed by means of Student's t test. The 5% level ($p < 0.05$) was adopted as a criterion of significance.

3. RESULTS

Table 1 lists the basal rates of metabolite release of livers from 24-hours fasted rats perfused with substrate-free medium. Under such conditions the livers are dependent solely on endogenous sources. The rates were referred to the wet liver weights which were not different in control and diabetic rats, namely 3.31 ± 0.09 and 3.53 ± 0.13 g per 100 g body weight, respectively. As revealed by Table 1 most basal rates were low. This did not occur with oxygen uptake and it should be noted that it was 13.6% lower in the liver of diabetic rats. Ammonia production, however, was 610% higher in the diabetic condition. All other parameters were similar in both the control and the diabetic condition.

Figure 1 illustrates the experimental protocol that was employed in the present study as well as the time course of the changes in three selected parameters. Sampling of the effluent perfusate for metabolite determination was always initiated after oxygen uptake stabilization (zero time). Alanine infusion was initiated at 12 minutes in the time scale of **Figure 1**. The time courses of the changes

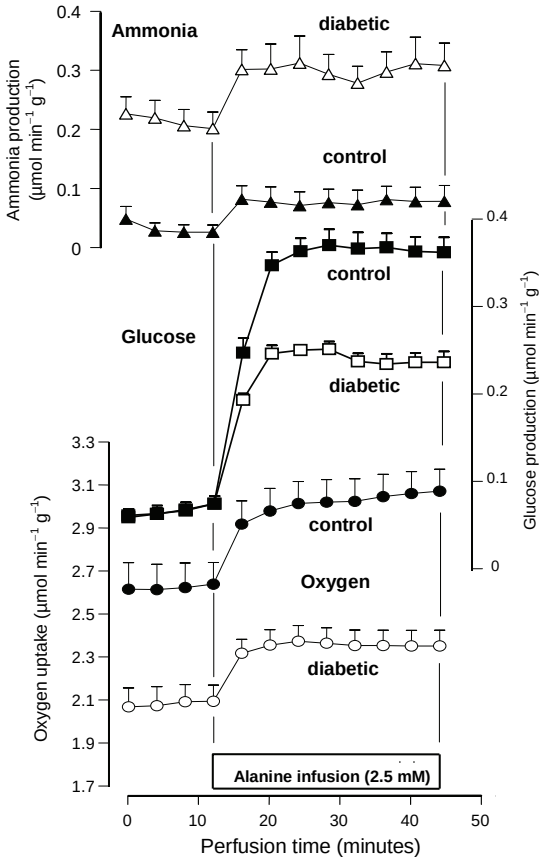


Figure 1. Time course of changes in oxygen uptake, glucose production and ammonia production in livers from fasted control and type 2 diabetic rats. Livers were perfused as described in Material and Methods. Alanine was infused as indicated by the horizontal bar. Results obtained with livers of control rats were represented by full symbols and those of diabetic rats with empty symbols. Data are means ± SEM of 9 (control) and 5 (diabetic) liver perfusion experiments.

in ammonia and glucose production and oxygen uptake were represented. The basal values of ammonia production, oxygen uptake and glucose release correspond to those ones listed in **Table 1**. The introduction of alanine caused increases in all parameters which tended to new steady-states during the following 30 minutes. Differences between the control and diabetic conditions were not only maintained but even accentuated because glucose production from alanine was smaller in the diabetic condition.

Figure 1 reveals that new steady-state levels were reached in consequence of alanine infusion. The steady-state rates and the increments caused by alanine infusion were evaluated and listed in **Table 2**. In addition to the three parameters that were represented in **Figure 1**, the rates of urea, lactate, pyruvate, glutamate and glutamine production were also shown in **Table 2**. Alanine infusion caused increases in all parameters and not only in those ones shown in **Figure 1**. The increment in ammonia production caused by alanine was higher in the diabetic condition. The increments in oxygen uptake, urea, glucose and glutamine productions, however, were smaller in the diabetic condition. Gluconeogenesis from alanine was, thus, smaller in the liver of diabetic rats (–43.5%). The total nitrogen flux generated by alanine infusion can be approximated by the sum of ammonia production + (2 × urea production) + (2 × glutamine production) + glutamate production, after subtracting the basal rates (before alanine infusion). In livers of control rats this calculation yielded a total nitrogen flux of 1.18 µmol min^{-1} g^{-1}; in the diabetic condition the corresponding value was 0.88 µmol min^{-1} g^{-1}, the difference amounting to 25.6%.

Glucose production from lactate, pyruvate and glycerol was investigated by using essentially the same experimental protocol illustrated for alanine in **Figure 1**. The results are summarized in **Tables 3** to **5**. **Table 3** shows that gluconeogenesis from 2.5 mM lactate was 28.3% smaller in livers from diabetic rats. The final oxygen uptake was also considerably smaller in the diabetic condition. Pyruvate production from lactate tended to be more pronounced in the diabetic condition, but there was no statistical significance at the 5% level. For pyruvate metabolism, on the other hand, no significant differences were found in glucose production, oxygen uptake and lactate production, as revealed by **Table 4**. There was a strong tendency, however, for higher values of glucose production in the diabetic condition ($p = 0.061$). Glucose production from glycerol however, was 30.5% smaller in the diabetic condition as revealed by **Table 5**. Lactate production from glycerol, however, was not statistically different. Pyruvate production from glycerol was negligibly small.

4. DISCUSSIONS

The results reveal that the metabolism of livers from neonatal type 2 diabetic rats presents a few differences when compared to livers from normal and also type 1 diabetic rats. These differences encompass both ammonia detoxification and carbohydrate metabolism. The liver of type 1 diabetic rats has been reported to present considerably higher rates of urea production [11]. This makes a clear contrast with the liver of type 2 diabetic rats where ureogenesis was found to be close to normal (**Tables 1** and **2**). Consistently, the plasma urea level of type 1 diabetic rats is very high, but normal in type 2 diabetic rats [2-18]. The perfused liver of type 2 diabetic rats, on the other hand, presented higher rates of ammonia production under both conditions examined in the present work, *i.e.*, during substrate-free perfusion and during alanine infusion (**Tables 1** and **2**). In relative terms the difference was more pronounced in the

Table 1. Basal rates of metabolites release and oxygen uptake in perfused livers from control and type 2 diabetic rats. Significant differences are indicated by an asterisk ($p < 0.05$). The results come from experiments in which various substrates were infused at 12 minutes perfusion time as illustrated by **Figure 1**.

Metabolic flux	Control	Diabetic
	μmol min^{-1} (*g liver wet weight*)$^{-1}$	
Ammonia production	0.029 ± 0.012 (n = 9)	0.206 ± 0.028* (n = 5)
Urea production	0.140 ± 0.016 (n = 9)	0.171 ± 0.003 (n = 5)
Glutamine production	0.064 ± 0.007 (n = 9)	0.062 ± 0.006 (n = 5)
Glutamate production	0.034 ± 003 (n = 9)	0.031 ± 0.007 (n = 5)
Oxygen uptake	2.470 ± 0.065 (n = 18)	2.135 ± 0.063* (n = 17)
Glucose production	0.080 ± 0.010 (n = 17)	0.064 ± 0.006 (n = 16)
Lactate production	0.052 ± 0.007 (n = 13)	0.051 ± 0.014 (n = 11)
Pyruvate production	0.007 ± 0.002 (n = 13)	0.009 ± 0.006(n = 13)

Table 2. Metabolic fluxes in livers from control and type 2 diabetic rats caused by alanine infusion (2.5 mM). The data were obtained from experiments in which alanine was infused during 30 minutes. Asterisks (*) and crosses (†) indicate values in the diabetic condition that are statistically different from the corresponding control values according to Student's t test ($p < 0.05$).

Parameter	Control (n = 9)		Diabetic (n = 5)	
	μmol min^{-1} (*g liver wet weight*)$^{-1}$			
	Rate in the presence of alanine	*Increment caused by alanine*	*Rate in the presence of alanine*	*Increment caused by alanine*
Ammonia production	0.081 ± 0.025	0.052 ± 0.016	0.312 ± 0.041†	0.106 ± 0.013*
Urea production	0.499 ± 0.030	0.359 ± 0.021	0.416 ± 0.019	0.245 ± 0.011*
Glutamine production	0.255 ± 0.015	0.191 ± 0.011	0.191 ± 0.008†	0.129 ± 0.005*
Glutamate production	0.060 ± 0.005	0.026 ± 0.002	0.053 ± 0.010	0.022 ± 0.004
Oxygen uptake	3.064 ± 0.102	0.434 ± 0.048	2.349 ± 0.073†	0.258 ± 0.026*
Glucose production	0.364 ± 0.017	0.292 ± 0.014	0.238 ± 0.011†	0.165 ± 0.013*
Lactate production	0.307 ± 0.019	0.255 ± 0.015	0.285 ± 0.024	0.234 ± 0.019
Pyruvate production	0.122 ± 0.028	0.115 ± 0.026	0.160 ± 0.018	0.151 ± 0.017

Table 3. Metabolic fluxes in livers from control and type 2 diabetic rats caused by lactate infusion (2.5 mM). The data were obtained from experiments in which lactate was infused during 30 minutes. Asterisks (*) and crosses (†) indicate values in the diabetic condition that are statistically different from the corresponding control values according to Student's t test ($p < 0.05$).

Parameter	Control (n = 4)		Diabetic (n = 5)	
	μmol min^{-1} (*g liver wet weight*)$^{-1}$			
	Rate in the presence of lactate	*Increment caused by lactate*	*Rate in the presence of lactate*	*Increment caused by lactate*
Oxygen uptake	3.195 ± 0.228	0.629 ± 0.042	2.631 ± 0.070†	0.531 ± 0.029
Glucose production	0.998 ± 0.119	0.922 ± 0.118	0.737 ± 0.021†	0.661 ± 0.018*
Pyruvate production	0.200 ± 0.101	0.182 ± 0.100	0.334 ± 0.097	0.324 ± 0.101

Table 4. Metabolic fluxes in livers from control and type 2 diabetic rats caused by pyruvate infusion (1.0 mM). The data were obtained from experiments in which pyruvate was infused during 20 minutes.

Parameter	Control (n = 4)		Diabetic (n = 3)	
	$\mu mol\ min^{-1}$ (*g liver wet weight*)$^{-1}$			
	Rate in the presence of pyruvate	*Increment caused by pyruvate*	*Rate in the presence of pyruvate*	*Increment caused by pyruvate*
Oxygen uptake	2.657 ± 0.101	0.361 ± 0.045	2.658 ± 0.014	0.434 ± 0.059
Glucose production	0.399 ± 0.049	0.340 ± 0.042	0.515 ± 0.013	0.463 ± 0.014
Lactate production	1.143±0.063	1.103 ± 0.059	1.232 ± 0.047	1.158 ± 0.022

Table 5. Metabolic fluxes in livers from control and type 2 diabetic rats caused by glycerol infusion (2.0 mM). The data were obtained from experiments in which glycerol was infused during 20 minutes. Asterisks (*) and crosses (†) indicate values in the diabetic condition that are statistically different from the corresponding control values according to Student's t test ($p < 0.05$).

Parameter	Control (n = 4)		Diabetic (n = 4)	
	$\mu mol\ min^{-1}$ (*g liver wet weight*)$^{-1}$			
	Rate in the presence of glycerol	*Increment caused by glycerol*	*Rate in the presence of glycerol*	*Increment caused by glycerol*
Oxygen uptake	2.696 ± 0.126	0.173 ± 0.066	2.077 ± 0.078†	0.061 ± 0.020
Glucose production	0.609 ± 0.049	0.518 ± 0.051	0.423 ± 0.036†	0.362 ± 0.035*
Lactate production	0.140 ± 0.049	0.091 ± 0.009	0.103 ± 0.036	0.056 ± 0.016

absence of alanine, a condition where ammonia comes solely from endogenous catabolic reactions. Even the second route of ammonia detoxification, namely glutamine production [25], seems to be impaired in the liver of type 2 diabetic rats, as can be judged from its lower rates in the presence of alanine (**Table 2**). The higher rates of ammonia production can be indicating that nitrogen catabolism in livers of type 2 diabetic rats surpasses the capacity of the urea cycle when this route depends solely on endogenous substrates and on endogenous substrates plus alanine. In principle this conclusion receives support from the higher plasma ammonia levels in type 2 diabetic rats [18]. It must be remarked, however, that the difference in the plasma ammonia levels between diabetic and control rats, as reported previously [18], is relatively small (+ 28%) when compared to the difference in ammoniogenesis (+ 285% in the presence of alanine). Extrapolation to the in vivo conditions is always subject to error, but it principle one would expect a more severe hyperammonemia in type 2 diabetic rats. Possibly there is some in vivo mechanism or mechanisms that avoid the development of severe hyperammonemia. One of these mechanisms could be higher rates of renal excretion. Alternatively, there could be a more efficient transformation of ammonia into urea and glutamine in vivo than that one found in the isolated perfused liver due to the presence of factors capable of stimulating ureogenesis.

With reference to the main hypothesis of the present work, it is apparent from the results of the present study that hepatic gluconeogenesis in neonatal type 2 diabetic rats is lower than that in non-diabetic rats, at least with the relevant precursors lactate, alanine and glycerol. This can also be interpreted as meaning, in principle at least, that the enzymatic machinery of the liver from neonatal type 2 diabetic rats is not adapted to higher rates of gluconeogenesis but much more to the lower rates of glucose uptake by peripheral tissues [26]. No significant difference was found when pyruvate was the substrate. There was a tendency toward higher rates of glucose synthesis in the diabetic condition without statistical significance, however, possibly due to the small number of rats used in these experiments. However, pyruvate is the only substrate used in the present work that induces, in the once-through perfused liver at least, an oxidizing state in the liver cells when it is present alone (*i.e.*, very low NADH/NAD ratios) [27]. This is a situation that does not normally occur in vivo where the pyruvate concentrations are normally very low and the lactate to pyruvate ratios high [27]. These results with livers of type 2 diabetic rats are in sharp contrast with those obtained with the liver of type 1 diabetic rats, where increased gluconeogenesis with virtually all substrates was observed [10-15]. Although the rates of oxygen uptake were always lower in the liver of diabetic rats even in the presence of gluconeogenic substrates, it is unlikely that ATP availability could be limiting glucose synthesis.

As shown previously, the ATP content of the hepatic tissue of type 2 diabetic rats under the same conditions as those used in the present work (24-hours fast) is even higher than that of normal rats [18]. Furthermore, there was a clear correlation between the increments in oxygen uptake caused by lactate, alanine and pyruvate and the gluconeogenic activity. This reinforces the general notion that gluconeogenesis controls the extra oxygen uptake and not the contrary.

It should be stressed with reference to the contribution of gluconeogenesis to the fasting hyperglycemia in animals and patients with type 2 diabetes that conflicting results have been reported. There are studies proposing a significant, though relatively small, contribution of gluconeogenesis to the fasting hyperglycemia [4,6,8] while others claim that the contribution is not significant [6,26]. Our results thus agree much more with those studies in which no enhanced gluconeogenesis was found in type 2 diabetic patients.

Extrapolations of the observations of the present work to the in vivo conditions or to different species (mice, humans) must always be done carefully. Even so, it should be stressed that in vivo lower rates of gluconeogenesis in humans or animals bearing type 2 diabetes have never been reported. Consequently it seems worth to discuss the possible factors that could lead to an at least normal or slightly above normal in vivo gluconeogenesis in spite of impaired enzymatic machinery. If one assumes that the enzymatic machinery is the result of long- or even medium-term regulation, efficient mechanisms of short-term up-regulation must be operative in type 2 diabetes, at least in the rat. There are several possibilities to be considered: 1) different concentrations of hormones able to stimulate gluconeogenesis; 2) higher substrate concentrations in the diabetic condition; 3) increased plasma concentrations of free fatty acid, which are known to estimulate gluconeogenesis [24-28]. With reference to the hormonal factors, it is known that glucagon, in addition to its medium- and long-term effects [16] can also promote short-term up-regulation of gluconeogenesis [29,30]. Insulin, in contrast, does not exert short-term effects in the liver [10,11]. Elevated glucagon levels have been found in type 2 diabetic humans [9-31] and in neonatal streptozotocin type 2 diabetic rats at least during certain stages after streptozotocin injection [17]. Consequently a short-term up-regulation by glucagon must be considered as a real possibility. It must be mentioned, however, that the stimulatory effect of glucagon on gluconeogenesis is relatively modest unless the cytosolic $NADH/NAD^+$ ratio is very high [29,30]. Concerning the possible contribution of increased substrate concentrations one cannot expect a significant contribution from lactate. The latter is by far the most important gluconeogenic substrate, but its normal concentration in blood (around 2 mM) is already saturating for gluconeogenesis [28,32] so that increments would not enhance glucose synthesis. Some positive effect favouring gluconeogenesis in the diabetic state could be expected, however, by a shift in the redox potential of the cytosolic NAD-NADH couple towards a more oxidized state (lower $NADH/NAD^+$ ratios). This would have the consequence of increasing the pyruvate concentration by virtue of the near-equilibrium of the lactate dehydrogenase reaction [27]. This positive effect is to be expected from the observation that when pyruvate was the sole substrate, a condition which means a strong oxidizing state for the cytosolic NAD-NADH couple [27], the difference between gluconeogenesis in the diabetic and the normal state was practically abolished. In a specific study with type 2 diabetic patients [31], plasma glycerol was increased by a factor of 1.46 and glycerol gluconeogenesis was increased more than twofold. From the glycerol concentration increase one would expect maximally a 1.46-fold increase in gluconeogenesis from this substrate. This disproportion, two-fold versus 1.46-fold, can be regarded as an indication that another factor or factors are contributing to the enhanced gluconeogenesis. One of these factors could be the more elevated glucagon concentration, as already mentioned. However, the more elevated fatty acid concentrations, which seem to be a frequent phenomenon in type 2 diabetes [4,31], could be equally contributing as stimulatory effectors.

In conclusion, the enzymatic machinery of the gluconeogenic pathway of neonatal type 2 diabetic rats seems to be adapted to low rates of glucose removal by extrahepatic tissues rather than to enhanced glucose production. Gluconeogenesis at rates high enough to contribute significantly to the fasting hyperglycemia can be generated only by short-term up-regulation which could, in principle, be produced by high glucagon, glycerol and fatty acids concentrations.

5. ACKNOWLEDGEMENTS

The authors wish to thank Dr. Ciomar Bersani Amado for supplying the diabetic rats.

REFERENCES

[1] Kraus-Friedmann, N. (1984) Hormonal regulation of hepatic gluconeogenesis. *Physiology Reviews*, **64**, 170-259.

[2] Pilks, S.J. and Granner, D.K. (1992). Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. *Annual Review of Physiology*, **54**, 885-909.

[3] Petersen, K.F., Price, T.B. and Bergeron, R. (1993) Regulation of net hepatic glycogenolysis and gluconeogenesis during exercise: Impact of type 1 diabetes. *Journal of Clinical Endocrinology and Metabolism*, **89(9)**, 4656-4664.

[4] Boden, G., Chen, X. and Stein, T.P. (2001) Gluconeo-

genesis in moderately and severely hyperglycemic patients with type 2 diabetes mellitus. *American Journal of Physiology*, *Endocrinology and Metabolism*, **280(1)**, E23-E30.

[5] Diraison, F., Large, V., Brunengraber, H. and Beylot, M. (1998) Non-invasive tracing of liver intermediary metabolism in normal subjects and in moderately hyperglycaemic NIDDM subjects: Evidence against increased gluconeogenesis and hepatic fatty acid oxidation in NIDDM. *Diabetologia*, **41**, 212-220.

[6] Andrikopoulos, S. and Proietto, J. (1995) The biochemical basis of increased hepatic glucose production in a mouse model of type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia*, **38**, 1389-1396.

[7] Puhakainen, I., Koivisto, V.A. and Yki-Jarvinen, H. (1992) Lipolysis and gluconeogenesis from glycerol are increased in patients with noninsulin-dependent diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism*, **75**, 789-794.

[8] Magnusson, I., Rothman, D.L., Katz, L.D., Shulman, R.G. and Shulman, G.I. (1992) Increased rate of gluconeogenesis in type II diabetes mellitus. *Journal of Clinical Investigation*, **90(4)**, 1323-1327.

[9] Basu, R., Schwenk, W.F. and Rizza, R.A. (2004) Both fasting glucose production and disappearance are abnormal in people with "mild" and "severe" type 2 diabetes. *American Journal of Physiology-Endocrinology and Metabolism*, **287(1)**, E55-E62.

[10] Söling, H.D., Koschel, R., Dragert, W., Kneer, P. and Creutzfeldt, W. (1966) The effect of insulin on the metabolism of isolated perfused livers of normal and alloxan-diabetic rats. I. The metabolism of isolated perfused livers in normal and alloxan-diabetic rats under various experimental conditions. *Diabetologia*, **2(1)**, 20- 31.

[11] Rudorff, K.H., Albrecht, G. and Staib, W. (1970) Über den Einfluβ von Insulin und Proinsulin auf die Gluconeogenese aus alanin in der isoliert perfundierten leber normaler und alloxandiabetischer ratten. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie*, **351**, 975-982.

[12] Cook, D.E. (1978) The effects of phenformin in normal vs. diabetic isolated perfused rat liver. *Research Communications in Chemical Pathology and Pharmacology*, **22**, 119-134.

[13] Wagle, S.R., Ingebretsen, W.R. and Sampson, L. (1975) Studies on gluconeogenesis and stimulation of glycogen and protein synthesis in isolated hepatocytes in alloxan diabetic, normal fed and fasted animals. *Acta Diabetologica Latina*, **12**, 185-198.

[14] Lombardo, Y.B., Hron, W.T. and Menahan, L.A. (1978) Effect of insulin in vitro on the isolated, perfused alloxan-diabetic rat liver. *Diabetologia*, **14(1)**, 47-51.

[15] Akimoto, L.S., Pedrinho, S.R., Lopes, G. and Bazotte, R.B. (2000) Rates of gluconeogenesis in perfused liver of alloxan-diabetic fed rats. *Research Communications in Molecular Pathology and Pharmacology*, **107(1-2)**, 65-77.

[16] Barthel, A. and Schmoll, D. (2003) Novel concepts in insulin regulation of hepatic gluconeogenesis. *American Journal of Physiology*, *Endocrinology and Metabolism*, **285(4)**, E685-E692.

[17] Arulmozhi, D.K., Veeranjaneyulu, A. and Bodhankar, S.L. (2004) Neonatal streptozotocin-induced rat model of type 2 diabetes mellitus: A glance. *Indian Journal of Pharmacology*, **36(4)**, 217-221.

[18] Oliveira, D.S., Bersani-Amado, C.A., Martini, M.C., Suzuki-Kemmelmeier, F. and Bracht, A. (2007) Glycogen levels and energy status of the liver of fasting rats with diabetes types 1 and 2. *Brazilian Archives of Biology and Technology*, **50(5)**, 785-791.

[19] Portha, B., Levacher, C., Picon, L. and Rosselin, G. (1974) Diabetogenic effect of streptozotocin in the rat during the perinatal period. *Diabetes*, **23(11)**, 889-895.

[20] Cuman, R.K.N., Bersani-Amado, C. and Fortes, Z.B. (2001) Influence of type 2 diabetes upon the inflammatory response in rats. *Inflammation*, **50(9)**, 460-465.

[21] Scholz, R. and Bücher, T. (1965) Hemoglobin-free perfusion of rat liver. In: Changce, B., Estabrook, W. and Williamson, J.R., Eds., *Control of Energy Metabolism*, Academic Press, New York, 393-414.

[22] Bracht, A., Ishii-Iwamoto, E.L. and Kelmer-Bracht, A.M. (2003) O estudo do metabolismo no fígado em perfusão. In: Bracht, A. and Ishii-Iwamoto, E.L. Eds., *Métodos de Laboratório em Bioquímica*, Editora Manole, São Paulo, 275-289.

[23] Bergmeyer, H.U. (1974) Methods of Enzymatic Analysis, Verlag Chemie-Academic Press, Weinheim-London.

[24] Bazotte, R.B., Constantin, J., Hell, N.S. and Bracht, A. (1990) Hepatic metabolism of meal-fed rats: Studies in vivo and in the isolated perfused liver. *Physiology and Behavior*, **48(2)**, 247-253.

[25] Häussinger, D., Gerok, W. and Sies, S. (1983) Regulation of flux through glutaminase and glutamine synthetase in isolated perfused rat liver. *Biochimica et Biophysica Acta*, **755(2),** 272-278.

[26] Beck-Nielsen, H., Hother-Nielsen, O. and Staehr, P. (2002) Is hepatic glucose production increased in type 2 diabetes mellitus? *Current Diabetes Reports*, **2**, 231-236.

[27] Sies, H. (1982) Nicotinamide nucleotide compartmentation. In: Sies, H., Ed., *Metabolic Compartmentation*. Academic Press, New York, 205-231.

[28] Veiga, R.P., Silva, M.H., Teodoro, G.R., Yamamoto, N.S., Constantin, J. and Bracht, A. (2008) Metabolic fluxes in the liver of rats bearing the walker 256 tumor: Influence of the circulating levels of substrates and fatty acids. *Cell Biochemistry and Function*, **26(1)**, 51-63.

[29] Constantin, J., Ishii-Iwamoto, E.L., Suzuki Kemmelmeie, F. and Bracht, A. (1994) Zonation of the action of glucagon on gluconeogenesis studied in the bivascularly perfused rat liver. *FEBS Letters*, **352(1)**, 24-26.

[30] Marques da Silva, A.C., D'Ávila, R.B., Ferrari, A.G., Kelmer-Bracht, A.M., Constantin, J. and Bracht, A. (1997) Ca^{2+}-dependence of gluconeogenesis stimulation by glucagon at different cytosolic NAD^{+}-NADH redox potentials. *Brazilian Journal of Medical and Biological Research*, **30(7)**, 827-836.

[31] Nurjhan, N., Conoli, A. and Gerich, J. (1992) Increased lipolysis and its consequences on gluconeogenesis in non-insulin-dependent diabetes mellitus. *Journal of Clinical Investigation*, **89(1)**, 169-175.

[32] Argilés, J.M. and López-Soriano, F.J. (1991) The energy state of tumorbearing rats. *Journal of Biological Chemistry*, **266**, 2978-2982.

Vol.2, No.5, 484-492 (2010)
doi:10.4236/health.2010.25072

Health

The lytic mechanism of *Escherichia coli* α-hemolysin associated to outer membrane vesicles

——Lytic action mechanism of OMVs-associated HlyA

Vanesa Herlax[1]*, Maria Florencia Henning[1], Ana María Bernasconi[1], Felix María Goñi[3], Laura Bakás[1,2]

[1]Instituto de Investigaciones Bioquímicas La Plata (INIBIOLP), CCT-La Plata, CONICET, UNLP, Facultad de Ciencias Médicas, La Plata, Argentina; *Corresponding Author: vherlax@atlas.med.unlp.edu.ar
[2]Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina
[3]Unidad de Biofísica (Centro Mixto CSIC-UPV/EHU), and Departamento de Bioquímica, Universidad del País Vasco, Bilbao, Spain

Received 2 January 2010; revised 1 February 2010; accepted 2 February 2010.

ABSTRACT

Alpha-hemolysin (HlyA) is an extracellular toxin secreted by *Escherichia coli*, targeting to plasma membranes of eukaryotic cells. Recently it was found that this toxin is released to external media associated to bacterial outer membrane vesicles (OMVs), but the hemolytic mechanism in this way has not been studied yet. Our results report that HlyA is the only protein present in OMVs that is responsible for erythrocyte lysis, and show that no fusion event is involved in the lytic mechanism of OMVs-HlyA. Furthermore, the specific hemolytic activity is approximately 10 fold higher than that of purified free-HlyA, showing the same relative lysis efficiency and specificity for erythrocytes from different species. OMVs could be an important auxiliary way of secretion, acting mainly as a concentration mechanism of HlyA near the target cells. Cell lysis would occur after toxin transfer from OMVs to target membranes, as demonstrated by hemolysis kinetic studies, lipid mixing and western blot assays.

Keywords: OMVs; Toxin; Lipid Mixing; Hemolytic Activity; Erythrocytes

1. INTRODUCTION

The hemolytic toxin α-hemolysin (HlyA), member of the RTX toxin family [1], is an important virulence factor produced by several strains of *Escherichia coli*. It is involved in human extraintestinal diseases, such as urinary tract infections, peritonitis, meningitis and septicemia [2]. As in most RTX proteins, an operon composed of 4 genes (*hlyABCD*) is involved in the polypeptide synthesis, postraslational modification and secretion of the active HlyA to extracellular media [3,4]. HlyA is synthesized in an inactive form (ProHlyA), which is activated in the cytoplasm to the hemolytically active form by HlyC, a fatty acid acyltransferase [5]. Then HlyA is secreted across both membranes by the type I export process employing an uncleaved C-terminal recognition signal [6,7]. Although HlyA has its own machinery to be export from the bacteria, Balsalobre *et al.* [8] demonstrated the presence of physiologically active HlyA in outer membrane vesicles (OMVs) of hemolytic strains of *Escherichia coli*.

Outer membrane vesicles (OMVs) are constantly being discharged from the surface of Gram negative bacteria during bacterial growth. All Gram negative bacteria studied up to date, including *Escherichia coli*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Shigella flexneri and Actinobacillus actinomycetemcomitans* produce outer membrane vesicles and their release is increased when bacteria are exposed to stressful conditions such as antibiotics or serum. One can be tempted to think that OMVs production is the result of membrane instability, but recent studies have demonstrated that mutations that affect protein synthesis, localization, envelope structure and envelope stress response pathway alter the vesiculation levels. In this way the release of OMVs offers to the cell an effective mechanism for removal of material as a macromolecular complex, allowing to discard unwanted material or alter the composition of the envelope under conditions where remodeling would be advantageous [9,10].

Despite that *in vivo*, release of OMVs could not be monitored, the presence of particles resembling these vesicles has been detected in plasma from patients with different infectious processes [11-18]. OMVs serve as secretory vehicles for proteins and lipids of Gram negative bacteria and play roles in establishing a colon-

ization niche carrying or transmitting virulence factors into host cells or modulating host defense and response, acting also as long-range virulence factors that can protect luminal cargo from extracellular host proteases and penetrate into tissues more readily than larger bacteria [19]. An extensive review about the significance of bacterial OMVs in the host-pathogen interaction was published by Kuehn and Kesty [20]. Besides the toxin o protein delivery, other roles were characterized for OMVs, such as: interaction interspecies during multispecies infections, DNA transfer, DNA uptake, communication interspcies [21].

OMVs are primarily composed of lipopolysaccharide (LPS), phospholipids and various outer membrane (OM) proteins and periplasmic components, but they do not contain intra membrane (IM) or cytoplasmic components [22], although OMVs isolated from *E. coli* O157:H7 contain DNA [23].

Considering that HlyA was found associated to OMVs of some strain of *E. coli* and that erythrocytes have been the cell type classically used in studies on the action mechanism of this toxin, in this research we studied the hemolytic mechanism of OMVs-associated HlyA. The results demonstrate that the lytic action of OMVs-associated HlyA like free-HlyA does not involve fusion events, instead the toxin is transferred from OMVs to target cells, as demonstrated by hemolysis kinetic studies, lipid mixing and western blot assays.

2. EXPERIMENTAL PROCEDURES

2.1. Isolation of Outer Membrane Vesicles OMVs

E. coli strains used as a source of HlyA were WAM 1824, an overproducing hemolysin strain [24], and WAM 783, for the unacylated inactive toxin ProHlyA [25]. Both of them were kindly provided by R. A.Welch (University of Wisconsin, Madison, Wisconsin).

OMVs were isolated from bacterial culture supernatants. Briefly, bacteria were grown at 37ºC in Luria Bertani broth (LB). Culture samples from the decelerating growth phase were centrifuged at 10000 rpm for 20 min at 4ºC. The resulting suspension was then centrifuged at 47500 rpm for 2 h at 4ºC to collect the pellet containing OMVs.

2.2. Protein Purification

Free-HlyA was purified from the supernatants resulting from OMVs. It was concentrated and partially purified by precipitation with 20% cold ethanol. A precipitate containing HlyA was collected by centrifugation (1 h, 10000 rpm in a Sorvall rotor SSA 34), then it was resuspended in 20 mM Tris, 150 mM NaCl, pH 7.0 (TC buffer). This preparation showed, on SDS-PAGE, a main band at 110 kDa corresponding to more than 90% of the total protein. This band was assigned to free-HlyA. The protein could be stored at –70°C.

2.3. OMVs Composition

2.3.1. Quantitative Assays

Quantitative assays were performed using Lowry method for proteins [26], KDO 2-keto-3-deoxyoctonate for LPS [27] and inorganic phosphorous for phospholipids [28].

2.3.2. Analysis of Proteins by SDS-PAGE

Samples were electrophoresed on 10% acrylamide gels in the presence of SDS according to Laemmli *et al.* [29]. Protein bands were visualized by Coomassie Blue staining [30].

2.3.3. Immunoblotting Analysis

Samples from 10% SDS-polyacrylamide gels were transferred to nitrocellulose by the method of *Towbin et al.* [31]. Blots were blocked with 3% skim milk in TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) at room temperature for 2 h. They were then incubated with a solution containing a polyclonal rabbit anti-hemolysin antibody (1:1000) in 3% skim milk-TBS at 4°C overnight, washed with TBS buffer, and finally reacted with peroxidase-conjugated anti-rabbit Ig antibody (Sigma) (1:1000) in TBS buffer with 3% skim milk at room temperature for 2 h. After incubation and washing as stated above, the nitrocellulose was transferred to a peroxidase substrate solution containing 6 mg 4-chloro-1-naphthol (Sigma) in 1 ml methanol, 1 ml TBS, 3 ml H_2O and 8 µl H_2O_2 for the detection of horseradish peroxidase-conjugate antibodies on the membrane.

2.4. Hemolytic Assays

For the hemolytic assays, free-HlyA or OMVs associated-HlyA were serially diluted in TC cold buffer containing 10 mM $CaCl_2$ on a 96-well microtiter plate. One hundred µL of the diluted suspensions were mixed with 100 µl of standardized horse or rabbit erythrocytes. The mixture was incubated at 37°C for 30 min. The absorbance of supernatants was read at 412 nM [32]. One hemolytic unit/mL (HU/mL) is defined as 10 fold the dilution of toxin preparation producing 50% lysis of the erythrocyte suspension.

The standardization of erythrocytes was done just before the assay. The erythrocytes were washed in 0.9% NaCl and then diluted to 12.5 µL in 1 mL of distilled water to give a reading of 0.6 absorbance unit at 412 nM [33].

2.5. Hemolysis Kinetics

Hemolysis kinetics was determined by measuring the decrease in turbidity (absorbance at 650 nM) of a standardized horse erythrocyte suspension exposed to free-HlyA or OMVs associated-HlyA as a function of time at 37ºC. A series of hemolytic reactions were set up starting with an amount of free-HlyA or OMVs associated-HlyA that would produce 100% of hemolysis in 96-well plate dilution assays. The real time kinetics assays for hemolysis were performed in the following manner: 500 µL of standardized erythrocytes and 500 µL of hemolysis buffer containing 10 mM $CaCl_2$ were placed into a cuvette. Hemolysis started when the toxin was injected into the cuvette, and the absorbance at 650 nM was measured.

2.6. LUVs Preparation

Large unilamellar vesicles (LUVs) composed of PC/PE/Chol 2:1:1 molar ratio were prepared by extrusion using 0.1 µM pore size membranes as described by Mayer *et al.* [34].

2.7. Ghost Erythrocytes Preparation

Five mililitres of horse erythrocytes were washed with TC buffer and osmotically lysed in 10 mM tris-HCl PH = 7.4 buffer at 4ºC for 30 min. The membranes were pelleted by centrifugation (10 min at 10000 rpm), and washed until the supernatant remained clear. The membranes were finally resuspended in 3 mL of TC buffer. The phosphate concentration of these samples was 0.53 ± 0.05 mg phospholipid/mL (n = 3).

2.8. Labeling of Ghost Erythrocytes with Rhodamine-Phosphatidylethalonamine (Rh-Pe) and N-(7-Nitro-2, 1, 3-Benzoxadiazol-4-Yl)-Phosphatidylethanolamine (NBD-PE)

Ghost erythrocytes were labeled with Rh-PE and NBD-PE (Molecular Probes, Eugene, O.R.). Both fluorophores are coupled to the free amino group of phosphatidylethanolamine to provide analogues which can be incurporated into a lipid bilayer. 20 µL of a fresh solution of each probe (1 mg/ml in ethanol) was added to 75 µL of ghost erythrocytes. After incubating the suspension for 1 hr at room temperature, 6 washes with TC were performed so as to separate unbound labeled lipids. Finally the labeled ghost erythrocytes were resuspended in 5.4 ml of TC.

2.9. Lipid Mixing

Membrane fusion was analyzed by lipid mixing between OMVs and ghost erythrocytes labeled with NBD-PE and Rh-PE. The fusion assay involves resonance energy transfer between NBD as donor and Rh as acceptor. When both fluorescent lipids are in ghost erythrocytes, efficient energy transfer is observed [35]. After lipid mixing by fusion with a population of unlabeled membranes, in this case OMVs, a decrease in efficiency of resonance energy transfer should be observed followed by an increase of the donor fluorescence or decrease of the acceptor fluorescence. In our experimental design, we followed the increase of donor (NBD-PE) fluorescence as a function of time using the excitation and emission monochromators set at 465 nM and 530 nM, respectively. Slits for excitation and emission were 4 nM.

The extent of lipid mixing was determined according to the following equation:

$$\% \text{ lipid mixing} = [(F_t - F_0) / (F_{max} - F_0)] \times 100$$

where F_0 is the initial fluorescence value of erythrocytes labeled with both probes, F_t is the value of fluorescence after t minutes of incubation with OMVs and F_{max} (100% fluorescence) is the value of fluorescence after addition of Triton × 100 to disperse maximally the probes.

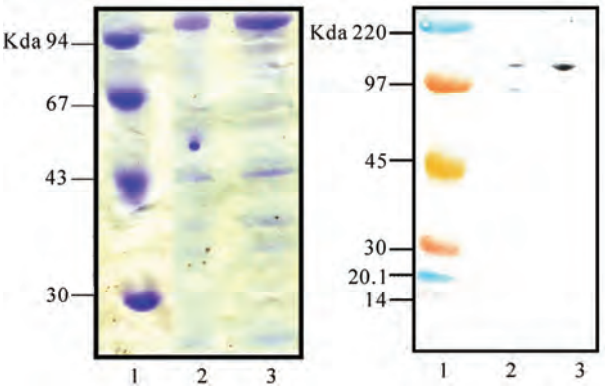
3. RESULTS

3.1. Characterization of OMVs Produced by *E. coli* WAM 1824

Recently, Balsalobre *et al.* demonstrated that physiologically active HlyA is associated with OMVs produced by laboratory strains and also from natural and clinical *E. coli* isolates. However, as the amount of OMVs and the percentage of the soluble and OMVs associated HlyA were likely to vary markedly depending on the strain [8], we characterized OMVs obtained from *E. coli* WAM 1824 strain.

OMVs were purified from the culture filtrate of *E. coli* WAM 1824. Many bilayered spherical vesicles with diameters ranging from 50 to 200 nm were observed by electron microscopy (data not shown). The lipid composition of these vesicles was analyzed by thin layer chromatography, showing that the two predominant lipid species are phosphatidylethanolamine (PE) and cardiolipin (CL), demonstrating that these vesicles arise indeed from the bacterial outer membrane (data not shown).

The presence of HlyA in OMVs produced by *E. coli* WAM 1824 is shown in **Figure 1(a)**. This figure shows the SDS-PAGE polypeptide profile of OMVs. A band with an electrophoretic mobility corresponding to HlyA (MW 110 kDa) was observed whose identity was confirmed by Western blot analysis employing polyclonal antibodies directed against purified HlyA (**Figure 1(b)**). The others bands that appear in SDS-PAGE stained by Comassie stain, correspond to periplasmic and outer membrane proteins also present in OMVs.



(a) (b)

Figure 1. HlyA is present in WAM 1824 outer membrane vesicles. (a) SDS-PAGE 10%, stained by Coomasie. 1: LMW, 2: WAM 1824 OMVs (10µg prot), 3: WAM 783 OMVs (10 µg); (b) Western-Blot, using anti-HlyA polyclonal antibodies. 1: Rainbow molecular weight, 2: OMV's (100 ng prot), 3: Standard HlyA (50 ng).

3.2. Comparison of Hemolytic Activity between Free-HLYA and OMVs Associated-HlyA

The following experiments were done so as to compare the hemolytic activity between the OMVs associated-HlyA and free toxin, which was quantified by serial dilution assays against horse erythrocytes. The hemolytic activity was 40960 and 2560 HU/ml for OMVs associated-HlyA and free toxin, respectively. If we consider that total protein concentration in the OMVs is 2.7 mg/mL and 70% corresponds to HlyA (estimated from the intensity of the 110 kDa band in a 10% SDS-PAGE gel, analyzed by Kodak digital Science 1D Software), so the approximate concentration of OMVs associated- HlyA is 1.7 10^{-5} M. These values allow us to calculate the specific activity which is 2.37 10^{12} HU/mol and 2.04 10^{11} HU/mol for OMVs associated-HlyA and free-HlyA, respectively.

In order to confirm that the hemolytic activity of OMVs is solely due to HlyA and not to other proteins present in the OMVs, *E. coli* WAM 783 (strain with a deletion of *hlyC* gen that encodes HlyC, the protein responsible for HlyA acylation) was used as a source of OMVs. The presence of a protein corresponding to 110 kDa in 10% SDS-PAGE confirms that inactive unacylated toxin is also associated to OMVs (**Figure 1**, lane 3). OMVs obtained from this strain do not have any hemolytic activity confirming that this activity in OMVs is a particular property due largely or totally to the presence of HlyA.

3.3. Lipid Mixing Assays

In order to determine whether the hemolytic action mechanism of OMVs associated-HlyA implies membrane fusion, we analyzed the lipid mixing between OMVs and ghost erythrocytes labeled with NBD-PE (donor) and Rh-PE (acceptor). The ghost erythrocytes concentration used in lipid mixing assays is equal to the erythrocyte suspension used in hemolytic experiments.

6.9% FRET decrease was detected between OMVs and labeled erythrocytes promoted by OMVs associated-HlyA. When free-HlyA was added to labeled erythrocytes, 8.1% FRET decrease was obtained (**Figure 2**), which cannot be assigned to lipid mixing because unlabeled erythrocytes were absent in this experiment, so it should be attributed to protein insertion into lipid bilayer, increasing the distance between donor and acceptor [36].

The decrease in energy transfer in both experiments was similar, indicating that fusion events are not involved in the action mechanism of this toxin when it is OMVs associated; instead, a transfer process from OMVs to the target membrane must be involved. The presence of a high affinity specific receptor is not strictly necessary, as the same experiments were repeated with labeled large unilamellar vesicles (LUVs), giving similar FRET decrease (data not shown).

3.4. HlyA Transfer from OMVs to Erythrocytes

To study HlyA transfer from OMVs to erythrocytes, a serial diluted hemolytic assay was carried out as described previously in Experimental Procedures. Samples were centrifuged at 10,000 rpm to separate ghost erythrocytes from OMVs. The pellets containing the ghosts were resuspended in SDS sample buffer, and electrophoresed in a 10% SDS-PAGE. Finally this gel was trans-

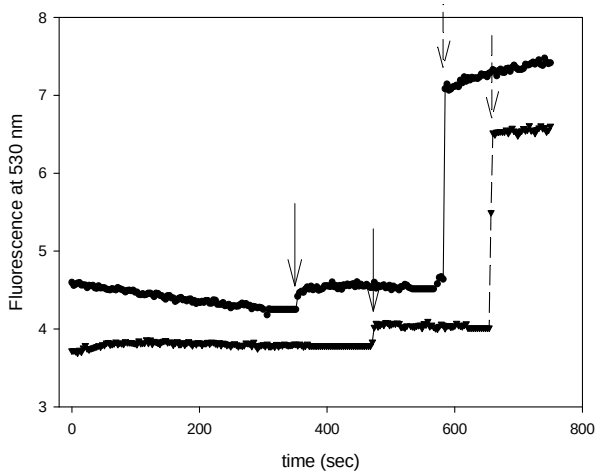
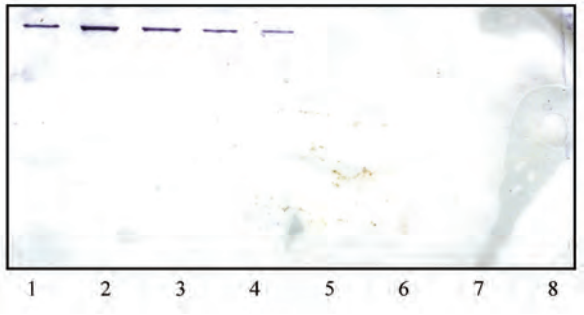


Figure 2. OMVs associated-HlyA does not induce membrane fusion. Lipid mixing assays between labeled erythrocytes with Rh-PE/NBD-PE and OMVs (●) or free-HlyA (▼). Solid arrows correspond to the addition of OMVs or free-HlyA. Dotted arrows correspond to the addition of 0.2% Triton X-100 (F_{max}). Total lipid concentration was 0.1 mM. 40 HU for free-HlyA and OMVs associated-HlyA were used.

ferred to nitrocellulose and HlyA was detected using a polyclonal rabbit anti-HlyA antibody. **Figure 3(a)** shows that HlyA is effectively transferred to erythrocytes. The amount of HlyA decreases with the decrease of the OMVs concentration, according to the decrease in the hemolytic activity observed (data not shown).

Another fact that confirms that fusion events are not involved in the hemolytic process mediated by OMVs is the absence of LPS in the ghost erythrocytes membranes after their incubation with OMVs. The detection of LPS was analyzed by a specific silver stain for LPS (BioRad silver stain kit, catalog 161-0445) of a 16% SDS-PAGE. A typical LPS pattern is not observed in this sample (**Figure 3(b)**, lane 1), instead some bands of glycoproteins of erythrocytes are observed.



(a)

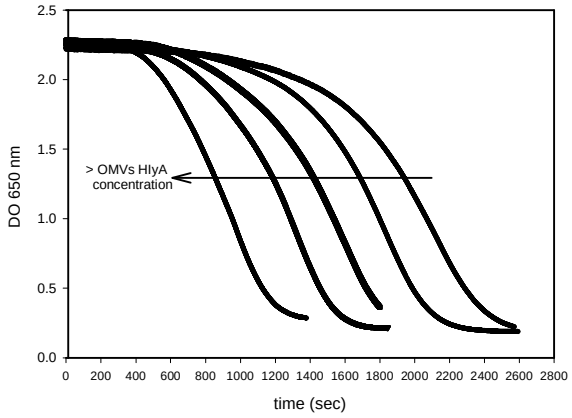
(b)

Figure 3. HlyA is transfered from OMVs to erythrocytes. (a) HlyA transference from OMVs to erythrocytes analyzed by western blot: lane 1: standard HlyA, 2-7: samples of ghosts erythrocytes previously incubated at 37°C during 30 min with serially diluted OMVs, 8: ghost erythrocytes; (b) Analysis of LPS content in ghost erythrocytes: lane 1: ghosts obtained from the hemolysis assay with OMVs, 2: ghosts, 3: OMVs and 4: Standard LPS from *E. coli* 011:B4.

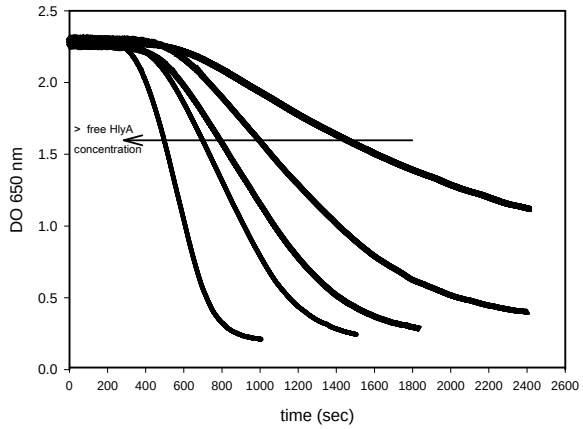
3.5. Hemolysis Kinetics

Typical hemolysis time courses are shown in **Figure 4**. For both, OMVs associated-HlyA and free-HlyA, the time to reach 50% of hemolysis increases as the toxin concentration decreases. The hemolytic curves exhibit a define lag period for both free-HlyA and OMVs associated-HlyA. This lag period lengthens noticeably at decreasing concentrations of OMVs associated-HlyA, but it remains practically constant for free-HlyA as seen in **Figure 5(a)**, indicating that the rate limiting step in the hemolysis process induced by OMVs associated-HlyA is the diffusion of vesicles in the aqueous medium, due to their higher molecular weight and volume compared to free-HlyA.

Initial rate of hemolysis obtained from the linear portion of the kinetic curve are shown in **Figure 5(b)**. Vir-

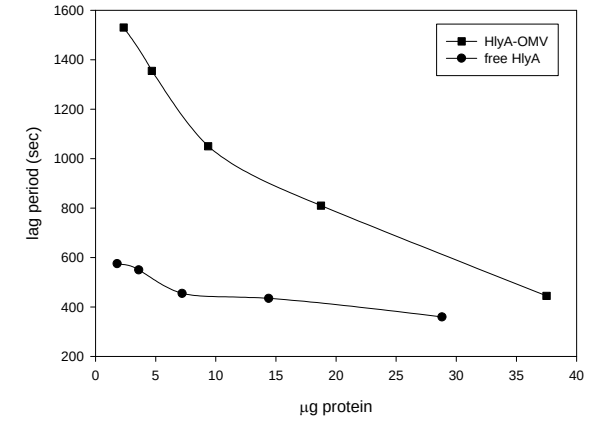


(a)

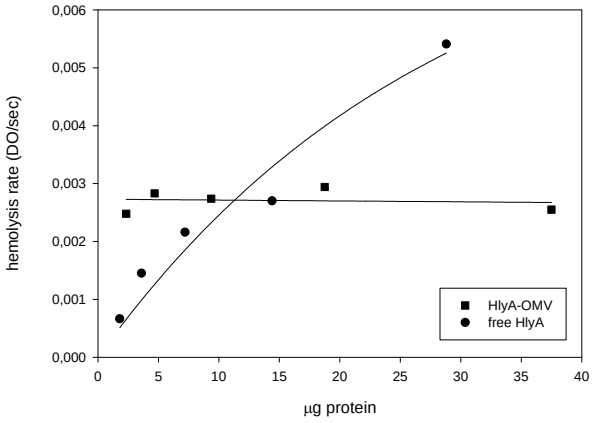


(b)

Figure 4. Kinetics of hemolysis induced by OMVs associated-HlyA (a) and free-HlyA (b). The real time kinetic assays for hemolysis were performed in the following manner: 500 µl of standardized erythrocytes and 500 µl of hemolysis buffer containing 10 mM $CaCl_2$ were placed into a cuvette. Hemolysis started when the toxin was injected into the cuvette, and the absorbance at 650 nm was measured. The arrow indicates toxin concentration increase.



(a)



(b)

Figure 5. Initial hemolytic rate (a) and lag time (b) as a function of OMVs associated-HlyA (●) and free-HlyA (■) concentration. Initial rates (linear portion) and lag time were obtained from **Figure 5** at different toxin concentrations.

tually constant values were obtained with OMVs associated-HlyA for all the concentrations studied, while a decrease in initial rates as concentrations decrease was observed for free-HlyA.

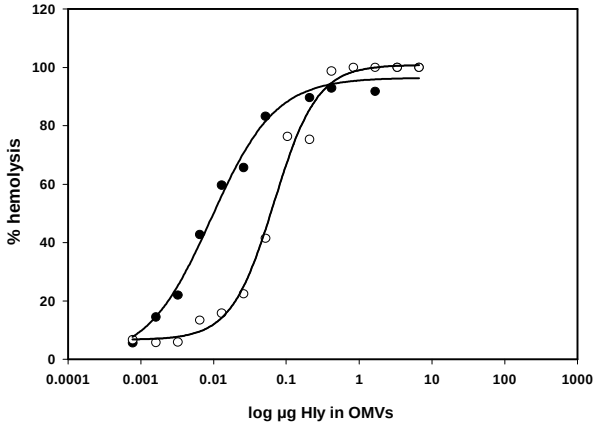
3.6. Erythrocytes Specificities of Free-HLYA and OMVs Associated-HlyA

To characterize the hemolytic efficiency of OMVs associated-HlyA on different species of mammalian cells, we chose rabbit and horse erythrocytes, which were the most commonly used and characterized in previous studies related with the hemolytic action mechanism of this toxin. In **Figures 6(a)** and **(b)** it can be seen that in both cases, rabbit erythrocytes are more sensitive than those of horse, as demonstrated in earlier results on the characterization of the lytic action of HlyA [37]. The D_{50} values (amount of toxin that produces 50% of hemolysis) are 0.46 and 2.81 nM for OMVs associated-HlyA for rabbit and horse erythrocytes respectively.

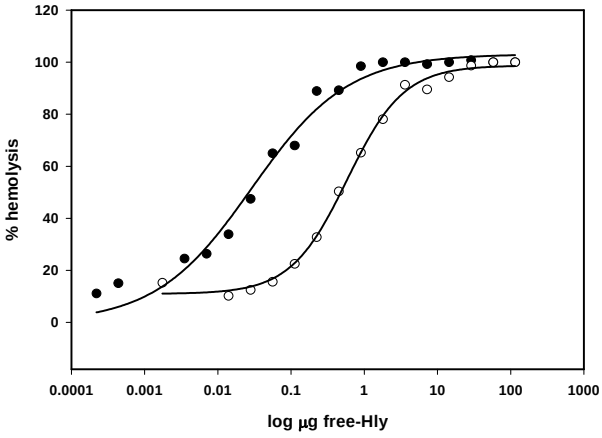
The dose-response curves indicate a higher lytic efficiency of OMVs associated-HlyA (**Figure 6(a)**) as compared to free-HlyA (**Figure 6(b)**), shown by the D_{50} on horse erythrocytes of 2.81 and 22.5 nM, respectively. Moreover, analysis of curves of hemolytic activity against concentration using SigmaPlot (Jandel Scientific, San Rafael, CA) fitted a sigmoidal curve in both cases. In the case of horse cells, data fit a sigmoid 4 parameter curve, while the rabbit ones fit a logistic 3 parameter curve, demonstrating a different cooperativity in the hemolysis process. The same behavior is observed for OMVs associated-HlyA as for free-HlyA.

4. DISCUSSIONS

Recently, it was demonstrated the presence of active HlyA associated with OMVs produced by natural and clinical *E. coli* isolates. Despite a great deal of published



(a)



(b)

Figure 6. Hemolysis induced by OMVs associated-HlyA and free-HlyA on erythrocytes of different species. Hemolytic activity of OMVs associated-HlyA (a) and free-HlyA (b), tested with rabbit erythrocytes (●) and horse erythrocytes (○).

data regarding the activity of soluble HlyA secreted by *E. coli*, nothing was known regarding the action mechanism of OMVs associated HlyA. First, we demonstrated in this research, the presence of HlyA associated to OMVs produced by a laboratory *E. coli* strains (WAM 1824). We demonstrated the presence of HlyA associated to these vesicles as seen in SDS-PAGE polypeptide profile and Western blot analysis employing polyclonal antibodies directed against purified HlyA (**Figure 1**). The toxin associated to OMVs has a specific activity one order of magnitude higher than the free-HlyA. It is important to note that this protein is the only one responsable of the hemolytic activity as OMVs produced by strain WAM 783 (strain encoding the inactive form of HlyA, ProHlyA) are not hemolytic.

In order to determine whether the hemolytic action mechanism of OMVs associated-HlyA implies membrane fusion, we analyzed the lipid mixing between OMVs and labeled ghost erythrocytes. The small FRET decrease obtained could be attributed to protein insertion into lipid bilayer, as similar values were obtained in the presence of free-HlyA (**Figure 2**). These values are similar to those obtained in the study of HlyA insertion area using also FRET experiments with labeled membranes [36]. As for free-HlyA [38], fusion events are not involved in the action mechanism of the toxin associated with OMVs; instead, a transfer process from OMVs to the target membrane must be involved. The transference of HlyA as a mechanism was confirmed by the presence of HlyA and by the absence of LPS in the pellet obtained after incubation of ghost erythrocytes with OMVs (**Figure 3**). These results effectively demonstrate that the hemolysis is caused by the transference of HlyA and not OMVs-cell membranes fusion.

Although fusion events have been described for leukotoxin from *A. actinomycetemcomitans*, another RTX toxin, this could be specific for this RTX toxin [39], as the secretion of leukotoxin differs from that of other RTX proteins in that it remains associated with intact cells even in the presence of functional *ltxBD* genes [40].

The common mechanism of the lytic action of HlyA implies the diffusion of a soluble secreted toxin from bacteria, through aqueous medium, to reach the target cells. Now, our results demonstrate that a large number of toxin molecules are concentrated and transported by OMVs and then transfer to target cells.

The different behavior in the kinetics of hemolysis observed in **Figure 4** for OMVs associated-HlyA and free-HlyA can be explained considering that, in the first case, the limiting step is the slow diffusion of OMVs in the aqueous medium that increases the lag time. Once OMVs reach the erythrocyte, a large number of toxin molecules which are concentrated on OMVs, are all together transferred to target cells either as a monomer or as a preassembled oligomer. On the contrary, the limiting step for free-HlyA is the number of toxin molecules that reach the target cells, due to the fact that they decrease as a function of toxin concentration. This can explain the high specific hemolytic activity for OMVs-associated HlyA in comparison with the hemolytic activity of free-HlyA. The fact that HlyA can be transfer in an oligomeric structure is possible because we have recently demonstrated that an oligomer is necessary for the occurrence of hemolysis [41]. What's more, the adoption of a cytolytically active oligomeric conformation in OMVs was described for enterobacterial ClyA cytotoxin [18].

The transference is direct from the OMVs to the erythrocytes without the delivery of the toxin to the medium. This is demonstrated by the differences found in the hemolysis kinetic studies (**Figure 4**). This conclusion is also supported by results published by Balsalobre *et al.*, who demonstrated that HlyA is tightly associated to vesicles appearing in a soluble form only after the membrane structure of the OMVs is disrupted by detergents [8]. This transfer process does not need the presence of a specific high affinity receptor because the same effect was observed when LUVs labeled with Rh-PE and NBD-PE were used (data not shown).

Rabbit and horse erythrocytes, were the most commonly erythrocytes used and characterized in previous studies related with the hemolytic action mechanism of this toxin. In **Figures 6(a)** and **(b)** it can be seen that in both cases, rabbit erythrocytes are more sensitive than those of horse, as demonstrated in earlier results on the characterization of the lytic action of HlyA [37]. For rabbit erythrocytes a smoother hemolytic curve was obtained in comparison to that of horse erythrocytes. This fact may be due to the presence of high proportion of high affinity receptors in the horse erythrocytes in comparison to those of rabbit. The higher lytic efficiency of the toxin with rabbit cells could be due to the presence of a large number of low affinity binding sites *i.e.* membrane phospholipids, which facilitate the concentration of the toxin in the membrane (Herlax *et al.*, unpublished results).

Currently, the presence of a receptor is a contradictory point in the elucidation of the action mechanism of this toxin, probably due that experiments were done with erythrocytes from different mammalian species. Glycophorin was described as a receptor in horse and human erythrocytes [42], while another group proposed that the hemolytic process does not depend on the receptor presence [43]. Anyway, the same behavior for HlyA against to erythrocytes of different species is observed for OMVs associated-HlyA as for free-HlyA.

Finally, it is important to remember that our previous results support the hypothesis that HlyA is secreted to the external medium as a LPS-HlyA complex, in which the main action of LPS is to maintain the protein stability in solution, while it only indirectly affects HlyA lytic

activity [44]. The presence of LPS in the toxin sample used in those studies, which was obtained by precipitation techniques from a culture supernatant without the ultracentrifugation step, could be due to some OMVs contamination. This is also supported by the presence of phospholipid in toxin samples purified by methods such as ammonium sulphate precipitation and exclusion chromatography [45]. Due to the fact that OMVs associated-HlyA represents a very high percentage of toxins in the culture supernatants, results on biological effects of HlyA published up to date, mainly those in which sublytic concentrations were used, should be revised as small amounts of LPS present in the OMVs can stimulate the release of inflammatory mediators [46].

In conclusion, our results demonstrate that OMVs constitute an alternative secretion mechanism for HlyA that result in the toxin reaching higher concentrations without altering its lytic action mechanism.

The observation that certain virulence factors are enrichhed in vesicles suggests that OMVs may have a key role in bacterial pathogenesis by mediating transmission of active virulence factors and other bacterial envelopment component to host cells. Numerous OMVs associated virulence factors have been shown to induce cytotoxicity confer vesicle binding to and invasion to host cells and modulate the immune response. On the other hand, similar to bacterial-host cell interactions, OMVs-cell interactions can be altered by manipulating the expression of outer membrane proteins in bacteria. The manipulation of OMVs adherence and the possibility to create chimeras between the N-terminal half of HlyA, which contains the pore forming domain, and binding domains to specific proteins in the surface of a cell of interest (e.g. cancerous cells) should be useful for numerous applications, redirecting this engineering OMVs to specific cell types in order to achieve a desired therapeutic response.

5. ACKNOWLEDGEMENTS

This work was partially supported by grants from UNLP, CICPBA and ANPCyT-Argentina. LSB is a member of the "Carrera del Investigador", CICPBA, Argentina; VH is a member of the "Carrera del Investigador", Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina and MFH is a Fellow from CONICET, Argentina. AMB is a member the "Carrera del Técnico y Profesional de Apoyo", CONICET.

REFERENCES

[1] Coote, J.G. (1992) Structural and fuctional relationships among the RTX toxin determinants of gram-negative bacteria. *FEMS Microbiology Reviews*, **88**, 137-162.

[2] Cavalieri, S., Bohach, G. and Synder, I. (1984) *Escherichia coli* a-hemolysin characteristics and probable role in pathogenicity. *Microbiology Reviews*, **48**, 326-343.

[3] Welch, R.A. (1991) Pore-forming cytolysins of gram-negative bacteria. *Molecular Microbiology*, **5(3)**, 521-528.

[4] Ludwig, A. and Goebel, W. (1999) The family of the multigenic encoded RTX toxin. In: Alouf, J.E. and Freer, J.H., Eds., *The Comprehensive Source-Book of Bacterial Protein Toxins*, Academic Press, 330-348.

[5] Stanley, P., Koronakis, V. and Hughes, C. (1998) Acylation of *Escherichia coli* hemolysin: A unique protein lipidation mechanism underlying toxin fuction. *Microbiology and Molecular Biology Reviews*, **62(2)**, 309-333.

[6] Jarchau, T., Chakraborty, T., Garcia, F. and Goebel, W. (1994) Selection for transport competence of C-terminal polypeptides derived from *Escherichia coli* hemolysin: The shortest peptide capable of autonomous HlyB/HlyD-dependent secretion comprises the C-terminal 62 amino acids of HlyA. *Molecular and General Genetics*, **245**, 53-60.

[7] Koronakis, V., Koronakis, E. and Hughes, C. (1989) Isolation and analysis of the C-terminal signal directing export of *Escherichia coli* hemolysin protein across both bacterial membranes. *European Molecular Biology Organization Journal*, **8(2)**, 595-605.

[8] Balsalobre, C., Silvan, J.M., Berglund, S., Mizunoe, Y., Uhlin, B.E. and Wai, S.N. (2006) Release of the type I secreted alpha-haemolysin via outer membrane vesicles from *Escherichia coli*. *Molecular Microbiology*, **59(1)**, 99-112.

[9] McBroom, A.J., Johnson, A.P., Vemulapalli, S. and Kuehn, M.J. (2006) Outer membrane vesicle production by *Escherichia coli* is independent of membrane instability. *The Journal of Bacteriology*, **188(15)**, 5385-5392.

[10] McBroom, A.J. and Kuehn, M.J. (2007) Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Molecular Microbiology*, **63(2)**, 545-558.

[11] Beveridge, T.J. (1999) Structures of Gram-negative cell walls and their derived membrane vesicles. *The Journal of Bacteriology*, **181(16)**, 4725-4733.

[12] Horstman, A.L. and Kuehn, M.J. (2000) Enterotoxigenic *E. coli* secretes active heat-labile enterotoxin via outer membrane vesicles. *Journal of Biological Chemistry*, **275(17)**, 12489-12496.

[13] Devoe, I.W. and Gilchrist, J.E. (1973) Release of endotoxin in the form of cell wall blebs during in vitro growth of *Neisseria meningitis*. *Journal of Experimental Medicine*, **138**, 1156-1167.

[14] Keenan, J., Day, T., Neal, S., Cook, B., Perez-Perez, G., Allardyce, R. and Bagshaw, P. (2000) A role for the bacterial outer membrane in the pathogenesis of *Helicobacter pylori* infection. *FEMS Microbiology Letters*, **182(2)**, 259-264.

[15] Fiocca, R., Necchi, V., Sommi, P., Ricci, V., Telford, J. Cover, T.L. and Solcia, E. (1999) Release of *Helicobacter pylori* vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium. *Journal of Pathology*, **188**, 220-226.

[16] Kato, S., Kowashi, Y. and Demuth, D.R. (2002) Outer membrane-like vesicles secreted by *Actinobacillus actinomycetemcomitans* are enriched in leukotoxin. *Microbial Pathogenes*, **32**, 1-13.

[17] Wai, S.N., Takade, A. and Amako, K. (1995) The release of outer membrane vesicles from the strains of enterotoxigenic *Escherichia coli*. *Microbiology and Immunology*, **39(7)**, 451-456.

[18] Wai, S.N., Lindmark, B., Soderblom, T., Takade, A., Weste-

rmark, M., Oscarsson, J., Jass, J., Richter-Dahlfors,V., Mizunoe, Y. and Uhlin, B.E. (2003) Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. *Cell*, **115(1)**, 25-35.

[19] Chi, B., Qi, M. and Kuramitsu, H.K. (2003) Role of dentilisin in Treponema denticola epithelial cell layer penetration. *Research in Microbiology*, **154(9)**, 637-643.

[20] Kuehn, M. and Kesty, N. (2005) Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes and Development*, **19(22)**, 2645-2655.

[21] Mashburn-Warren, L. and Whiteley, M. (2006) Special delivery: Vesicle trafficking in prokaryotes. *Molecular Microbiology*, **61(4)**, 839-846.

[22] McBroom, A. and Kuehn, M.J. (2005) Outer membrane vesicles. In: Curtiss, R. III, *et al*., Eds., *EcoSal-Escherichia coli and Salmonella: Cellular and Molecular Biology*, ASM Press, Washington, DC.

[23] Kolling, G.L. and Matthews, K.R. (1999) Export of virulence genes and shiga toxin by membrane vesicles of *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*, **65(5)**, 1843-1848.

[24] Moayeri, M. and Welch, R. (1997) Prelytic and lytic conformation of erythrocyte-associated *Escherichia coli* hemolysin. *Infection and Immunity*, **65(6)**, 2233-2239.

[25] Boehm, D., Welch, R. and Snyder, I. (1990) Calcium is requiered for binding of *Escherichia coli* hemolysin (HlyA) to erythrocyte membrane. *Infection and Immunity*, **58(6)**, 1951-1958.

[26] Markwell, M.A., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) A modification of the lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry*, **87(1)**, 206-210.

[27] Karkhanis, Y.D., Zeltner, J.Y., Jackson, J.J. and Carlo, D.J. (1978) A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolisaccharide of Gram-negative bacteria. *Annals of Clinical Biochemistry*, **85**, 595-601.

[28] Chen, P., Toribara, T. and Warner, H. (1956) Microdetermination of phosphorus. *Annals of Chemistry*, **28**, 1756-1758.

[29] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227(5259)**, 680-685.

[30] Fomsgaard, A., Freudenberg, M. and Galanos, C. (1990) Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. *Journal of Clinical Microbiology*, **28(12)**, 2627-2631.

[31] Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences*, **76(9)**, 4350-4354.

[32] Snyder, I.S. and Zwadyk, P. (1969) Some factors affecting production and assay of *Escherichia coli* hemolysin. *Journal of General Microbiology*, **5**, 133-143.

[33] Soloaga, A., Ramírez, J.M. and Goñi, F.M. (1998) Reversible denaturation, self-aggregation, and membrane activity of *Escherichia coli* alpha-hemolysin, a protein stable in 6 M urea. *Biochemistry*, **37(18)**, 6387-6393.

[34] Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986) Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochimica et Biophysica Acta*, **858**, 161-168.

[35] Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Use of resonance energy transfer to monitor membrane fusion. *Biochemistry*, **20**, 4093-4099.

[36] Herlax, *et al*, unpublished results.

[37] Rennie, R.P. and Arbuthnott, J.P. (1974) Partial characterization of *Escherichia coli* haemolysin. *Journal of Medical Microbiology*, **7(2)**, 179-188.

[38] Ostolaza, H., Bartolome, B., Ortiz de Zarate, I., de la Cruz, F. and Goñi, F.M. (1993) Release of lipid vesicle contents by the bacterial protein toxin alpha-haemolysin. *Biochimica et Biophysica Acta*, **1147**, 81-88.

[39] Demuth, D.R., James, D., Kowash, Y. and Kato, S. (2003) Interaction of *Actinobacillus actinomycetemcomitans* outer membrane vesicles with HL60 cells does not require leukotoxin. *Cellular Microbiology*, **5(2)**, 111-121.

[40] Lally, E.T., Golub, E.E. and Kieba, I.R. (1991) Structure and function of the B and D genes of the *Actinobacillus actinomycetemcomitans* leukotoxin complex. *Microbial Pathogenesis*, **11**, 111-121.

[41] Herlax, V., Maté, S., Rimoldi, O. and Bakás, L. (2009) Relevance of fatty acid covalently bound to Escherichia coli alpha-hemolysin and membrane microdomains in the oligomerization process. *Journal of Biological Chemistry*, **284**, 25199-25210.

[42] Cortajarena, H., Goñi, F. and Ostolaza, H. (2001) Glycophorin as a receptor for Escherichia coli alphahemolysin in erythrocytes. *Journal of Biological Chemistry*, **276(16)**, 12513-12519.

[43] Valeva, A., Walev, I., Kemmer, H., Weis, S., Siegel, I., Boukhallouk, F., Wassenaar, T., Chavakis, T. and Bhakdi, S. (2005) Binding of *Escherichia coli* hemolysin and Activation of the target cells is not receptor-dependent. *Journal of Biological Chemistry*, **280(44)**, 36657-36663.

[44] Herlax, V., Tacconi de Alani, M. and Bakás, L. (2005) Role of lipopolysaccharide on the structure and function of alpha-hemolysin from *Escherichia coli*. *Chemistry and Physics of Lipids*, **135(2)**, 107-115.

[45] Ostolaza, H., Bartolome, B., Serra, J.L., Cruz, F. and Goñi, F.M. (1991) Alpha-haemolysin from *E. coli*. Purification and self-aggregation properties. *Microbiology Letters*, **280(2)**, 195-198.

[46] Czuprynski, C.J. and Welch, R.A. (1995) Biological effects of RTX toxins: The possible role of lipopolysaccharide. *Trends in Microbiology*, **3(12)**, 480-483.

Vol.2, No.5, 493-498 (2010)
doi:10.4236/health.2010.25073

Long-term administration of traditional kampo medicine shimotsuto, juzentaihoto and unseiin inhibits experimental thrombosis in mice

Yoshinobu Ijiri[1,2], Hiroko Anzai[3], Weifua Gao[4], Kunio Takahashi[5], Naemi Kajiwara[3], Masahiro Murakami[2,4], Junichiro Yamamoto[2,6]*

[1]Department of Food Nutritional Science, Faculty of Liberal Arts, Osaka Shoin Women's University, Osaka, Japan
[2]Antithrombotic Diet Discussion Group, Laboratory of Physiology, Faculty of Nutrition, Kobe Gakuin University, Kobe, Japan
[3]Laboratory of Nutrition Physiology, Faculty of Health and Welfare, Kobe Women's University, Kobe, Japan
[4]Laboratory of Pharmaceutics, Faculty of Pharmacy, Osaka-ohtani University, Osaka, Japan
[5]Zaiseido Pharmaceutical Co Ltd, Wakayama, Japan
[6]Laboratory of Physiology, Faculty of Nutrition and Cooperative Research Center of Life Sciences, Kobe Gakuin University, Kobe, Japan; *Corresponding Author: yamamoto@nutr.kobegakuin.ac.jp

Received 13 January 2010; revised 27 January 2010; accepted 30 January 2010.

ABSTRACT

Traditional Chinese herbal medicines (Kampo) are used to improve flow characteristics of blood (Oketsu). We assumed that by preventing stagnation of blood, these medicines may be beneficial not only in venous but in arterial thrombotic conditions. The present study aimed to assess the antithrombotic effect of three Kampo, using well-established in vitro and animal models of thrombosis. Western-style highfat diet containing 1% Kampo (Shimotsuto, Juzentaihoto or Unseiin) was administered to C57BL/6 mice for 12 weeks. The effect on thrombus formation by laser irradiation of the carotid artery of mice was assessed. In addition the ex-vivo technique of shear-induced platelet reactivity measurement (haemostatometry) and the in vivo test of endothelial function (flowmediated vasodilation) were also used to assess the mechanism of antithrombotic effect. All three medicines have significantly inhibited arterial thrombus formation in mice. According to our studies, the mechanism of antithrombotic effect is based on the inhibition of shear-induced platelet reactivity and stimulation of endothelial function (Unseiin). It is assumed that the common ingredients Japanese Angelica Root, Cnidium Rhizome, Peony Root and Rehmannia Root could be responsible for the observed antithrombotic effect.

Keywords: Kampo; Chinese Medicine; Thrombosis; Platelet; Endothelial Function; Stroke; Cardiovascular Disease

1. INTRODUCTION

Prevention of lifestyle-related atherothrombotic diseases such as myocardial infarction and stroke is an important and urgent social task in many developed countries. Epidemiological studies have provided evidence for the causative role of inappropriate diet in the development of atherothrombotic diseases and the regular life style and exercise in the prevention of such diseases.

Chinese herbal medicine (Kampo) has a long history of treating various diseases. Currently used Kampo is believed to prevent stagnation thus improve the flow characteristics of blood. In our earlier studies, we used sensitive animal models of arterial thrombosis to assess the actual thrombotic status. We succeeded overcoming the main difficulty of the relative resistance of rodents to the prothrombotic effect of high fat diet. We showed that in apolipoprotein E and low-density lipoprotein receptor double deficient C57BL/6J mice, high fat diet induced a prothrombotic state, similar to humans [1]. By inducing and measuring the rate of arterial thrombus formation in response to laser irradiation in such spontaneously atherogenic mice, we could test the antithrombotic effect of various substances and diets. In the past, the use of this in vivo test together with other global in vitro thrombosis tests enabled us to find various fruits and vegetables with significant experimental antithrombotic activity [2,3].

2. MATERIALS AND METHODS

2.1. Chinese Herbal Medicines (Kampo) for Preventing Stagnation of Blood (Oketsu)

Shimotsuto, Juzentaihoto and Unseiin were used (**Table 1**). These were supplied by Zaiseido Pharmaceutical Co Ltd, Wakayama, Japan.

2.2. Diets Containing Kampo

Experimental diets containing the three Kampos in 1% (w/w) were prepared by adding Kampo to the Western-style high fat model diet (**Table 2**). Diets were stored at –30℃ until use.

2.3. Animals and Administration of Diet

Five week old male C57BL/6J mice were purchased from SLC Co Ltd (Hamamatsu, Japan) one week before the experiments and raised for one week on standard solid chow (CE-2, Clea Japan Inc., Tokyo, Japan) and drinking water ad libitum. Subsequently the chow was changed to the experimental diet, starting at age of 6 week, and lasted for 12 weeks. Mice were kept in the Animal Unit of Kobe Women's University, had free access to both diets and water, the room was air-conditioned (22.5 ± 2℃ and humidity 40-60%) having 12-h light and dark cycle. Animals were fasted overnight before the test and kept in compliance with the "Guiding Principles for the Care and Use of Animals in the field of Physiological Sciences," published by Physiological Society of Japan. The experiments were approved by the Animal Experiment Committee of Kobe Women's University.

Table 1. Composition of the three Kampo for blood stagnancy (Oketsu).

Ingredient	Content (g)		
	Shimotsuto	Juzentaihoto	Unseiin
Japanese Angelica	4.0	3.0	4.0
Cnidium Rhizome	4.0	3.0	4.0
Peony Root	4.0	3.0	4.0
Rehmannia Root	4.0	3.0	4.0
Atractylodes Rhizome	0	3.0	0
Poria Sclerotium	0	3.0	0
Cinnamon Bark	0	3.0	0
Ginseng	0	3.0	0
Glycyrrhiza	0	1.0	0
AStragalus Root	0	3.0	0
Coptis Rhizome	0	0	1.5
Scutellaria Root	0	0	3.0
Phellodendron Bark	0	0	1.5
Gardenia Fruit	0	0	2.0

2.4. He-Ne Laser-Induced Carotid Artery Thrombosis Test

This technique has been described in detail [1-4]. Briefly, the left femoral artery and the carotid artery (450-500 μM in diameter) of Nembutal anaesthetized mouse were exposed. The mouse was placed on a special microscope stage and through the femoral artery, a heat-absorbing dye Evans blue was injected. Subsequently the centre of the exposed carotid artery was irradiated with He-Ne laser. Thrombus formation at the site of irradiation was monitored under epi-illumination and recorded on videotape using CCD camera.

2.5. Calculation of Thrombus Size

From the start of laser irradiation, the computer-image of the forming thrombus was recorded in every 10 seconds for 10 minutes. The thrombus mass was delineated and its size was calculated by a software (Image Processing and Analysis Java version 1.30, National Institutes of Health, Bethesda, Maryland, USA). Due to frequent embolisation, the size of thrombus increased (building up the thrombus) or decreased (partial embolisation). Thrombotic status was defined by the total sum of thrombus sizes. An increase of such total sum indicated a prothrombotic state (enhanced thrombus formation), while decrease of such sum indicated an antithrombotic effect.

2.6. Endothelial Function Test

The endothelium-dependent flow-mediated vasodilation (FMV) technique originally described for rat was adapted to mice [5,6]. The anaesthetized mouse was kept on a heated pad to maintain body temperature; the left femoral artery was exposed, isolated and covered with gauze saturated with 37℃ saline. Blood flow in the artery was stopped by clamping for 3 minutes and then the flow was restored by releasing the clamp. Diameter of the artery was monitored 2-4 mm distal from the site of clamping by a CCD camera (Model CS900, Takenaka System Co. Ltd., Kyoto, Japan). Baseline images were taken before clamping and then in every 10 seconds over 60 seconds and further in 30 seconds intervals over 450 seconds after restoration of blood flow. Nitroglycerin-mediated vasodilation was induced by placing 70 microliters of 2.2 mM nitroglycerin/saline solution on the artery. The recorded images of the artery were transferred to a computer and the diameter changes were calculated with a software (Image Processing and Analysis; Java version 1.30). Changes in vessel diameter after restoration of flow were expressed as percentage of the baseline values (before clamping or nitroglycerin).

Table 2. Experimental diets containing Chinese herbal prescriptions (Kampo) for blood stagnancy (Oketsu).

Ingredient	Control diet (High fat diet)	Shimotsuto	Juzentaihoto	Unseiin
		g/kg		
Casein	232	232	232	232
Cystine	3	3	3	3
Corn starch	362	362	362	362
Sucrose	99.5	99.5	99.5	99.5
Soy oil	30	30	30	30
Butter	75	75	75	75
Beef tallow	100	100	100	100
Cellulose	50	50	50	50
Mineral mix	35	35	35	35
Vitamin mix	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
tert-Butylhydroquinon	0.041	0.041	0.041	0.041
Cholesterol	0.5	0.5	0.5	0.5
Shimotsuto	-	10	-	-
Juzentaihoto	-	-	10	-
Unseiin	-	-	-	10
Energy, KJ/100g	1956			

2.7. Platelet Function Test

The shear-induced platelet reactivity test (haemostatometry) was performed with a three-channel purpose-built Haemostatometer constructed in the Physiology Laboratory of the Faculty of Nutrition at Kobe Gakuin University. Details of haemostatometry have been described in detail elsewhere [7,8]. In brief, non-anticoagulated blood withdrawn from the abdominal aorta was perfused through polyethylene tubing by oil displacement technique and the perfusion pressure was continuously monitored. When the pressure stabilized at 60 mmHg, through-holes were pierced in the tubing by a fine needle. Escape of blood ("bleeding") through the pierced holes into the surrounding saline resulted in a sharp drop and then a gradual return of the perfusion pressure to the baseline level. The recovery of pressure after the initial drop reflected platelet-rich haemostatic plug formation in the pierced holes. The area of pressure changes (mmHg.s) were calculated (H1 and H2) and used as an index of platelet reactivity. An increase or decrease of H1 and H2 over controls indicated suppressed or enhanced platelet reactivity, respectively. The time from the start of the test until the first decrease of perfusion pressure of at least 10 mmHg (CT1) and to a level not higher than 10 mmHg (CT2) reflected the initial and completed coagulations, respectively. Prolongation of CT1 and CT2 indicated inhibition of dynamic coagulation, whereas shortening of CT1 and CT2 suggested hypercoagulation.

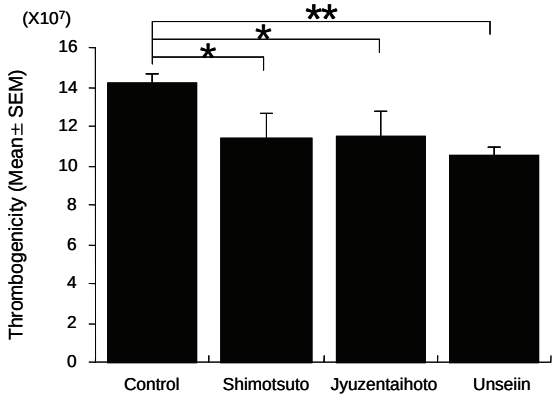
2.8. Statistical Analysis

The effect of Shimotsuto, Juzentaihoto and Unseiin in the applied tests groups was analysed by factorial ANOVA, followed by the post hoc test of Fisher's PLSD using commercially available statistical package Stat View (v. 5.0; SAS Institute Inc., North Carolina, USA). Logarithmic H1, H2 data and FMV data were analyzed by Student's unpaired t-test; CT1 and CT2 values were analyzed without logarithmic conversion. Results were expressed as mean ± SEM. $P < 0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSIONS

3.1. Antithrombotic Effect of Diets Containing Kampos

Results are shown in **Figure 1**. Long-term administration



N = 5-6, *P < 0.05, **P < 0.01.

Figure 1. Antithrombotic effect of diets containing various Kampo.

of all Kampo significantly decreased experimental thrombogenicity.

Shimotsuto contains four components common in three antithrombotic Kampos (**Table 2**): Japanese angelica, Cnidium rhizome, Peony root and Rehmannia root. In contrast, Juzentaihoto and Unseiin contain other additional components than the above common four. These components are added to adjust the efficacy of medicine to individual personality considering the physical and mental characters. The present results show that antithrombotic activity of the tested three Kampos derives from Japanese angelica, Cnidium rhizome, Peony root and Rehmannia root. As Unseiin showed the strongest antithrombotic activity, its effect was further investigated.

3.2. Effect of Unseiin on Endothelial Function

The effect of diet containing Unseiin on endothelial function was investigated after 12 weeks feeding by the flow-mediated or endothelium-mediated vasodilation (FMV) and nitroglycerin-mediated vasodilation (NMV) techniques. Results are shown in **Figure 2**. Endothelium-mediated vasodilation was significantly higher in mice with Unseiin feeding than in the controls but Unseiin did not affect nitroglycerin-induced vasodilation. Thus our finding suggests that Unseiin enhances endothelial function but not medial or muscular layer of the blood vessel.

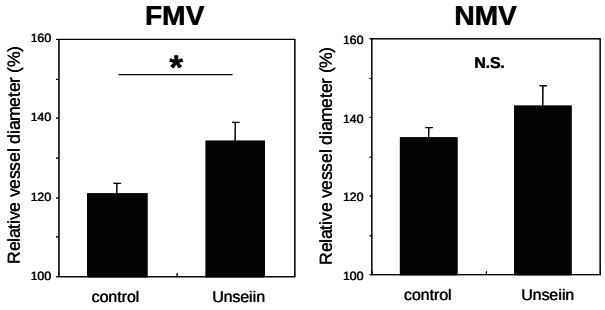
Keishi-bukuryo-gan (Gui-zhi-fu-ling-wan) has been used for the improvement of blood circulation and recently it is often used to prevent arteriosclerosis. One of the mechanisms involved is thought to be the improvement of endothelial dysfunction [9]. Choto-san has also been shown to improve blood circulation by protecting endothelium [10].

Flow-mediated vasodilation test was developed for diagnosing arteriosclerosis in humans [11-15] and we have employed this test to animal experiments [5,6]. Publications using FMV test are tremendously increasing in humans and demonstrating that nitric oxide and prostacyclin are involved in FMV [16]. Unseiin enhances endothelial function by nitric oxide and prostacyclin generation, and this could be a mechanism of the inhibition of arterial thrombosis.

3.3. Effect of Unseiin on Shear-Induced Platelet Reactivity

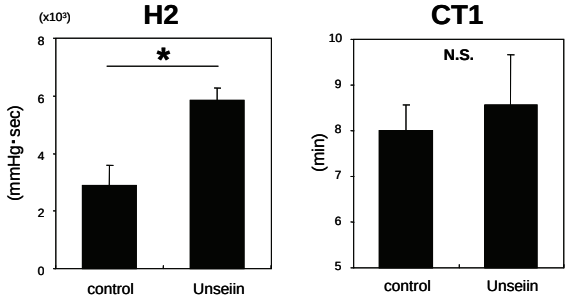
The diet containing Unseiin was given to mice for 12 weeks and the effect of Unseiin on platelet function was assessed by shear-induced platelet function test (haemostatometry) using non-anticoagulated blood. Results are shown in **Figure 3**. Unseiin inhibited shear-induced platelet reactivity, but no effect on blood coagulation [7,8].

Kangen-karyu (KGK) is a traditional Chinese herbal medicine to invigorate circulation. It contains 6 herbs, peony root, cnidium rhizome, safflower, cyperus rhizome, saussurea root (JP XIV), and Salvia miltiorrhiza root. KGK significantly extended tail-bleeding time and suppressed exvivo platelet aggregation in mice three days after oral administration, while it did not extend prothrombin time. These findings suggest that the antithrombotic effect of KGK may be due to the inhibition



FMV: n = 8, *P = 0.023, NMV: n = 7, N.S.: not significant (P = 0.199).

Figure 2. Effect of diets containing Unseiin on flow-mediated vasodilation (FMV) and nitroglycerin-mediated vasodilation (NMV).



H2: n = 4, *P = 0.013; CT1: n = 4, N.S.: not significant (P = 0.643).

Figure 3. Effect of diets containing Unseiin on shear-induced platelet reactivity and dynamic coagulation.

of platelet aggregation but not to anticoagulation [17]. The components, peony root and cnidium rhizome are common in KGK and in the currently investigated three Kampos. Peony root and cnidium rhizome might play a major role in antithrombotic activity of Kampo.

Agonist-induced platelet aggregation tests were used to assess of antithrombotic effect of Gosha-jinki-gan [18]. However, there are differences in the effects obtained by agonist-induced and shear-induced platelet function test [19]. We have compared agonist-induced platelet function test using anticoagulated whole blood and shear-induced platelet function test using non-anticoagulated whole blood, and concluded that shear-induced platelet function test using non-anticoagulated whole blood is physiologically more relevant to platelet function existing in vivo.

In conclusion, long-term intake of Shimotsuto, Juzentaihoto and Unseiin inhibited arterial thrombogenesis by their anti-platelet activities and by stimulating the vascular endothelium.

4. ACKNOWLEDGEMENTS

We thank Mrs Kinuyo Matsumoto, Ms Miho Hayashi and Ms Ikue Kusuda, Faculty of Health and Welfare, Kobe Women's University for their help of animal care.

REFERENCES

[1] Ijiri, Y., Miura, M., Hashimoto, M., Fukunaga, C., Watanabe, S., Kubota, A., Oiwa, K., Okuda, T. and Yamamoto, J. (2002) A new model to evaluate the diet-induced prothrombotic state, using He-Ne laser-induced thrombogenesis in the carotid artery of apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. *Blood Coagul & Fibrinolysis*, **13(6)**, 497-504.

[2] Yamamoto, J., Taka, T., Yamada, K., Ijiri, Y., Murakami, M., Hirata, Y., Naemura, A., Hashimoto, M., Yamashita, T., Oiwa, K., Seki, J., Suganuma, H., Inakuma, T. and Yoshida, T. (2003) Tomatoes have natural anti-thrombotic effects. *British Journal of Nutrition*, **90(6)**, 1031-1038.

[3] Yamamoto, J., Naemura, A., Ijiri, Y., Ogawa, K., Suzuki, T., Shimada, Y. and Giddings, J.C. (2008) The antithrombotic effects of carrot filtrates in rats and mice. *Blood Coagul & Fibrinolysis*, **19(8)**, 785-792.

[4] Kovacs, I.B., Tigyi-Sebes, A., Trombitas, K. and Gorog, P. (1975) Evans blue: An ideal energy-absorbing material to produce intravascular microinjury by He-Ne gas laser. *Microvascular Research*, **10**, 107-124.

[5] Taka, T., Yoshio, Y., Seki, J., Giddings, J.C. and Yamamoto, J. (2002) Impaired flow-mediated vasodilation in vivo and reduced shear-induced platelet reactivity in vitro in response to nitric oxide in prothrombotic, stroke-prone spontaneously hypertensive rats. *Pathophysiology of Haemostasis & Thrombosis*, **32**, 184-189.

[6] Tamura, Y., Naemura, A., Inoue, A., Ijiri, Y., Seki, J., Yada, T., Goto, M., Shinohara, M., Kawashima, S., Giddings, J.C. and Yamamoto, J. (2009) Impaired endothelial function may be due to decreased aortic tetrahydrobiopterin, assessed by a new flow-mediated vasodilation in vivo in hypercholesterolemic/atherogenic mice. *Blood Coagul & Fibrinolysis*, **20(8)**, 699-705.

[7] Ratnatunga, C.P., Edmondson, S.F., Rees, G.M. and Kovacs, I.B. (1992) High-dose aspirin inhibits shear-induced platelet reaction involving thrombin generation. *Circulation*, **85(3)**, 1077-1082.

[8] Yamamoto, J., Taka, T., Nakajima, S., Ueda, M., Sugimoto, E., Sasaki, Y., Muraki, T., Seki, J. and Watanabe, S. (1999) A shear-induced in vitro platelet function test can assess clinically relevant anti-thrombotic effects. *Platelets*, **10(2)**, 178-184.

[9] Goto, H., Shimada, Y., Sekiya, N., Yang, Q., Kogure, T., Mantani, N., Hikiami, H., Shibahara, N. and Terasawa, K. (2004) Effects of keishi-bukuryo-gan on vascular function and hemorheological factors in spontaneously diabetic (WBN/kob) rats. *Phytomedicine*, **11(2-3)**, 188-195.

[10] Yang, Q., Goto, H., Shimada, Y., Kita, T., Shibahara, N. and Terasawa, K. (2002) Effects of choto-san on hemorheological factors and vascular function in stroke-prone spontaneously hypertensive rats. *Phytomedicine*, **9**, 93-98.

[11] Moens, A.A., Goovaerts, I., Claeys, M.J. and Vrints, C.J. (2005) Flow-mediated vasodilation: A diagnostic instrument, or an experimental tool? *Chest*, **127(6)**, 2254-2263.

[12] Soga, J., Nishioka, K., Nakamura, S., Umemura, T., Jitsuiki, D., Hidaka, T., Teragawa, H., Takemoto, H., Goto, C., Yoshizumi, M., Chayama, K. and Higashi, Y. (2007) Measurement of flow-mediated vasodilation of the brachial artery: A comparison of measurements in the seated and supine positions. *Circulation Journal*, **71(5)**, 736-740.

[13] Matsuo, S., Matsumoto, T., Takashima, H., Ohira, N., Yamane, T., Yasuda, Y., Tarutani, Y. and Horie, M. (2004) The relationship between flow-mediated brachial artery vasodilation and coronary vasomotor responses to bradykinin: Comparison with those to acetylcholine. *Journal of Cardiovascular Pharmacology*, **44(2)**, 164-170.

[14] Tarutani, Y., Matsumoto, T., Takashima, H., Yamane, T. and Horie, M. (2005) Brachial artery flow-mediated vasodilation is correlated with coronary vasomotor and fibrinolytic responses induced by bradykinin. *Hypertension Research*, **28(1)**, 59-66.

[15] Anderson, T.J., Uehata, A., Gerhard, M.D., Meredith, I.T., Knab, S., Delagrange, D., Lieberman, E.H., Ganz, P., Creager, M.A., Yeung, A.C. and Selwyn, A.P. (1995) Close relation of endothelial function in the human coronary and peripheral circulations. *Journal of the American College of Cardiology*, **26(5)**, 1235-1241.

[16] Engelke, K.A., Halliwill, J.R., Proctor, D.N., Dietz, N.M. and Joyner, M.J. (1996) Contribution of nitric oxide and prostaglandins to reactive hyperemia in human forearm. *Journal of Applied Physiology*, **81(4)**, 1807-1814.

[17] Makino, T., Wakushima, H., Okamoto, T., Okukubo, Y., Saito, K. and Kano, Y. (2002) Effects of Kangen-karyu on coagulation system and platelet aggregation in mice. *Biological & Pharmaceutical Bulletin*, **25(4)**, 523-525.

[18] Suzuki, Y., Goto, K., Ishige, A., Komatsu, Y. and Kamei, J. (1998) Effect of Gosha-jinki-gan, a Kampo medicine, on enhanced platelet aggregation in streptozotocin-induced

diabetic rats. *Japanese Journal of Pharmacology*, **78(1)**, 87- 91.

[19] Nakajima, S., Noguchi, T., Taka, T., Ueda, T., Kaizu, K., Fukamizu, M., Fujita, S., Tabuchi, M. and Yamamoto, J. (2000) A global platelet test of thrombosis and thrombolysis detects a prothrombotic state in some patients with non-insulin dependent diabetes and in some patients with stroke. *Platelets*, **11**, 459-466.

Vol.2, No.5, 499-503 (2010)
doi:10.4236/health.2010.25074

Efficacy of Miswak (salvadora persica) in preventing dental caries

Fatemeh Ezoddini-Ardakani

Department of Oral and maxillofacial radiology, School of Dentistry, Shahid Sadoughi University of Medical Sciences, Yazd, Iran; ezoddini@gmail.com, afsan40@yahoo.co.uk

Received 1 November 2009; revised 5 December 2009; accepted 7 December 2009.

ABSTRACT

The rate of dental caries and periodontal diseases in the world is still high, regardless of progress in the oral and dental hygiene. The natural toothbrush or chewing stick called "Miswak" has been used since ancient history. In this research the efficacy of Miswak in preventing dental caries was investigated and compared with the efficacy of toothbrush and toothpaste. The analytical and clinical trial method was applied for this research among high school's students in the city of Yazd, Iran, 2006. Three hundred eighty second year's students (190 cases and 190 controls) were examined dentally. Then the Miswak was distributed to the case group and required trainings were given to both groups. After one year, the examinations were repeated. For analyzing the data one-way variance analysis test, Kai square, Paired t-test and two variable analyses were used. In the beginning of this study, there were no significant differences between two groups (cases and controls) regarding their dental situation and the frequency of brushing their teeth (p-value = 0.162). In addition, there were no significant differences in DMFT between the two groups. The data collected at the end of the study showed a significant increase in DMFT in the control group (p-value = 0.000). There was 55% increase in the rate of dental caries in control group compared to case group (0.89 before the study and 1.38 after the study). The risk of dental caries for each tooth in control group was 9.35 times more than case group (9.14% and 0.98% respectively). Dental caries rate was detected slightly less in the case group at the end of this study. This might be as a result of the antimicrobial effects of Miswak. A longer study with more cases is needed to prove this suggestion.

Keywords: Miswak; Dental Caries; Salvadora Persica; Toothbrush

1. INTRODUCTION

Salvadora Persica (S.P.) is a plant that grows in the deserts of the area from west India to Africa. The roots and sticks of S.P. are used widely for cleaning the teeth in these areas. The other names for this plant are Arak tree, Chewing stick, Natural toothbrush and Miswak [1].

Regardless of daily improving dental and oral health in the world the dental caries and gingivitis is increasing due to the widely use of sugar in food, fluoride and calcium deficiency and finally ignoring health care [2]. There are nearly seven plants for this meaning but the most commonly used one is Miswak which is derived from S.P. plant mostly in Saudi Arabia and parts of the Middle East [3]. The values of these sticks are due to their components and cleaning mechanisms. Recently these sticks were recommended as an effective tool for oral health by the World Health Organization (WHO).

The sticks have usually 15 cm length and 1 cm diameter (**Figure 1**). This is why these sticks are called chewing sticks as well. Their pleasant hot taste made them easily chewable. Sometimes a small part of the stick is used as a tooth stick.

Primary analysis showed that S. Persica contains Tri-Methyamin, Salvadrin, Chloride, Fluoride, Silica, Sulfur, Mustard, Vitamin C and a small amount of Saponine Tanin. These components have antibacterial and antifever effects in addition to be against gingival irritation [4]. There are many studies showing that Miswak have strong anti caries effects due to large amounts of fluoride in it. Many dental studies reported antimicrobial activity in derivates from Miswak. In addition, the mechanical cleaning effects of Miswak were investigated and it was reported that the value of chewing sticks is due to their mechanical cleaning [5]. There are many reports showing Miswak is effective in decreasing gingivitis and dental plaques. Elvin-Lewis *et al.* [6] showed that the dental loss in adults is very low in the countries

Figure 1. The shape of some chewing sticks.

that Miswak is used widely. Almas, [7] showed amazing antibacterial effects of Miswak on Streptococcus Mutant and Fecalis. Olsson [8] reported that chewing sticks are more effective in decreasing dental caries than toothbrush. Al-lafi and Ababneh [9] investigated antibacterial effects of Miswak in three ways and showed that derivatives if these sticks have strong effects on the growth of Streptococcus and staphylococcus aurous. Danielsen, *et al.* [10] studied two groups of students in Kenya. One group used chewing sticks plus toothpaste and the other group used only chewing sticks. There were no extra effects in removing dental plaques in the group that used toothpaste in addition to chewing sticks.

The aims of this study were to investigate the effects of Miswak in preventing tooth caries comparing with toothbrush.

2. MATERIALS AND METHODS

This study was a clinical trial with before and after design. It was done in 190 cases and 190 controls (95 girls and 95 boys) in the second year students of Yazd high schools. Cluster sampling was used to choose 6 boys and 6 girls high schools in Yazd. Then these high schools were randomly divided into two groups of three high schools each using as case and control groups.

The investigators were chosen among final year dental students. Required training regarding filling questionnaires and dental examination were given to them. The author supervised all the investigation's steps. The materials used by the investigators were explorer, mirror, light, gloves, upper and lower teeth models, Oral-B toothbrush and Miswak. Dentists examined all of the students teeth at the beginning of the study and the number and degree of DMF teeth were recorded in the questionnaires. The degrees of caries were measured by explorer and persons complaints. Even small insertion of the explorer in the teeth recorded as a caries. The degrees of the caries were divided to class I to class VI. After recording the primary data Miswak were given to the case group and the way to use it were trained to the group and ask them to use it two to three times per day. The Miswaks were controlled every three months and the new Miswaks were given to them if it was necessary. While, the toothbrushes were given to control group, and the training was given to them to brush their teeth two to three times per day exactly as it done for the case group.

After one year, the teeth examinations were done the same as the beginning of the study and the data were recorded in the questionnaire. Our data showed the caries rate in the beginning and at the end of the study and because DMFT have no improvement so the differences were considered to be as the results of disease development in the study year.

The data were imported in the EPI 6 software and were analyzed by SPSS 13 version software. For comparing the data one way variance analysis test, Chi-Square, Paired t-test and two variable analyses were used. For comparing the relative risk of disease development in two groups the Relative risk index was used.

3. RESULTS

In this research, from the initial 380 students that started this study, 330 students continued it until the end. In case group there were 174 students which used Miswak for one year and 156 students were in the control group which used toothbrush for one year. The trainings and number of brushing teeth per day were the same for two groups. At the beginning of the study the numbers of case and control students were the same (190). This means that 16 students in the case group (8.4%) but 34 students in the control group (17.9%) did not continue the experiments until the end. The difference is significant (p-value = 0.006). This means that the cases which used Miswak have more interest in brushing their teeth than the controls. The odds ratio to discontinue brushing was 2.37 times in control group than this ratio in case group with the confidence interval of 1.21 to 3.72 which is different from number one significantly (p-value < 0.05).

The same numbers of male and female were chosen at the beginning of this study, but after one year 159 male and 171 female continue the study. This means that the number of cases which abandon the study was more in the male group but this difference is not significant (p-value = 0.068) and the sex ratio in two groups is nearly the same. In addition, the age of all the cases and controls were nearly the same with maximum 6 months difference from the median.

The number of brushing the teeth in two group were the same at the beginning of the study (p-value = 0.162) (**Table 1**). There were no significant differences regarding DMFT rate between two groups of cases and controls at the beginning of the study (**Table 2**).

In this study DMFT was used as a dependent variable to investigate the effectiveness of Miswak. The mean DMFT in two groups at the beginning of the study was 3.691 ± 2.797 (mean ± S.D.) with the confidence interval of 95% from 3.39 to 3.99. These data shows the efficacy of Miswak in preventing tooth decay (**Table 3**).

Table 4 shows that the rate of increased DMF in the case group was nearly 10 times this rate in the control group.

Our data showed that at the beginning of the study the incidence of DMF in the case group was more than this in the control group, but after one year (at the end of the study) this incidence in the case group became less than this in the control group (**Table 5**). The prevalence ratio of decayed teeth in control group compare with case group was 0.89 before the study, but this was 1.38 after the study. These data shows a 55% increase in the rate of decay in control group compare with the case group. In addition, the relative risk of decay incidence in the control group was 9.35 times this in the case group.

4. DISCUSSIONS

The importance of chewing sticks or Miswak was investigated in several studies (11, 12, 13 and 14). Almas, [7] showed that Miswak has antimicrobial effects against Streptococcus Mutants and Fecalis. In addition, Al-Lafi and Ababnch [9] and Almas, *et al.* [15] studies showed that Streptococcus Fecalis is the most sensitive microorganism affected by Miswak. Our results showed that the rate of carries decreases after using Miswak and this can be due to these antimicrobial effects. Almas and Al-Zeid [16] in a study investigated the antimicrobial effects of Miswak and it's extract specially on Streptococus Mutan and Lacto Basilus. These effects were compares with the effects of toothbrush and normal saline. Their results showed that in Miswak users there was a significant decreases in streptococcus (p = 0.013) but not in Lacto Basilus (p = 0.147).

Table 1. The number of brushing per day in the two groups at the beginning of the study.

Group / Brushing/day	Cases		Controls		Total	
	No	%	No	%	No	%
3 times	2	1.1	2	1.3	4	1.2
2 times	12	6.9	18	11.5	30	9.1
1 time	84	48.3	71	45.5	155	47
Rarely	62	35.6	43	27.6	105	31.8
Never	14	8	22	14.1	36	10.9
Total	174	100	156	100	330	100

P-value = 0.162.

Table 2. The teeth situation in the students at the beginning of the study.

Group / No	Cases (No = 174)		Controls (No = 156)		p-value
	Mean	S.D.	Mean	S.D.	
Filled teeth					
One side	0.79	1.81	0.54	1.47	0.176
Two sides	0.1	0.52	0.08	0.32	0.767
Three sides	0.06	0.375	0	0	0.036
Decay teeth					
Class I	2.36	2.18	2.41	2.14	0.366
Class II	0.16	0.47	0.16	0.461	0.99
Class III	0.02	0.13	0.05	0.3	0.17
Class IV	0	0	0.02	0.18	0.156
Class V	0.01	0.08	0.02	0.18	0.364
Class VI	0.01	0.11	0	0	0.18
Missing teeth	0.13	0.44	0.17	0.41	0.39

Table 3. Comparison of the differences between DMF in two groups before and after the study.

Group / Time	Case No = 174				Control No = 156				
	X	S.D.	Min	Max	X	S.D.	Min	Max	p-value
Before	3.9	2.89	0	14	3.46	2.68	0	13	0.147
After	4.14	3.05	0	17	5.7	3	0	16	0.000
p-value	0.000				0.000				

Table 4. The difference between DMF in each group.

Group	No	Mean of increase during 1 year	S.D.	p-value
Case	174	0.2356	0.523	0.000
Control	156	2.2436	1.188	0.000

Table 5. Prevalence and incidence of decay in two groups.

Prevalence	Teeth No in Case group			Teeth No in Control group			p-value
	Total	Decayed	%	Total	Decayed	%	
Before	4872	679	13.94	4368	539	12.34	0.059
After	4872	720	14.78	4368	889	20.35	0.00
One year incidence	4193	41	0.98	3829	350	9.14	0.00

Wolinsky *et al.* [17] showed that S.P. decreases the ability of some Streptococcus to colonize on teeth surfaces. Bearing in mind that the cause of tooth decay is acid secretion by microorganisms, the decreases in the rate of caries in this study and also low rate of DMF in the countries which use Miswak can be due to this ability of S.P.

Elvin-lewis *et al.* [6] and Almas [7] suggested that the antibiotic effects detected in S.P. may have interaction with bacteria and prevent their attachment. The other component of Miswak with possible interaction with bacterial glycolytic enzyme and their acids or intracellular polysaccharides products is Fluoride. Furthermore, Benzylisothiocyanate (BIT) that is naturally a component of S.P. acts as inhibitor of bacterial growth and their acidic products [18]. Chawla [19] showed that some kinds of chewing sticks such as Neem, S.P. and Acaccia Arabica have reasonable amounts of fluoride. They showed that Miswak sticks from S.P. tree have significant antimicrobial effects on streptococcus mutants, streptococcus mitis and staphylococcus. Darout *et al.* [20] studied the caries and periodontal situation of adults Sudanese using either habitual Miswak or Toothbrush. They suggested using Miswak in developing world because of its availability and cheap price. In our study as well the effects of Miswak were significant and every three months that we were going to the schools to deliver new Miswak sticks to the students their excitement in requesting Miswak was interesting.

At the end of our study, the Miswak has been used more frequently in the Miswak users than toothbrush in the other group. This difference cannot be due to the training, because both groups have the same trainings. In addition our study showed that the rate of DMF which was the same in both groups increased significantly in the toothbrush users compared with Miswak users. This difference can be because of two reasons; 1/Miswak users used Miswak more frequently and 2/Miswak has antimicrobial effects plus fluoride and calcium.

Faiez [21] reviewed the effects of Miswak. Many researchers believed that the efficacy of S.P. is because of its mechanical action while the others believe that Miswak is a natural source for topical fluoride.

About half of the people in Saudi Arabia use Miswak. Magbool [22] showed that only 12.5% of students are Caries free in Saudi Arabia and the rate of tooth decay is 48% in 6 and 7 years old age group but this rate decreases and is only 1.03% in 16 and 17 years old age group. We showed that the prevalence of caries in case group is less than this rate in control group and this can be as a result of using Miswak.

Norton and Addy [23] did a pilot and cross sectional study among adults in Ghana. They showed that the rate of plaque and carries in Miswak users was less than this rate among non users.

In the other studies among Ethiopian and Nigerian students and Saudi Arabian dental students, using Miswak and toothbrush was compared. These comparisons showed that Miswak was more effective than toothbrush in removing plaque [6,23,24]. These differences can be related to the frequency and duration of brushing, experiences in using Miswak, motivation and supervision.

One of the specifications of Miswak is its straight shape that is a disadvantage because it cannot clean linguinal sides of the teeth.

Miswak contains nearly 1.02 μg/g total fluoride. Chawla showed that the chewing sticks of S.P., Neemkikar, Walnut and Pekujebu have 2.8, 1.0, 0.5 and 0.2 μg/ml fluorides respectively [19]. Miswak releases a significant amount of calcium and phosphor in water and these elements are necessary for remineralization. Crystallographic studies with fluorescence and x-ray microanalysis showed that S.P. sticks have more calcium and phosphor [24].

Recently S.P. extract was used in some toothpastes like Ouali Meswak, Pharba, Sarakan, Backenham UK, Basaraj and Epident [25]. Miswak extract and 0.8 % monoflourophosphate sodium) compared with Oral-B toothpaste (containing only 0.8 % monoflourophosphate sodium) in dental students. Their results showed that the toothpaste containing Miswak extract significantly was more effective in removing dental plaques compare with Oral-B toothpaste. It was also showed that Miswak has antidecay effects because of its fluoride contents. In addition, the hot taste of Miswak plus the chewing effects of the stick can increase the salvia secretion in the mouth and therefore increase its buffering capacity [21]. In our study these factors can be effective as well.

Sofrata reported that the chewing stick (Miswak) is used for oral hygiene in many parts of the world. In addition to the mechanical removal of plaque, an antibacterial effect has been postulated. Miswak embedded in agar or suspended above the agar plate had strong antibacterial effects against all bacteria tested. The antibacterial effect of suspended Miswak pieces suggests the presence of volatile active antibacterial compounds [26].

Almas concluded that toothbrushes and Miswak (chewing sticks) are widely used for the mechanical removal of plaque [27].

Sofrata reported that the difference in plaque pH between Miswak extract and water rinse was statistically significant at 30 min ($p < 0.001$) [28]. Rinsing with Miswak extract stimulated parotid gland secretion ($p < 0.01$). Miswak extract raised the plaque pH, suggesting a potential role in caries prevention

5. CONCLUSIONS

Our study showed that Miswak effectively prevented dental caries in high school students. Conducting some studies on antimicrobial and silicate effects of Miswak are suggested.

REFERENCES

[1] Galati, E.M., Monforte, M.T., Forestieri, A.M., Miceli, N., Bade, A. and Trovato, A. (1999) Salvadora persica L: Hypolipidemic activity on experimental hypercholesterolemia in rat. *Phytomedicine*, **6(3)**, 181-185.

[2] Kimery, M.J. and Stallard, R.E. (1992) The evolutionary development and contemporary utilization of various oral hygiene procedures. *Periodontal Abstracts*, **16**, 90-97.

[3] Abderahim, M. and Jurner, J.E. (1983) In vitro evaluation of Saudi Arabian tooth tree (Salvadoran Persica). *Odontostomatological Tropicale*, **613**, 145-148.

[4] Wolinsky, L.E. and Sote, E.O. (1984) Isolation of natural plaque inhibiting substances from Nigerian chewing sticks. *Caries Research*, **18(3)**, 216-225.

[5] Akpata, E.S. and Akinrimisi, E. (1977) Antibacterial activity of extracts from some African Chewing sticks. *Oral Surgery*, **44(5)**, 717-722.

[6] Elvin-Lewis, M., Hall, J.B. and Adu-uta, M. (1980) The dental health of chewing stick users of southern Ghana, preliminary finding. *Journal of Preventive Dentistry*, **6**, 151-154.

[7] Almas, K. (1999) The antimicrobial effects of extracts of Azadirachta Indica (NEEM) and Salvadora Persica (Arak) chewing sticks. *Indian Journal of Dental Research*, **10(1)**, 23-26.

[8] Olsson, B. (1978) Efficiency of traditional chewing sticks in oral hygiene programs among Ethiopian Schoolchildren. *Community Dentistry and Oral Epidemiology*, **6**, 105-109.

[9] Al-lafi, T. and Ababneh, H. (1995) The effect of the extract of the Miswak (chewing sticks) used injordan and the middle east on oral bacteria. *International Dental Journal*, **45(3)**, 218-222.

[10] Danielsen, B.O., Vibeke, B., Manji, F. and Fejerskov, O. (1989) Chewing sticks, tooth paste and plaque removal. *Acta Odontol Scand*, **47**, 121-125.

[11] Akhtar, M. and Ajmal, M. (1981) Significance of chewing sticks (Miswak) in oral hygiene from a pharmacological viewpoint. *Journal of Pakistan Medical Association*, **31(4)**, 89-95.

[12] Farooqi, M.I.H. and Srivastava, J. (1983) The tooth tree (Salvadora persica). *Quarterly Journal of Crude Drug Research*, **81(4)**, 1297-1299.

[13] El-Mostehy, M.R., Al-Jassem, A.A., Al-Yassin, I.A., *et al*. (1983) Miswak as an oral health device preliminary chemical and clinical evaluation. *Hamdard*, **26**, 41-50.

[14] Khoory, T. (1999) The use of chewing sticks in preventive oral hygiene. *Clinic Preventive Dentistry*, **5**, 11-14.

[15] Almas, K., Al-Bagieh, N. and Akpata, E.S. (1997) In vitro antimicrobial effects of freshly cut and 1-month old miswac (chewing stick). *Biomedical Letters*, **56**, 145-149.

[16] Almas, K. and Al-Zeid, Z. (2004) The immediate antimicrobial effect of a toothbrush and Miswak on cariogenic bacteria: A clinical study. *Journal of Contemporary Dental Practice*, **5(1)**, 105-114.

[17] Wolinsky, L.E., Mania, S., Nachnani, S., *et al*. (1996) The inhibiting effect of aqueous Azadirechta indica (Neem) extract upon bacterial properties influencing in vitro plaque formation. *Journal of Dental Research*, **75(2)**, 816-822.

[18] Al-Bagieh, N.H. and Weinberg, E. (1989) Benzylisothicyanate: A possible agent for controlling dental caries. *Microbios*, **39**, 143-151.

[19] Chawla, H.S. (1983) A new natural source for topical fluoride. *Journal of the Indian Dental Association*, **55 (10)**, 419-422.

[20] Darout, I.A., Albandar, J.A. and Nils, S. (2000) Periodontal status of adult Sudanese habitual users of Miswac chewing sticks or toothbrushes. *Acta Odontol Scand*. **58**, 31-37.

[21] Faiez, N.H. (1995) Miswac: The natural toothbrush. *The Journal of clinical Dentistry*, **8(5)**, 125-129.

[22] Magbool, G. (1992) Prevalence of dental caries in school children in Al-Khobar Saudi Arabia. *Journal of Dentistry for Children*, **59(5)**, 384-386.

[23] Norton, M.R. and Addy, M. (1989) Chewing sticks versus toothbrushes in West Africa. A pilot study. *Clinical Preventive Dentistry*, **11(3)**, 11-13.

[24] Char, D.C.N., Dogao, A.U. and Dogan, M.M. (1987) SEM, XRF. and EMPA evaluation of Middle Eastern toothbrush "Salvadora persica". *Journal of Electron Microscopy Technology*, **5**, 145.

[25] Guile, E.E., Al-Shammery, A.R. and El-Backly, M.N. (1996) Oral health survey of Saudi Arabia: Oral health knowledge attitudes and practice among adults. *Journal of Dental Research*, **75**, 1276.

[26] Sofrata, A.H., Claesson, R.L., Lingström, P.K. and Gustafsson, A.K. (2008) Strong antibacterial effect of Miswak against oral microorganisms associated with periodontitis and caries. *Journal of Periodontology*, **79(8)**, 1474-1479.

[27] Almas, K. (2002) The effect of Salvadora persica extract (Miswak) and chlorhexidine gluconate on human dentin: A SEM study. *The Journal of Contemporary Dental Practice*, **3(3)**, 27-35.

[28] Sofrata, A., Lingström, P., Baljoon, M. and Gustafsson, A. (2007) The effect of Miswak extract on plaque pH. An in vivo study. *Caries Research*, **41(6)**, 451-454.

Vol.2, No.5, 504-510 (2010)
doi:10.4236/health.2010.25075

Estimating the effect of early discharge policy on readmission rate. An instrumental variable approach

Eugenia Amporfu

Kwame Nkrumah University of Science and Technology, Kumasi, Ghana; eamporfu@gmail.com

Received 23 January 2010; revised 7 February 2010; accepted 8 February 2010.

ABSTRACT*

Early discharge policy, common in the developed countries, refers to the reduction of hospital length of stay as a way of reducing the cost of care. The effect of the policy on quality of care has received a lot of attention in the literature. Some of the earlier papers have ignored the endogeneity of length of stay in the readmission equation, an approach that could lead to inconsistent estimation. This study develops a statistical technique for the consistent estimation of the effect of the early discharge policy. An instrument that can be used extensively across different diagnostic groups is provided, hence solving the difficult problem of finding an instrument for length of stay. The exogeneity test in Gorgger (1990), the test for weak instruments in Staiger and Stock (1997) as well as the Hensen (1982) for over identification confirmed respectively that length of stay is endogenous the instrument is strong and the valid.

Keywords: Instrument; Length of Stay; Early Discharge; Endogeneity; Instrumental Variable Estimation

1. INTRODUCTION

Instrumental variable estimation is a two stage estimation technique that first purges endogenous regressors, and hence exogenizes them before including them as regressors in a regression equation for estimation. An important reason why instrumental variable estimation is becoming less popular is the difficulty of finding an instrument. This difficulty is encountered in models that measure the impact of early discharge policy on quality of care. Many studies have thus ignored the need for instrumental variable estimation and hence the consistency of their estimated impact of the policy is questionable. The objective of this paper is to develop a statistical technique for consistent estimation of the impact of early discharge on quality of care regardless of the diagnostic group used. The British Columbia (Canada) Ministry of Health and Center for Health Services and Policy Research linked database provided the maternity data used for the study.

The models that measured the impact of the early discharge policy on quality of care used readmission and mortality rates as proxies for quality of care with length of stay as one of the independent variables. Length of stay, because it is correlated with an unobservable variable, the severity of illness, is endogenous in the regression equation. Severity of illness is unobservable to the researcher but observable to the patient (or doctor) and a severely ill patient is likely to stay long in the hospital and be readmitted. Ignoring the endogeneity of length of stay then can lead to inconsistent estimation and hence unreliable conclusions. Examples of papers that ignored the endogeneity of length of stay in the readmission equation can be cited [1].

Consistent estimation of the impact of early discharge policy on quality of care is important because the policy is implemented extensively across North America and Europe (e.g., Sweden, Norway in the 1990s), as a cost containment strategy. In the United States the policy was induced by the introduction of prospective payment [2] and capitation [3] in the 1980s. In Canada, the policy was introduced by provincial governments in the 1990s (example, 1994 in British Columbia and Alberta) and was often accompanied by home visits implemented through public health nursing programs. In general, the early discharge policy has raised concern about its impact on quality of care and has attracted a lot of studies in the area [2,4].

Information on the impact of the policy on quality of care will serve as an important guide to policy makers on the extent to which the policy is able to reduce the cost of care. Inconsistent estimation of the impact then can lead to misleading results and hence mislead policy makers on the efficiency of the allocation of health care

*Data used for this study was provided by British Columbia (Canada) Ministry of Health and Center for Health Services and Policy Research linked database.

resources. The endogeneity problem can be solved if severity of illness can be accurately measured and included in the regression equation. This requires clinical information from medical records [5]. Such an approach is however not used in the literature because it is extremely costly to gather the information [6].

Some studies such as [7] used the cost weight of patients diagnostic group, hospital entry type (*i.e.*, whether it was emergency or elective) and whether the diagnostic group was a surgical or non surgical type as proxies for severity of illness. These variables are reasonable proxies for the study's data: all elderly patients across different diagnostic groups. Such variables however cannot pass as proxies for all diagnostic groups especially maternity data. This is because for maternity, an emergency entry usually implies advancement in labor but does not necessarily imply severity of illness. Caesarean section could occur by choice, or as a result of the woman's pelvic or birth canal or for medical reasons. Thus a woman who undergoes caesarean section is not necessarily ill. Hence surgical delivery cannot be an indicator of severity of illness for a maternity patient and the difficulty of finding a proxy for the severity of illness remains. Finding an appropriate instrument for length of stay then would lead to a consistent estimation of the policy's impact on quality of care and nullify the need to find a proxy for severity of illness.

The first to alert of the endogeneity problem with length of stay and the need to use instrumental variables to ensure consistent estimation did not come till 2000 [8]. However, [8] used time of delivery and the method of delivery as instruments for length of stay. While these instruments might have produced consistent estimates, the instruments chosen are only restricted to the data set used, newborns, and so cannot be applied to other diagnostic groups. It is not surprising that more recent papers [1] that did not use infant data ignored the endogeneity problem. What is needed then is an instrument that is highly correlated with length of stay but uncorrelated with severity of illness regardless of the diagnostic group used for the analysis of effect of the early discharge policy. Such an instrument is very important because the early discharge policy is widely implemented for different diagnostic groups.

One such variable in this context is a policy instrument for the early discharge policy: the average number of patients per bed in a given year for a given hospital. To implement the early discharge policy hospitals may have to increase the number of patients per bed that is allocated to the diagnostic group targeted by the policy. This could be done by either increasing the size of the population served by the hospital through the closure of other hospitals or by reducing the number of beds in the hospital or both. Because some hospitals are likely to attract the severely ill patients the average number of patients per bed in the hospital of delivery could be correlated with the severity of illness. Thus for each observation (patient) I computed the average number patients per bed whether or not it was the choice hospital. The number of patients per bed is a better instrument than the year of policy used in an earlier work [9] because year of policy is only valid as an instrument if no other policy that could affect readmission rate was implemented in the years of and after the policy. Weak instrument test was used to test for the strength of the instrument [10].

The paper is organized as follows. Section 2 describes the model, its estimation and various tests performed. Section 3 describes the data while Section 4 reports the results and Section 5 discusses the results.

2. METHODS

2.1. The Model

The model for the analysis has two regression equations, an OLS regression **Eq.1**, and a discrete time duration hazard **Eq.2**:

$$LOS_i = \theta + \delta\Im + \phi Z + u_i \quad (1)$$

$$y_{it} = \alpha + \varphi L\hat{O}S + \eta Z + \vartheta_{it} \quad (2)$$

where LOS represents length of stay in days, $\Im$ is a vector of the instruments, the annual average number of patients per bed for each hospital; Z represents the observable characteristics of the patient: age, income, method of delivery, complications, Indian status, education and birth rate of patient's neighborhood; u_i is the unobserved characteristic, severity of illness, of the patient that affects length of stay. Complications is a dummy variable indicating whether the patient's diagnostic short list falls between 147 and 151 which includes hemorrhage of pregnancy, care during pregnancy as well as complications in labor, delivery and pueperium. Note that complication is observable and it differs from severity of illness in that two patients may both have hemorrhage but differ in severity.

In (2), y_{it} is a binary variable indicating whether or not patient i is readmitted t days (t is from 1 to ninety) after discharge. Thus y_{it} = 1 if the patient is readmitted and zero otherwise. Even though the body takes about 60 days to return to its pre-pregnancy state, when psychological adjustment is taken into account, the woman needs at least 90 days to adjust. Thus 90 days is long enough for any impact of the early discharge policy to be found. The error term $\vartheta_{it} = v_{it} + \varepsilon_{it}$, where v_{it} is the severity of illness component of ϑ_{it} and ε_{it} is identically and independently distributed. Thus it is assumed that apart from the severity of illness the error term, ϑ_{it}, is identically and independently distributed. The presence of severity of illness means that v_{it} is correlated

with u_{it} which implies that *LOS* is endogenous and the estimates of α, φ and η are inconsistent.

The hazard equation specifies the probability of being readmitted conditional on not being readmitted. Following the standard data transformation for discrete duration hazard, each patient contributed several observations to the data depending on the number of days, after discharge, it takes before she is readmitted. Thus a patient that is readmitted 18 days after discharge contributes 18 observations to the model. The observations are truncated after 90 days making those not readmitted after 90 days the censored group, each with 90 observations. The transformed data was then estimated by logit.

2.2. Estimation and Tests

To technically confirm the endogeneity of length of stay in the readmission equation, the exogeneity test for logit was used to test for the exogeneity of length of stay [11]. The test statistic, nR^2, was obtained from a regression of a vector of units on $[y_i - F(Z_i\hat{\beta}^{ML})X_i]$ and $[y_i - F(Z_i\hat{\beta}^{NLIV})LOS_i]$ where $\hat{\beta}^{ML}$ is the vector of the estimated parameters in (2) without IV estimation and $\hat{\beta}^{NLIV}$ is the vector of estimated parameters of (2) using IV estimation by nonlinear least squares. Under the null of exogeneity, the test statistic follows χ^2_G where *G* is the number of instrumental variables and *n* is the sample size.

To ensure the consistent estimation of the coefficients a test for weak instruments as shown in [10] was performed. It is basically a Wald test for the significance of the instruments in (1), *i.e.*, the null hypothesis is $\delta = 0$. The test statistic is $\hat{\lambda}'(\hat{C}'\hat{\Omega}\hat{C})^{-1}\hat{\lambda}$, where $\hat{\lambda}$ is a vector of *V* restrictions, $\delta = 0$, evaluated at $\hat{\beta}^{IV}$, $\hat{C}$ is a *K*X*V* matrix of the derivatives of the restrictions with respect to β^{IV}, and $\hat{\Omega}$ is the asymptotic Cramer-Rao lower bound variance both evaluated at $\hat{\beta}^{IV}$. The test statistic is χ^2_V where *V* is the number of instruments, which is 16 in this context.

Over identification test [12] was performed to test for the appropriateness of the instruments for length of stay. The null hypothesis states that the instruments are not significant in the readmission equation implying that they are appropriate instruments for length of stay. The test statistic here is also nR^2 from the least square regression of the residual of (2) on the instruments and the exogenous variables. nR^2 is also χ^2_V where *V* again is the number of instruments.

2.3. Data

The study used a four year (1993-1996) maternity data on all deliveries from sixteen acute care hospitals in British Columbia, Canada, provided by the British Columbia Ministry of Health and Center for Health Services and Policy Research linked database. For the purposes of this study, the data contained information on age in years, length of stay in days, method of delivery, complications, Indian status, local health area, dates of admission and discharge, transfers and hospital of delivery. Since transfers involve admission and discharges from different hospitals, a transferred patient that returns to her original hospital can easily be mistaken for a readmitted patient. Thus transferred patients were removed from the data. This reduced the sample size from 92,595 to 90,658 deliveries. Readmissions were also reduced from 3492 to 3326. To ensure that readmissions are related to the initial hospitalization only those readmissions caused by obstetric problems were included.

Information on patients' income and educational background were not available and so patients' neighborhood information was used. Neighborhood characteristics of the patients' local health areas for education, income and fertility rates were obtained from the website of the government of British Columbia. The number of maternity beds in each hospital which was needed for the construction of the instruments was obtained from the Directory of Canadian Hospitals. Since the data covered all deliveries in each of the hospitals, the number of patients per bed was computed by dividing the total deliveries in each hospital in a year by the number of maternity beds in the hospital for the year.

As shown in **Table 1** the average age of the mothers remained at about 29 over the four years. Length of stay decreased gradually over the years. The number of maternity beds in all the hospitals decreased relative to the number of patients who delivered hence the number of patients per bed increased over time. There was a significant (17.4%) reduction in the number of maternity beds in 1994, the year of the policy, followed by smaller (0.05% and 3%) reduction in the two years that followed. The number of maternity beds was therefore reduced over time to implement the early discharge policy hence making it correlate with length of stay.

Table 1. Data summary.

	1993	1994	1995	1996
Sample size	23149	23325	22795	22583
Average length of stay	3.71	3.47	3.29	3.22
Average age	29.2	29.3	29.5	29.7
Number of maternity beds	432	357	355	344
Patients per bed	53.6	65.3	64.2	92.6

The data used had several advantages in aiding with the consistent estimation of the parameters. First, the omission of breastfeeding and home visit are not likely to affect the consistency of the estimation. In addition to the severity of illness, home visit and breastfeeding are also correlated with both readmission rate and length of stay [8]. As noted in [13], newborns who receive home visits have short stays. Since mothers are unlikely to outstay their babies in the hospital, at least during the period under study, the availability of home care should in general, reduce length of stay for mothers. Home visit also reduces readmissions because the home visit nurse is likely to detect a developing infection and treat before it develops to require readmission. Thus, the omission of home visit can result in inconsistent estimation of the readmission equation. The source of the data used in this study however minimizes any such problem because the early discharge policy in British Columbia was implemented through the introduction of home visit program funded by the Closer to Home Fund available to all hospitals. All patients in the data then had access to home visits and so the impact on readmission is captured by the intercept and not the error term.

Mothers with no lactation problem have short stay and are less likely to be readmitted. Thus the omission of breastfeeding from the estimation equation could render length of stay inconsistent. However, breastfeeding is not likely to affect the consistency of the estimates in the study because lactation problems are captured under complications as care during puerperium. Thus the assumption that without the severity of illness the error term in (2) is identically and independently distributed is valid.

Second, maternity data is appropriate for the study because the early discharge policy has in general being applied to maternity patients across North America and Europe and so the resulting impact on readmission rates of maternity patients have received a lot of attention in the literature [1,3].

Lastly, studies have shown that severity adjusts well when it is disease specific [14]. This is to ensure that the clinical parameters in the model such as complications have similar effect on outcome hence validating the expectation that hemorrhage, for example, increases the probability of readmission rate.

3. RESULTS

The test statistic of the endogeneity test was 97835.29 with 16 degrees of freedom and a p-value of zero. Thus the null of no endogeneity was rejected. This confirms the expectation that length of stay is endogenous in the readmission equation; hence previous studies that ignored the endogeneity problem could have produced inconsistent estimates, making their conclusions unreliable.

The test for weak instruments yielded a test statistic of 104458.8 with 16 degrees of freedom and a p-value of zero, leading to the rejection of the null hypothesis of weak instruments. As stated in [10] weak instruments yield inconsistent estimates. The test results then confirm that the instruments used, number of patients per bed, are highly correlated with length of stay and uncorrelated with the severity of illness hence they are not weak instruments for length of stay. Finally the over identification test yielded a test statistic of 14.67 with 16 degrees of freedom and a p-value of 0.547. Thus, the null was not rejected implying that the instruments are appropriate for length of stay. Having been assured of consistent estimation of the coefficients, attention is now turned to the results of the regression.

As shown in **Table 2**, all the instrumental variables had negative signs confirming the expectation that length of stay falls as the number of patients per bed increases. The results also showed that length of stay dropped gradually even after 1994, the year of the policy.

The results from the hazard estimation, in **Table 2**, show that all the estimated coefficients in the IV and the non IV estimation had the same signs. Both show that there was no significant change in readmission rate in, 1995, the year after the policy but readmission rate increased in 1994 and 1996. As expected the readmission rate of those who lived in local health areas with high education level and/or high income were less likely to be readmitted conditional on not yet been readmitted. Patients with complications as well as those with Native Indian status were more likely to be readmitted conditional on not yet been readmitted, than those without complications and/or Native Indian status. As expected, Native Indians were more likely to be admitted than non-native Indians. In general, the IV estimates were less efficient than the non-IV estimates.

Since readmission rates are considered often as unconditional, unconditional readmission rates were computed to compare the impact of the policy on readmission rate using the IV and the non-IV estimations. The computation involves calculating, for each patient, the survival rate, subtracting it from one and then averaging over all the patients. The coefficient for length of stay under the non-IV was $-0.051 + 0.0008*LOS$ and that of the IV was $-0.115 + 0.006*LOS$. The average length of stay of 1994 in the above coefficients and unconditional readmission rates were used to compute the marginal effect of length of stay on readmission rate. The results showed a marginal effect of –1.54% under the IV estimation and –1.1% under the non-IV estimation, *i.e.*, an increase in length of stay by a day reduced readmission rate by 1.54% under the IV estimation and 1.1% under

Table 2. Regression results (numbers are estimated coefficients).

Dependent Variables / Independent Variables	Length of Stay	Readmission Equation (with IV estimation)	Readmission Equation (without IV estimation)
Number of patients per bed 1	–0.00003		
Number of patients per bed 2	0.0002		
Number of patients per bed 3	–0.015		
Number of patients per bed 4	–0.007		
Number of patients per bed 5	–0.028		
Number of patients per bed 6	–0.016		
Number of patients per bed 7	–0.019		
Number of patients per bed 8	–0.032		
Number of patients per bed 9	–0.043		
Number of patients per bed 10	–0.167		
Number of patients per bed 11	–0.026		
Number of patients per bed 12	–0.150		
Number of patients per bed 13	–0.00005		
Number of patients per bed 14	–0.091		
Number of patients per bed 15	–0.015		
Number of patients per bed 16	–0.047		
1994	–0.174	0.104	0.105
1995	–0.344	0.002	0.008
1996	–0.404	0.092	0.103
Income	0.841	–0.174	–0.175
Native Indians	0.130	0.277	0.283
Fertility	0.239	0.097	0.103
Age	–0.122	–0.073	–0.092
Age*age	0.002	0.001	0.002
Education	–0.897	–0.142	–0.154
Complication	0.450	0.544	0.523
Length of stay		–0.115	–0.051
Length of stay * length of stay		0.031	0.0001
T		–0.59	–0.059
T2		0.001	0.001
constant	5.288	–5.477	–5.431

All estimates are significant at 5% significance level.

the non-IV estimation. Thus, there is a greater marginal impact of length of stay on readmission rate under the IV than the non IV estimation. This result is similar to that in [8] as well as in [6].

An LM test was run to check if the coefficients of length of stay in the IV estimation were statistically different from those of non-IV. This null hypothesis is $\varphi^{IV} = \varphi^{nonIV}$. The test involves running the residuals of the restricted regression on the derivatives of the logit with respect to each of the coefficients in the model. The test statistic is nR^2 which follows the chi square distribution with two degrees of freedom (number of restrictions). The resulting test statistic was 2445.7 with a p-value of zero, implying that the coefficients were statistically different from each other. Hence the earlier studies that ignored the endogeneity of length of stay could be flawed.

The extend to which the policy contributed to the increase in readmission rates was found by rerunning the readmission regression after including interactions of length of stay with the year dummies. The resulting estimated coefficients for the interactions of the year dummies with the square of length of stay were not significant for either the IV or the non-IV estimates so the regressions were reran without those interactions. The results are reported in **Table 3** and they show that the interaction coefficients for the years and length of stay were negative for the IV results and positive but close to zero for the non-IV results. This implies that according to the IV results, the reduction in readmission rate from a day's increase in length of stay is greater after the period of the policy. The opposite is the case under the non-IV results.

To translate these into readmission rates, the results in **Table 3** were used to compute the contribution of the policy to any change in readmission rate as the difference between the readmission rates with and without the interactions. The readmission rates without the interaction were the readmission rates for each year without any contribution from the early discharge policy. The readmission rates that included the interactions represented the readmission rates for each year including the impact of the policy. The difference between the two then is the change in readmission rate as a result of the policy. To compare the impact of the policy with the overall change in readmission rate over time, the results in **Table 2** were used to compute the change in readmission rate for each year after the policy.

The results, reported in **Table 4**, show that readmission rates increased over the years under both the IV and the non-IV estimation. However, the increase was consistently higher under the IV than the non-IV estimation. The results under IV also showed that with the exception of 1994, where other factors must have contributed to the increase in readmission rate, any increase in readmission rate in the years that followed was due to the policy and other factors must have reduced the impact of the policy. Such information was not captured by the non-IV results.

Table 3. Regression results from interacting length of stay with the year dummy variables.

	Readmission Rate (with IV)	Readmission rate (without IV)
1994*	0.128	0.095
1995*	0.111	–0.023
1996*	–0.212	0.050
1994*length of stay*	–0.004	0.002
1995*length of stay*	–0.027	0.007
1996*length of stay*	0.102	0.013
Income	–0.174	–0.177
Native Indians	0.275	0.283
Fertility	0.100	0.103
Age	–0.079	–0.092
Age*age	0.001	0.002
Education	–0.141	–0.155
Complication	0.494	0.523
Length of stay*	–0.036	0.045
Length of stay * length of stay*	0.017	–0.0004
T	–0.059	–0.059
T2	0.001	0.001
constant	–5.516	–5.406

*Significant at 10% level. All other variables are significant at 5% significance level.

Table 4. Effect of policy on readmission rates.

	1994		1995		1996	
	IV	Non-IV	IV	Non-IV	IV	Non-IV
Change in readmission rate (%)	2.8	2.6	2.1	0.19	2.6	2.5
Change in readmission rate due to policy (%)	1.5	1.1	2.4	0.23	5.2	.101
Number of readmissions due to policy	536	23	547	52	1174	23

As shown in **Table 4**, for 1994 the policy increased readmission rate by 0.1 percentage points under the non-IV estimation and 2.3 percentage points under the IV estimation. Considering the number of deliveries of 23,325 in the selected hospitals in 1994, it implies, according to the non-IV estimation about only 23 women were readmitted as a result of the early discharge policy.

The number however increased to about 536 women under the IV estimation.

4. CONCLUSIONS

The current study has one main weakness. The IV estimation, when the second stage regression is logit or probit, requires that the endogenous variable is continuous. This implies that length of stay should be measured in hours and not in days as was used. The requirement that the problem variable be continuous is consistent with probit estimation which is continuous. However, in the current study the second stage regression is discrete and so a discrete endogenous variable for the first stage may not be problematic.

The study however has several strengths. First, the instruments, average patients per bed, proposed to ensure consistent estimation of the impact of the early discharge policy on readmission rate are not weak. Second the instruments are not significant in the readmission equation implying that they are not one of the regressors for readmission. Hence they are strong and appropriate instruments. Third, the instruments are not restricted to any diagnostic group and so can widely be applied to any diagnostic group to find consistent estimation of the impact of the early discharge policy. Finally, information on the number of beds for a diagnostic group can easily be found for the computation of the average number of patients per bed.

The study has shown that the non-IV estimates are statistically different from the IV estimates implying that earlier studies that ignored the endogeneity of length of stay might have produced misleading results. The non-IV estimates underestimate the impact of the policy on readmission rate. That could explain why some of the studies that ignored the endogeneity problem found no impact of the policy on readmission rate. The results from the non-IV estimates inform policy makers that the policy as it was implemented did not deteriorate readmission rate and so provided no reason for policy makers to make any adjustment. The results from the IV estimates imply that, since readmissions are expensive, the policy as implement might not contain as much cost as was expected and so further amendments such as improvement in home care is necessary to make the policy more able to reduce the cost of care.

REFERENCES

[1] Thomspon, A.H., Sauders, A.L.D., Cumming D.C. and Thanigasalam, N. (2003) Post-maternity outcomes following health care reform in Alberta: 1992-1996. *Canadian Journal of Public Health*, **94(4)**, 104-108.

[2] Gazmararian, J.A. and Koplan, J.P. (1996) Length-of-stay after delivery: Managed care versus fee-for-service. *Health Affairs*, **15(4)**, 74-80.

[3] Kosecoff, J., Kahn, K., Rogers, W., Reinisch, E., Sherwood, M., Rubenstein, L., Draper, D., Roth, C., Chew, C. and Brook, R. (1990) Prospective payment system and impairment at discharge: 'The quicker and sicker' story revisited. *Journal of the American Medical Association*, **264(15)**, 1980-1983.

[4] Tai-Seale, M., LoSasso, A.T., Freund, D.A. and Gerber, S.E. (2001) The long-term effects of medicaid managed care on obstetric care in three California counties. *Health Services Research*, **36(4)**, 751-771.

[5] Rubenstein, L., Kahn, K., Reinisch, E., Sherwood, M., Rogers, W., Karnberg, Draper, D. and Brook, R. (1990) Changes in quality of care for five diseases measured by implicit review. *Journal of the American Medical Association*, **264(15)**, 1981-1986.

[6] Kahn, K., Rogers, W., Rubenstein, L., Sherwood, M., Reinisch, E., Keeler, E., Draper, D., Kosecoff, J. and Brook. R. (1990) Comparing outcomes of care before and after implementation of the DRG-based prospective payment system. *Journal of the American Medical Association*, **264(15)**, 1984-1988.

[7] Keeler, E., Kahn, K., Draper, D., Sherwood, M., Rubenstein, L., Reinisch, E., Kosecoff, J. and Brook, R. (1990) Changes in sickness at admission following the introduction of the prospective payment system. *Journal of the American Medical Association*, **264**, 1962-1968.

[8] Iezzoni, L.I., (1994) Risk adjustment for measuring health care outcomes. Health Administration Press, Ann Arbor, IM.

[9] Gowrinsandaran, G. and Town, R.J. (1999) Estimating the quality of care in hospitals using instrumental variables. *Journal of Health Economics*, **18(6)**, 747-767.

[10] Heggestad, T. (2002) Do hospital length of stay and staffing ratio affect elderly patient's risk of readmission? A nation-wide study of Norwegian hospitals. *Health Services Research*, **37(3)**, 647-665.

[11] Malkin, J.D., Broder, M.S. and Keeler, E. (2000) Do longer postpartum stays reduce newborn readmissions' analysis using instrumental variables. *Health Services Research*, **35(5)**, 1071-1091.

[12] Amporfu, E. (2008) Quality effect of early discharge of maternity patients: Does hospital specialization matter? Forum for Health Economics & Policy. *Health Econmics*, **11(2)**. http://www.bepress.om/fhep/11/2/11

[13] Staiger, D. and Stock, J.H. (1997) Instrumental variables regression. *Econometrica*, **65**, 557-586.

[14] Grogger, J. (1990) A simple test for exogeneity in probit and logit, and poisson regression models. *Economics Letters*, **33(4)**, 329-332.

[15] Hensen, L. (1982) Large sample properties of generalized method of moments estimators. *Econometrica*, **50(4)**, 1029-1054.

[16] Gazmararian, J.A., Koplan, J.P., Cogswell, M.E., Bailey, C.M., Davis, N.A. and Cutler, C.M. (1997) Maternity experiences in a managed care organization. *Health Affairs*, **16(3)**, 198-208

[17] Wray, N.P., Hollingsworth, J.C., Petersen, N.J. and Aston, C.M. (1997) Case-mix adjustment using administrative databases: A paradigm to guide future research, *Medical Care Research and Review*, **54(3)**, 326-356.

Vol.2, No.5, 511-512 (2010)
doi:10.4236/health.2010.25076

Diuretic activity of Phyllanthus niruri (Linn.) in rats

A. L. Udupa[1]*, Sanjeeva[2], Adarsh Benegal[2], Vinay Prusty[2], G. Prabhath Kodancha[2], M. C. Satish Kumar[2], Vinutha Bhat[3], U. P. Ratnakar[4]

[1]Department of Pharmacology, Faculty of Medical Sciences, University of the West Indies, Cave Hill, Barbados; *Corresponding Author: aludupa2002@yahoo.com
[2]Department of Pharmacology, Kasturba medical college, Manipal, India
[3]Department of Biochemistry, Kasturba medical college, Manipal, India
[4]Department of Pharmacology, Kasturba medical college, Mangalore, India

Received 10 December 2009; revised 1 February 2010; accepted 3 February 2010.

ABSTRACT

Aqueous extract of Phyllanthus niruri (200 mg/kg and 400 mg/kg. p.o. single dose) was tested for its diuretic activity and compared with the standard drug hydrochlorothiazide (10 mg/ kg p.o.; single dose). Significant increase in the volume of urine and excretion of sodium, potassium and chloride was recorded when aqueous extract of Phyllanthus niruri was administered to hydrated albino rats.

Keywords: Phyllanthus niruri; Diuretic action

1. INTRODUCTION

Phyllanthus niruri [1] is claimed to have diuretic and antilithiatic activity in indigenous system of medicine. A survey of the literature revealed the absence of any systematic study on diuretic activity of the plant. Hence a study has been taken up to verify the claims made in the indigenous Ayurvedic system of medicine.

2. MATERIALS AND METHODS

The plant material—phyllanthus niruri was collected locally during the month of July to December. The botanical identity was confirmed by the Department of Botany Sri Poornaprajna college Udupi. Institutional Ethical Committee clearance was obtained for the experiment.

3. EXTRACTION

The shade dried plant (2 kg) was boiled with water in batches of 600 gm each. The aqueous extract was concentrated and dried on water bath (yield = 10%).

4. ACUTE TOXICITY STUDY [2]

Aqueous extract of Phyllanthus niruri was administered orally in varying doses of 1, 2, 4 and 8 g/kg to Wister strain albino rats of either sex (n = 6/group. 180-250 g).

Animals were observed for acute toxic effect initially continuously for two hours and thereafter at frequent intervals for 24 hours and thereafter once daily for 14 days.

5. DIURETIC ACTIVITY [3,4]

Albino rats of either sex (180-250 g) fasted over night were used, each group consisting of 10 animals. The animals were orally hydrated with 5 mL water, immediately before starting the procedures. Rats were placed individually in a metabolic cage and the urine was collected in tubes containing two drops of liquid paraffin to prevent evaporation. The urine collected over a period of 24 hours was measured, pH was noted and sodium, potassium, chloride, magnesium, phosphate and uric acid concentrations were determined.

Each animal was given three trials at biweekly intervals and the average was taken as the reading for calculation. The tests were done with single oral dose of aqueous extract of Phyllanthus niruri (200 mg and 400 mg/kg p.o.) and hydrochlorothiazide (10 mg/kg p.o.). These results were then compared with the diuretic activity of the orally administered vehicle as control. This was repeated with single oral dose of aqueous extract of Phyllanthus niruri (200 mg and 400 mg/kg p.o.) and hydrochlorothiazide (10 mg/kg p.o.) and compared with that of the orally administered vehicle as control.

6. STATISTICAL ANALYSIS

Student ‘t’ test was used for statistical analysis.

7. RESULTS AND DISCUSSIONS

Acute toxicity studies did not show any toxic effect up to 4 g/kg p.o. in a single dose up to 14 days. In 8 g/kg p.o.

Table 1. Urine volume and concentration of electrolytes (mEq/L).

Drug & pH of urine	Dosage mg/kg	Urine volume ml/24hr	Electrolytes mEq/L		
			Na^+	K^+	Cl^-
Control pH – 8.24 ± 0.26	Vehicle 5 ml water	5.2 ± 0.12	35.5 ± 3.70	56.19 ± 5.88	50.3 ± 2.73
AEPN pH – 8.02 ± 0.08	5 ml water + 200 mg/kg p.o.	8.9 ± 0.01[c]	46.2 ± 2.13[a]	87.3 ± 3.81[c]	69.14 ± 6.4[a]
AEPN pH – 8.06 ± 0.12	5 ml water + 400 mg/kg p.o.	9.74 ± 0.08[c]	49.2 ± 4.54[a]	94.5 ± 10.74[b]	74.4 ± 9.31[a]
Hydrochlorothiazide pH – 8.5 ± 0.21	5 ml water + 10 mg/kg p.o.	10.2 ± 0.13[c]	51 ± 3.9[b]	79.10 ± 4.97[b]	79.0 ± 2.87[c]

n = 10; a = P < 0.05; b = p < 0.01; c = p < 0.001; AEPN = Aqueous extract of Phyllanthus niruri.

dose some animals showed drowsiness and reduced spontaneous activities. So one tenth of the highest tolerated dose (*i.e.* 400 mg/kg and one lower dose *i.e.* 200 mg/kg) was used for diuretic activity studies. The pH of the urine was not significantly altered with the drug and it varied between 8.2 in control to 8.5 with hydrochlorothizide. The results (**Table 1**) show that aqueous extract of Phyllanthus niruri has significant diuretic activity and it has significantly increased the excretion of sodium, potassium and chloride as compared to that of the vehicle control and the volume of urine and electrolyte excretion pattern was comparable to that of the standard drug chosen *i.e.* hydrochlorothiazide.

These results substantiate the claims made in indigenous system of medicine.

8. ACKNOWLEDGEMENTS

The authors are thankful to the Dean of Kasturba Medical college-Manipal and K.M.C.Trust, Manipal for the financial support rendered for this study.

REFERENCES

[1] Nadkarni, A.K. (2002) Indian Materia Medica. Popular Prakashan Private Limited, **1**, 947.
[2] Crosland, J. (1980) Lewis Pharmacology Churchill Livingstone Inc. New York, 137-146.
[3] Hwang, K. and Goldberg, M.E. (1959) *Federation proceedings*, **18**, 405.
[4] Schaumann, O. (1960), *Archaic experimental Pathology Pharmaceutical*, **238**, 219.

HEALTH, 2010, 2, 391-512
Published Online May 2010 in SciRes (http://www.scirp.org/journal/health).

TABLE OF CONTENTS

Volume 2, Number 5, May 2010

