ISSN: 1949-4998 (Print), 1949-5005 (Online) Volume 2, Number 1, January 2010



Health



Editor-in-Chief Kuo-Chen Chou

www.scirp.org/journal/health

Journal Editorial Board

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

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

Editor-in-Chief Prof. Kuo-Chen Chou	Gordon Life Science Institute, San Diego, California, USA					
Managing Executive Editor						
City Chen	Scientific Research Publishing, USA Email: tchen@scirp.org					
Managing Production Edi	tor					
Jane Xiong	Scientific Research Publishing, USA Email: health@scirp.org					
Editorial Board (Accordin	g to Alphabet)					
Dr. Yiqiang Cai Dr. James CS Chim Dr. Yohichi Kumaki Dr. Petr Kuzmic Dr. CHIH MING LIN Prof. Baoliang Lu Prof. George Perry Prof. Kenji Sorimachi Dr. Yuande Yang Prof. Jun Zhang Prof. Xuehong Zhang Prof. Qun Zhao Dr. Wei Zhong Prof. Yang Zhong	Yale University School of Medicine, USA University of Hong Kong, Hong Kong (China) Institute for Antiviral Research, Utah State University, USA BioKin Ltd., USA Johns Hopkins University, Singapore Shanghai Jiao Tong University, China University of Texas at San Antonio, USA Dokkyo Medical University, Japan Wuhan University, China University of Kentucky, USA Shanghai Jiao Tong University, China University of Georgia, USA University of South Carolina Upstate, USA Fudan University, China					
Editorial Advisory Board (According to Alphabet)						
Dr. Wade Adams Prof. Dmitrios Braddock Dr. Athel Cornish-Bowden Dr. Louis J. Denis Prof. Reba Goodman	Eurofins AvTech Laboratories, USA Yale University School of Medicine, USA Centre National de la Recherche Scientifique, France Clinical Research & Development, Pfizer, USA Columbia University, USA					
Dr. Robert L. Heinrikson Prof. Denise Kirschner Dr. Claude Klee Prof. Harold A. Scheraga Prof. Kai Wucherpfennig	Proteos, Inc., Michigan, USA University of Michigan Medical School, USA National Institutes of Health (Cancer), Maryland, USA Cornell University, USA Harvard Medical School, USA					

Global Research & Development, Pfizer, USA

Guest Reviewers

Anshu Agrawal
Eva C. Bonefeld-Jorgensen
Mario Cesarelli

Dr. W. Z. Zhong

Mario Giorgi Jan Iwaniszewski Kyong-Hoon LEE Yasumitsu Ogra Yehuda Shoenfeld

.

TABLE OF CONTENTS

Volume 2, Number 1, January 2010

A low dose of nicotine is sufficient to produce nicotine withdrawal in mice
B. Morgane, S. Sandra, C. Jean-Pierre, G. Sylvie1
THE role of intracellular sodium (Na ⁺) in the regulation of calcium (Ca ²⁺)-mediated signaling and toxicity
X. M. Yu, B. R. Groveman, X. Q. Fang, S. X. Lin
Translational research in acupuncture-teleacupuncture bridges science and practice
G. Litscher17
Does conventional abdominal preoperative ultrasonography have a role in the detection of pancreatic insulinoma? A case report
L. Spadaro, O. Magliocco, G. Trovato, F. Purrello
Applicability of the P19CL6 cells as a model of cardiomyocytes – a transcriptome analysis
I. Khodadadi, N. J. Plant, V. Mersinias, A. E. Thumser
Development of a high-throughput cell based 384-well influenza A quantification assay for interpandemic and highly pathogenic avian strains
M. R. Gainey, A. M. Wasko, J. N. Garver, D. J. Guistino, E. M. Vela, J. E. Bigger
Gait spectral index (GSI): a new quantification method for assessing human gait
R. Héliot, C. Azevedo-Coste, L. Schwirtlich, B. Espiau
The effect of high fat food on erythrocyte phospholipids, fatty acids composition and glutathione redox-system of rats with alimentary dyslipidemia
T. P. Novgorodtseva, Y. K. Karaman, N. V. Zhukova45
Bioengineering functional copolymers. XII. Interaction of boron-containing and peo branched derivatives of poly(MA- <i>alt</i> -MVE) with HeLa Cells
M. Türk, Z. M. O. Rzayev, G. Kurucu
The central nucleus of amygdala is involved in tolerance to the antinociceptive effect of NSAIDs
M. G. Tsagareli, N. Tsiklauri, G. Gurtskaia, I. Nozadze, E. Abzianidze
Paediatric gallstones and laparoscopic cholecystectomy
D. G. Samuel, N. N. Naguib, A. Y. Izzidien,67
Troponin based studies in search of a biomarker for cardiac arrest
P. Ghazal, K. F. Shad, Shadat, N. Sidduiqui70

HEALTH

Journal Information

SUBSCRIPTIONS

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

E-mail:service@scirp.org

Subscription rates: Volume 2 2010

Print: \$50 per copy. Electronic: free, available on <u>www.SciRP.org</u>. To subscribe, please contact Journals Subscriptions Department, E-mail: <u>service@scirp.org</u>

Sample copies: If you are interested in subscribing, you may obtain a free sample copy by contacting Scientific Research Publishing, Inc at the above address.

SERVICES

Advertisements Advertisement Sales Department, E-mail: <u>service@scirp.org</u>

Reprints (minimum quantity 100 copies) Reprints Co-ordinator, Scientific Research Publishing, Inc., USA. E-mail: <u>service@scirp.org</u>

COPYRIGHT

Copyright© 2009 Scientific Research Publishing, Inc.

All Rights Reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording, scanning or otherwise, except as described below, without the permission in writing of the Publisher.

Copying of articles is not permitted except for personal and internal use, to the extent permitted by national copyright law, or under the terms of a license issued by the national Reproduction Rights Organization.

Requests for permission for other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creating new collective works or for resale, and other enquiries should be addressed to the Publisher.

Statements and opinions expressed in the articles and communications are those of the individual contributors and not the statements and opinion of Scientific Research Publishing, Inc. We assumes no responsibility or liability for any damage or injury to persons or property arising out of the use of any materials, instructions, methods or ideas contained herein. We expressly disclaim any implied warranties of merchantability or fitness for a particular purpose. If expert assistance is required, the services of a competent professional person should be sought.

PRODUCTION INFORMATION

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

A low dose of nicotine is sufficient to produce nicotine withdrawal in mice

Besson Morgane^{1,2}, Suarez Sandra¹, Changeux Jean-Pierre¹, Granon Sylvie^{1,3*}

¹Département de Neuroscience, Institut Pasteur, Paris, France; <u>sylvie.granon@u-psud.fr</u>

²Department of Experiment Psychology, Downing Site, Cambridge, UK

³Centre de Neuroscience Paris Sud, Université d'Orsay, Orsay, France

Received 12 October 2009; revised 12 November 2009; accepted 13 November 2009.

ABSTRACT

The objective of our study was to investigate whether the chronic administration of a low dose of nicotine can be followed by a withdrawal syndrome at cessation of nicotine delivery. Previous studies showed various results, depending in the doses of nicotine, species, ways of administration and behavioural paradigms, but all emphasized a withdrawal effect on some or all of the following spontaneous behaviours: grooming, rearing, body shake or tremor, body scratching, abdominal constriction, jumping. However, it is not clear which behaviour is exactly altered, as a global behavioural index is most frequently used. This is not clear either if anxiety modulates the behavioral expression of withdrawal or which factors contribute to its locomotors effect, if any. To distant-angle these processes, we scored each of these behaviours individually before nicotine exposure, during continuous nicotine delivery and at cessation of nicotine delivery after precipitated withdrawal by mecamylamine injection. We also measured locomotor activity and anxiety levels in the same animals. We used a low dose of nicotine (2.4 mg/kg/day as free base) that has been previously shown to produce nicotinic receptors up-regulation, both in the brain and in blood cells. With such a low dose, nicotine withdrawal didn't affect locomotion nor anxiety levels but increased the number of rearing, jumping, and marginally, body-scratching. Other behaviours, classically considered to contribute to withdrawal syndrome, were unaffected, e.g., grooming, body or forelimb shakes. Our results show that anxiety may be dissociated from the behavioural withdrawal syndrome. Also, the severity of the syndrome produced by nicotine withdrawal is qualitatively and quantitatively different from the one induced by other drugs of

abuse and also by the one produced by nicotine at higher doses.

Keywords: Withdrawal; Anxiety; Locomotor Activity; Nicotinic Receptors

1. INTRODUCTION

Nicotine is known to be an addictive substance in humans as well as in other mammals [1-3]. As such, behavioral studies in animal models show that nicotine induces a withdrawal syndrome [4,5], associated with the increased exhibition of multiple heterogeneous behaviors, many of which being shared by other drugs of abuse [2,6]. These findings, however, are confounded by two major factors: 1) in most cases, nicotine withdrawal is not associated with the same severity of symptoms as other drugs of abuse [6]; 2) global scores alone are not highly informative as they may reflect the grouping of different processes and different origins. Indeed, withdrawal signs are constituted of exaggerated levels of behaviours that are part of the normal behavioural repertoire of the animal, and as any behaviour, they may be influenced by multiple parameters such as genetic strain, individual and group level of stress, experimenter manipulation, lightning level or noise.

Authors of previous studies targeted several behaviours that are affected by nicotine as well as by other addictive drugs, i.e., rearing, abdominal constriction, grooming, scratching, chewing, cage scratching, and head nodding and jumping [7-10], and established a global abstinence score. In order to match with what others previously established, we scored the same behaviours but individually. Further, we scored the frequency of these behaviours in the same animals, before, during and after nicotine administration, in order to question whether their frequency varies within and between individuals and/or represents robust indicators of withdrawal. We therefore expected to provide evidence regarding which of these behaviours are specifically affected by nicotine withdrawal. We chose to administer continuously a low dose of nicotine (2.4 mg/kg/day, as free-base), via osmotic mini-pumps, as it was previously shown that it is sufficient to produce nicotinic receptor up-regulation in the mouse [11] and to induce withdrawal syndrome in the rat [12]. In accordance with previous studies [7,8,10,12,13], we then used 1 mg/kg of mecamylamine to precipitate homogeneously the withdrawal the last day of mini-pump theoretical depletion.

Withdrawal from chronic use of nicotine also results in increased anxiety [14,15], and reduced locomotor activity [8,12,16-20], although these last processes were altered either after higher doses of nicotine than the one we chose, or in rats instead of mice. We have therefore tested the effects of nicotine withdrawal, after the low dose administered, on locomotor activity and anxiety behaviours of the mouse.

Experiments have been carried out following the guidelines of the European Communities Council (86/609/EEC). Twenty-two C57BL6/J male mice (3 to 5 months) supplied by Charles River Laboratories (France) were housed individually upon arrival under a 12h light-dark cycle in rooms at a controlled temperature (21°C). All experiments were carried out during the light phase, from 10 am to 6 pm. They were handled during approximately ten days before the beginning of the tests. They were slightly food deprived one week before the start of the experiments with their weight adjusted at 90% of their normal body weight, as the same animals were subsequently used in a learning experiment, in order to minimize the use of animals. However, in order to reduce anxiety potentially induced by food deprivation, animals were fed before the experiments.

After handling, mice were habituated 15 minutes/day for three days to the environment (a large 50 cm X 30 cm transparent box, containing clean bedding) in which the behaviours were scored. Two experimenters, blind to the drug condition of the animals, scored the behaviours on line. Before mini-pump implantation, we first established the baseline level of each of the following behaviours: rearing, grooming, body shakes, body tremor and abdominal constriction, jumping, scratching, forelimb shake, chewing and head shakes. After the baseline measurement, mini-pumps were implanted subcutaneously under general anesthesia (a combination of xylazine, 2.5%, and ketamine, 15% in 82.5% of PBS). Mini-pumps (Alzet) were filled with either nicotine (2.4 mg/kg/day free base) or saline delivered at a constant rate (0.25 µl/hour) for 28 days. In order to measure the effect of chronic nicotine administration, we scored the same behaviours a second time two weeks after mini-pump implantation. At the last day of mini-pump delivery, i.e., the 28th day, mecamylamine was injected 15-20 minutes before the beginning of the withdrawal test at a dose of 1 mg/kg, IP. The same behaviours were then scored subsequently to evaluate the effects of nicotine withdrawal.

Locomotor activity was measured for 30 minutes in an empty large open field (1m in diameter) made of white plastic. It was situated in a room containing distal visual cues with light level set between 80 and 100 Lux and was placed under a camera connected to a video-track system that allows the discrimination of navigatory -speed > 11.8cm/sec- and exploratory movements -speed < 6.8cm/sec- [17]. It has been shown that rodents spontaneously avoid the center of a large open area which is more brightly illuminated than the periphery [21]. We therefore used the number of visits and time spent in the center as an index of anxiety during free exploratory activity. The baseline level of anxiety is altered by novelty [22]. Therefore, in order to avoid the alteration of the anxiety level generated by the first visit of a new environment, we conducted exploratory experiments in distinct groups of mice under the chronic administration of nicotine or saline and during withdrawal of nicotine or saline.

Each behavior was analyzed using repeated measures analysis of variance (ANOVAs) with "Drug condition" as the main within-subject factor, with 3 levels (before implantation, under chronic treatment and withdrawal), and "treatment" (with 2 levels, saline and nicotine) as the main between-subjects factors. Upon significant main effect (i.e., for p<0.05), data was further analyzed with paired t-tests.

Statistical analysis revealed a significant effect of the main within-subject factor, drug condition, for rearing (F_(2.40)=15.38, p<0.0001), jumping (F_(2.40)=3.98, p<0.026), body-scratching (F_(2,40)=5.05, p=0.01), grooming (F_(2,40)= 3.23, p=0.05) and forelimb shakes (F_(2,40)=12.23, p< 0.0001), suggesting that, individually, each of these parameters were altered either by the treatment and/or the repetition of the test. We also observed a significant interaction drug condition X treatment for rearing ($F_{(2,40)}$) =3.96, p=0.027), suggesting that nicotine and saline treatment produced a different effect on rearing. By contrast, no significant effect of drug condition and no interaction drug condition x treatment were observed for abdominal constriction (respectively, F<1 and $F_{(2,40)}=1.61$, NS) suggesting that this variable was not altered by the drug condition and, therefore, may not contribute significantly to the withdrawal syndrome.

When measured under chronic treatment, rearing (t=-0.86, p=0.4, df=20) and jumping (t=0.34, p=0.74, df=20) levels were similar in animals receiving saline and those receiving nicotine, making both variables unaffected by the chronic administration of nicotine, as illustrated in **Figure 1.** Grooming was the only behaviour that showed a significant main effect of group ($F_{(1,20)}=7.44$, p=0.013) and a significant main effect of drug condition ($F_{(2,40)}=3.23$, p=0.05) with no significant interaction (F<1, NS), suggesting that the drug condition was identical for the group receiving saline and the one receiving nicotine. It further suggests that the group effect (i.e. nicotine effect) was not dependent of the drug condition, i.e., both groups



Figure 1. Quantification of spontaneous behaviours affected by chronic administration of nicotine and nicotine withdrawal precipitated by injection of mecamylamine. Rearing, jumping, scratching, grooming and forelimb shakes were scored before mini-pump implantation (baseline), under chronic nicotine (white) or saline (black) treatment, and the last day of mini-pump delivery, 20 min after mecamylamine injection (withdrawal). * indicates a significant difference between animals under saline and those under nicotine.

may show different levels of grooming before mini-pump implantation. Post-hoc tests confirmed that the level of grooming was marginally different between the saline and the nicotine groups at baseline condition (t=-1.95, p=0.065, df=20) and was significantly different under chronic treatment (t=-2.38, p=0.027, df=20). These results suggest that nicotine had no effect by itself on grooming but that both groups, analysed by experimenters blind to the drug condition of the animals, were different regarding this parameter from the beginning of the experiment.

Body-scratching was marginally affected by the chronic drug treatment (t=-2.05, p=0.054, df=20). As illustrated in **Figure 1**, and confirmed by paired t-tests, animals receiving saline showed significantly less body- scratching levels when tested on baseline than when tested under mini-pumps (p=0.026) whereas animals receiving nicotine did not show different level of body- scratching between the two testing conditions (p=1.0). This suggests that the repetition of the test affected only animals that were not under nicotine treatment.

Forelimb shakes were unaffected by nicotine administration as the number of paw tremor recorded was not significantly different between animals receiving saline and those receiving nicotine under chronic treatment (t=0.17, p=0.87, df=20).

Post-hoc tests confirmed that the levels of rearing (t=-7.03, p<0.0001, df=10), jumping (t=-2.33, p=0.042, df=10), body-scratching (t=-2.14, p=0.058, df=10) and forelimb shakes (t=-2.58, p=0.027, df=10) were significantly different under nicotine administration and during withdrawal. The levels of rearing, jumping and body scratching didn't change from chronic treatment to withdrawal condition in saline animals (respectively: t=0.4, p=0.7, NS; t=0.25, p=0.81, NS and t=-0.99, p=0.35, NS), suggesting that these behaviours were altered specifically by nicotine withdrawal, as illustrated in Figure 1 Forelimb shakes were significantly altered in mice under chronic administration of saline (paired t-test conducted for chronic condition versus withdrawal condition, t=-2.7, p=0.022, df=10), suggesting that this behaviour was affected by mecamylamine administration but not by nicotine withdrawal. Post-hoc tests showed that nicotine withdrawal did not affect grooming (t=-1.43, p=0.18, df=10).



Figure 2. Exploratory and navigatory activity recorded in the circular open field for 30min during chronic treatment (A) and during withdrawal (B). No significant difference was found between animals under saline (black) and those under nicotine (white).

Navigation and exploration were not affected by the chronic administration of nicotine, as illustrated in **Figure 2A** ANOVAs confirmed that there was no treatment effect on navigation nor on exploration (both Fs<1, NS). For both types of behaviour, there was a significant effect of habituation (for navigation: $F_{(5,85)}=44.6$, p<0.0001; for exploration: $F_{(5,85)}=31.85$, p<0.0001) and no significant interaction treatment X habituation (both Fs<1, NS) suggesting that subjects receiving saline as well as those receiving nicotine showed normal habituation.

Navigation and exploration (**Figure 2**) were not statistically different after withdrawal in saline and in nicotine animals, as there was no treatment effect neither for navigation ($F_{(1,12)}$ =1.05, NS) nor for exploration (F<1, NS). Within 30 minutes, there was a significant habituation for both navigation ($F_{(5,60)}$ =57.96, p<0.0001) and exploration ($F_{(5,60)}$ =25.02, p<0.0001) but no significant interaction treatment X habituation for either navigation ($F_{(5,60)}$ =1.1, p=0.37, NS) or exploration (F<1, NS). These results showed that the levels of exploratory and navigatory activity as well as habituation were not affected by nicotine withdrawal.

The chronic administration of nicotine didn't affect the number of entries in the centre (data not shown, ANOVAs for drug effect: $F_{(1,17)}=2.45$, NS) but produced a trend to

reduce the time spent in the centre of the arena (**Figure 3A**, ANOVAs for drug effect: $F_{(1,17)}$ =4.33, p=0.053), suggesting that, in an unfamiliar environment, chronic nicotine administration tends to increase anxiety level.

The total number of entries in the centre (data not shown) and the time spent in the centre of the open field in 30 minutes (**Figure 3B**) were not different after withdrawal between saline and nicotine animals, as confirmed by ANOVAs (no drug effect for number of entries: F<1, NS; no drug effect for time in the centre: F<1, NS). These results showed that the level of anxiety was not affected by nicotine withdrawal in an unfamiliar environment.

Our present results demonstrate that only rearing, jumping and body-scratching reflect specifically nicotine withdrawal syndrome in mice, when nicotine dependence is produced by the continuous administration of a low dose i.e., 2.4mg/kg/day. This dose has been shown to induce a significant up-regulation of nicotinic receptors both in the blood and in the brain [11]. We find that in these conditions nicotine withdrawal affects neither anxiety nor locomotion. Previous studies have used significantly higher doses, i.e., up to ten times higher, that induced withdrawal signs of different nature than the one we observed in the present study but that also increased anxiety level [9,14,15]. Interestingly, when using a dose







Anxiety level after nicotine withdrawal (Fig. 3B)



Figure 3. Time spent in the centre of the brightly illuminated open field over one single 30-minute session during chronic treatment (A) and during nicotine withdrawal (B). Under chronic nicotine, mice tended to decrease the time spent in the centre of the open field, as compared to animals receiving saline (A). No significant difference was found between animals receiving saline (black) and those receiving nicotine (white) during withdrawal (B).

in a range similar to our, but excluding rearing and jumping from their scores, Damaj and collaborators [14] did not find any withdrawal signs or anxiety alteration. Therefore, in agreement with previous studies using a large range of nicotine doses, our present results provide evidence that a low dose of nicotine given continuously also induces an increase in the frequency of some specific behaviour. Using a low dose of nicotine, we were able to show dissociation between anxiety and other behavioural processes that may influence the general withdrawal syndrome. This dissociation suggests that withdrawal signs may not be exclusively due to an increase in anxiety levels after nicotine abstinence. It must be noticed that our animals were under a slight food deprivation schedule and, although they were fed before behavioural recording, we cannot exclude that chronic food deprivation has modified the effects of nicotine withdrawal. We conducted experiments (unpublished results) showing that a slight chronic food deprivation doesn't alter by itself navigatory and exploratory processes. These experiments do not provide evidence that food deprivation does not interfere with the nicotine exposure, for example by increasing anxiety levels. Such data are not yet available, to our knowledge.

Rearing behaviours have been shown to be sensitive to manipulations of the dopaminergic system [23]. Hildebrand and collaborators [13] also showed in nicotinedependent rats that intra-Ventral Tegmental Area injection of mecamylamine induced a rearing frequency modification with a dose of nicotine similar to our dose range. Therefore, it is possible that chronic nicotine administration followed by precipitated withdrawal created a deficit in cholinergic transmission which, in turn, reduced dopamine (DA) release, leading to an increased level of rearing. Microdialysis studies indeed showed that in nicotine-dependent rats, precipitated withdrawal leads to a decrease in DA release in the nucleus accumbency and in the prefrontal cortex [24,25]. Considering the role of the DA system in addiction [1], it is therefore not surprising that behaviours that are influenced by its modulation are particularly affected during withdrawal. Forelimb shakes are generally considered as a behavioural manifestation associated with drug withdrawal [8,26]. Our results showed that their frequency was affected by mecamylamine administration but not by nicotine withdrawal, as it was observable also in mice that were administered saline chronically. Previous studies [14,27] showed that this measure as well as some other signs are affected by hexamethonium or chlorisondamine, nicotinic antagonists which penetrate poorly the blood-brain barrier. Therefore, our data, along with previous ones [19] suggests that forelimb shakes are from peripheral origin and cannot be considered as a centrally-mediated withdrawal sign. We further show here that some of the behaviours usually recorded as part of a withdrawal syndrome, i.e., grooming, may be affected by the repetition of testing, but not by the nicotine treatment or withdrawal. It is furthermore noticeable that a baseline difference between groups (Figure 1), uncorrected as the experimenters were blind to the animals' treatment, remains observable for weeks, despite the habituation of the animals. This suggests that grooming is a behaviour that varies strongly between individuals, reinforcing the interest of longitudinal studies that allow baseline measurements. We also showed that body scratching was affected by nicotine administration and, only in animals that received nicotine, by withdrawal. Although this effect is statistically evidenced, it must be noticed that in absolute numbers, it is not highly represented in our data and should therefore be confirmed, despite the fact that it is classically associated with drug withdrawal [8,10,20,27]. Together with grooming data, scratching data emphasized the necessity to give baseline levels of such spontaneous behaviours when scoring drug effects.

In the open field, our data showed that mice under chronic administration of nicotine tend to spend less time in the centre of the arena, suggesting that, in an unfamiliar environment, chronic nicotine administration tends to increase anxiety. Our results also show that nicotine withdrawal, at a low dose, had no effect on anxiety when the anxiogenic environment was not familiar. This result is compatible with the one of other authors who compared the withdrawal effect after different doses of nicotine on anxiety [14]. Locomotor activity was affected neither by chronic nicotine exposure, nor by withdrawal. It is noticeable that, in rats, a similar dose of nicotine induced a temporary decrease of locomotor activity that was observable between 16 to 24 hours after mini-pump removal [6,12], but not in mice after pharmacological precipitation [28]. It is not possible to conclude firmly from experimental data obtained in different species (rats versus mice) and with two different procedures (mini-pump removal versus precipitation). Indeed, it is known that the sensitivity to nicotine is different in rats and mice [29]. Also, it is possible that mini-pump removal, that allows enough time for producing alteration in striatal DA concentration [12], produces a higher impact on locomotor activity than pharmacological precipitation, via DA mechanisms.

In conclusion, our present results support the idea that a continuous administration of low dose of nicotine, followed by pharmacological precipitation of withdrawal, is sufficient to produce a withdrawal syndrome in the mouse represented by increased levels of rearing and jumping. With such a low dose, nicotine withdrawal didn't affect locomotion nor anxiety levels. Other behaviours, contributing to nicotine withdrawal after higher doses of nicotine, were unaffected. Therefore, in spite of the fact that anxiety may produce some of the behaviours, unrelated with nicotine withdrawal, other behaviours, unrelated with anxiety can also be affected. These results can be discussed in terms of a relatively dissociable role of nicotinic receptors in the reward *versus* somatic dependence systems.

The future determination of whether these behaviours rely on specific neurotransmitter systems or brain structures is expected to complement the current therapeutical proposal of the withdrawal syndrome [30, for review].

REFERENCES

- Chiara, Di G. (2000) Role of dopamine in the behavioural actions of nicotine related to addiction. *Eur. J. Pharmacol*, 393, 295-314.
- [2] Epping-Jordan, M.P., Watkins, S.S., Koob, G.F. and Markou, A. (1998) Dramatic decrease in brain reward function during nicotine withdrawal. *Nature*, **393**, 76-79.
- [3] Rose, J.E. and Corrigall, W.A. (1997) Nicotine self-administration in animals and humans: Similarities and differences. *Psychopharmacology*, **130**, 28-40.
- [4] Aston-Jones, G. and Harris, G.C. (2004) Brain substrates for increased drug seeking during protracted withdrawal. *Neuropharmacol*, **47**, 167-179.
- [5] Stolerman, I.P. and Jarvis, M.J. (1995) The scientific case that nicotine is addictive. *Psychopharmacology*, **117**, 2-10.
- [6] Malin, D.H., Lake, J.R., Newlin-Maultsby, P., Roberts, L.K., Lanier, J.G., Carter, V.A., Cunningham, J.S. and Wilson, O.B. (1992) Rodent model of nicotine abstinence syndrome. *Pharmacol. Biochem. Behav.*, 43, 779-784.
- [7] Castané, A., Valjent, E., Ledent, C., Parmentier, M.,

Maldonado, R. and Valverde, O. (2002) Lack of CB1 cannabinoid receptors modifies nicotine behavioural responses, but not nicotine abstinence. *Neuropharmacology*, **43**, 857-867.

- [8] Isola, R., Vogelsberg, V., Wemlinger, T.A., Neff, N.H. and Hadjiconstantinou, M. (1999) Nicotine abstinence in the mouse. *Br. Res.*, 850, 189-196.
- [9] Salas, R., Pieri, F., and Biasi, De M. (2004) Decreased signs of nicotine withdrawal in mice null for the 4 nicotinic acetylcholine receptor subunit. J. Neurosci., 24, 10035-10039.
- [10] Salmon, A.M., Evrard, A., Damaj, I. and Changeux, J.P. (2004) Reduction of withdrawal signs after chronic nicotine exposure of alpha-calcitonin gene-related peptide knock-out mice. *Neurosci Lett.*, **360**, 73-76.
- [11] Cormier, A., Paas, Y., Zini, R., Tillement, J.P., Lagrue, G., Changeux, J.P. and Grailhe, R. (2004) Long-term exposure to nicotine modulates the level and activity of acetylcholine receptors in white blood cells of smokers and model mice. *Molec. Pharmacol*, 66, 1712-1718.
- [12] Fung, Y.K., Schmid, M.J., Anderson, T.M. and Lau, Y.S. (1996) Effects of nicotine withdrawal on central dopaminergic systems. *Pharmacol. Biohem. Behav*, 53, 635-640.
- [13] Hildebrand, B.E., Nomikos, G.G., Bondjers, C., Nisell, M. and Svensson, T.H. (1997) Behavioural manifestations of the nicotine abstinence syndrome in the rat: Peripheral versus central mechanisms. *Psychopharmacology*, **129**, 348-356.
- [14] Damaj, M.I., Kao, W. and Martin, B.R. (2003) Characterization of spontaneous and precipitated nicotine withdrawal in the mouse. *J. Pharmacol. Exp. Ther.* 307, 526-534.
- [15] Jonkman, S., Henry, B., Semenova, S. and Markou, A. (2005) Mild anxiogenic effects of nicotine withdrawal in mice. *Eur. J. Pharmacol*, **516**, 40-45.
- [16] Gäddnäs, H., Pietilä, K. and Ahtee, L. (2000) Effects of chronic oral nicotine treatment and its withdrawal on locomotor activity and brain monoamines in mice. *Behav. Br. Res*, **113**, 65-72.
- [17] Granon, S., Faure, P. and Changeux, J.P (2003) Executive and social behaviours under nicotinic receptor regulation. *Proc Natl Acad Sci USA*, **100**, 9596-9601.
- [18] Helton, D.R., Modlin, D.L., Tizzano, J.P. and Rasmussen, K. (1993) Nicotine withdrawal: A behavioural assessment using schedule controlled responding, locomotor activity, and sensorimotor reactivity. *Psychopharmacology*, **113**, 205-210.

- [19] Hildebrand, B.E., Panagis, G., Svensson, T.H. and Nomikos, G.G (1999) Behavioural and biochemical manifestations of mecamylamine-precipitated nicotine withdrawal in the rat: Role of nicotinic receptors in the ventral tegmental area. *Neuropsychopharmacology*, **21**, 560-574.
- [20] Malin, D.H., Lake, J.R., Carter, V.A., Cunningham, J.S., Hebert, K.M., Conrad, D.L. and Wilson, O.B. (1994) The nicotine antagonist mecamylamine precipitates nicotine abstinence syndrome in the rat. *Psychopharmacology*, **115**, 180-184.
- [21] Lipkind, D., Sakov, A., Kafkafi, N., Elmer, GI., Benjamini, Y. and Golani, I. (2004) New replicable anxiety-related measures of wall vs centre behaviour of mice in the open field. *J. Appl. Physiol.*, **97**, 347-59.
- [22] Crawley, J.N. (2000) What's wrong with my mouse? Behavioural phenotyping of transgenic and knockout mice. John Wiley-Liss Publication.
- [23] Fujita, S., Adachi, K., Lee, J., Uchida, T., Koshikawa, N. and Cools, A.R. (2004) Decreased postsynaptic dopaminergic and cholinergic functions in the ventrolateral striatum of spontaneously hypertensive rats. *Eur. J. Pharmacol*, **484**, 75-82.
- [24] Carboni, E., Bortone, L., Giua, C. and Chiara, Di G. (2000) Dissociation of physical abstinence signs from changes in extra cellular dopamine in the nucleus accumbens and in the prefrontal cortex of nicotine dependent rats. *Drug Alcohol Depend*, **58**, 93-102.
- [25] Rada, P., Jensen, K. and Hoebel, B.G. (2001) Effects of nicotine and mecamylamine-induced withdrawal on extracellular dopmaine and acetylcholine in the rat nucleus accumbens. *Psychopharmacology*, **157**, 105-110.
- [26] Maldonado, R. (2002) Study of cannabinoid dependence in animals. *Pharm. Ther.*, 92, 153-164.
- [27] Kenny, P.J. and Markou, A. (2001) Neurobiology of the nicotine withdrawal syndrome. *Pharmacol. Biochem. Behav.*, **70**, 531-549.
- [28] Balerio, G.N., Aso, E., Berrendero, F., Murta, P. and Maldonado, R. (2004) D9-Tetrahydrocannabinol decreases somatic and motivations of nicotine withdrawal in mice. *Eur. J. Neurosci*, **20**, 2737-2748.
- [29] Spielewoy, C. and Markou, A. (2004) Strain-specificity in nicotine attenuation of phencyclidine-induced disruption of prepulse inhibition in mice: Relevance to smoking in schizophrenia patients. *Behav. Genet.*, 34, 343-354.
- [30] Berrettini, W.H. and Lerman, C.E. (2005) Pharmacotherapy and pharmacogenetics of nicotine dependence. *Am. J. Psychiatry*, **162**, 1441-1451.

The role of intracellular sodium (Na⁺) in the regulation of calcium (Ca²⁺)-mediated signaling and toxicity

Xian-Min Yu, Bradley R. Groveman, Xiao-Qian Fang, Shuang-Xiu Lin

Department of Biomedical Sciences, College of Medicine, Florida State University, Tallahassee, USA; xianmin.yu@med.fsu.edu

Received 15 October 2009; revised 1 December 2009; accepted 2 December 2009.

ABSTRACT

It is known that activated N-methyl-D-aspartate receptors (NMDARs) are a major route of excessive calcium ion (Ca²⁺) entry in central neurons, which may activate degradative processes and thereby cause cell death. Therefore, NMD-ARs are now recognized to play a key role in the development of many diseases associated with injuries to the central nervous system (CNS). However, it remains a mystery how NMDAR activity is recruited in the cellular processes leading to excitotoxicity and how NMDAR activity can be controlled at a physiological level. The sodium ion (Na⁺) is the major cation in extracellular space. With its entry into the cell, Na⁺ can act as a critical intracellular second messenger that regulates many cellular functions. Recent data have shown that intracellular Na⁺ can be an important signaling factor underlying the up-regulation of NMDARs. While Ca²⁺ influx during the activation of NMDARs down-regulates NMDAR activity, Na⁺ influx provides an essential positive feedback mechanism to overcome Ca²⁺-induced inhibition and thereby potentiate both NMDAR activity and inward Ca2+ flow. Extensive investigations have been conducted to clarify mechanisms underlying Ca²⁺mediated signaling. This review focuses on the roles of Na⁺ in the regulation of Ca²⁺-mediated NMDAR signaling and toxicity.

Keywords: NMDA Receptors; Sodium and Calcium Influx; Sodium and Calcium Signaling; Excitability; Toxicity

1. INTRODUCTION

Cytoplasmic Ca^{2+} is the most common signaling factor in all types of cells. Normal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is approximately 40,000-fold lower than extracellular $[Ca^{2+}]$, which ranges from 1 to 2 mM [1,2].

Ca²⁺ ions enter neurons via various pathways including voltage-gated Ca²⁺ channels, ligand-gated Ca²⁺ channels and the Ca^{2+} exchangers [2,3]. It is known that activated NMDAR channels are a major route of excessive Ca²⁺ entry in neurons [4-10]. While excessive intracellular Ca^{2+} may activate degradative processes and thereby cause toxic effects [2,10-12], NMDAR channel activity may be inhibited by intracellular Ca²⁺ through: 1) α actinin/cvtoskeleton dissociation from the NR1 subunit of NMDARs [13]; 2) calmodulin activation [13-16], and 3) activation of phosphatases, such as calcineurin which dephosphorylates NMDARs [17-19]. The Ca²⁺-induced down- regulation of NMDARs is considered an important negative feedback mechanism to control NMDAR activity [20-23]. Based on these findings we questioned: How do excessive amounts of Ca²⁺ get into neurons through NMDARs if NMDARs are inhibited by Ca^{2+} influx?

 Na^+ is the major cation in the extracellular space, and it can enter cells through a variety of routes including permeation through ligand- (e.g., glutamate) and voltagegated cation channels, uptake via membrane exchangers and gradient-driven co-transporters [24]. NMDAR channels are highly permeable to both Na^+ and Ca^{2+} . Short burst or tetanic stimulation of afferents that induces synaptic LTP increases [Na⁺] up to 40 or 100 mM in spines and adjacent dendrites [25,26]. These increases can essentially be prevented by the blockade of NMDARs, indicating that they are mainly mediated by Na^+ entry through NMDARs [25,26].

Our initial studies demonstrated that intracellular Na⁺ is an up-regulator of NMDARs, such that raising $[Na^+]_i$ or activating Na⁺ permeable channels may increase NMD-AR-mediated currents [27-29]. We then identified that in hippocampal neurons an increase of 5 ± 1 mM in $[Na^+]_i$ represents a threshold required to mask the down- regulation of NMDARs induced by Ca²⁺ influx. Further increases in Na⁺ influx not only significantly enhance Ca²⁺ influx induced by the activation of NMDARs, but also overcome the Ca²⁺- dependent inhibition of NMDARs [27,30]. This review focuses on the roles of Na⁺ in the development of tissue injury and in the regulation of Ca²⁺-mediated NMDAR signaling and toxicity.

2. Na⁺ IN THE PROCESS OF TISSUE INJURY

A significant increase in $[Na^+]_i$ is a characteristic event associated with tissue injury [31-37]. Application of voltage-gated Na⁺ channel blockers reduce both Na⁺ entry and apoptotic neuronal death [32] whereas increases of Na⁺ entry by application of the voltage-gated Na⁺ channel activator, veratridine, induce neuronal apoptosis and caspase-3 activation [32,33]. There is a report showing that during anoxia Na⁺ entry can occur through either Gd³⁺-sensitive channels or via Na⁺/K⁺/2Cl⁻ cotransporters in cultured hippocampal neurons [38], implying that multiple pathways for Na⁺ entry may be activated during tissue injury.

It is known that Na⁺ influx into the cell is accompanied by chloride ions (Cl⁻) and water, which can lead to acute neuronal swelling and damage [4,39]. Previous studies have shown that Na⁺ entry may cause an increase in cytosolic Ca²⁺ through either Na⁺/Ca²⁺ exchangers or activation of voltage-gated Ca^{2+} channels [40.41], thereby activating Ca²⁺-dependent signaling mechanisms. Moreover, Na⁺ entry via Na⁺/H⁺ exchange may cause changes in intracellular pH, and thereby regulate many cellular functions including enzyme activity, neuronal growth and death [38,42-46]. A recent study showed that Na⁺ influx plays an important role in the onset of anti-Fas-induced apoptosis and that blocking Na⁺ influx may rescue programmed cell death in Jurkat cells [47]. Cox and colleagues reported that the binding of agonists to opioid receptors on guinea pig cortical neuron membranes is significantly reduced by increases in [Na⁺]; of 10-30 mM [48]. Maximal inhibition of μ -, δ - and κ -opioid receptor binding by Na⁺ is approximately 60%, 70% and 20%, respectively [48]. Co-occurrence of Na⁺, K⁺-ATPase dysfunction and Na⁺ influx causes α-amino-3-hydroxyl-5-methyl-4-isoxa-zole-propionate receptor (AMPAR) proteolysis and a rapid reduction of AMPAR cell-surface expression [49]. Na⁺-mediated K⁺ channels such as Slo gene-encoded K⁺ channels [50-54], are widely distributed throughout the nervous system and are involved in both the regulation of the after-potential following action potentials [51,55], and the protection of neurons from hypoxic stimulation [51,52,54].

While the details of the mechanisms remain to be clarified, significant pharmacological data have demonstrated the protective effects of blocking Na⁺ influx during injuries to the nervous tissue. The blockade of voltage-gated Na⁺ channels can prevent neurons from traumatic spinal cord injury [33,56-60] and the loss of white matter [30,56,60,61], concurrently reducing the sensitization associated with pain [62-65] and preventing seizures during kindling development [66]. The inhibition of Na⁺/H⁺ exchange attenuates ischemia-induced cell death [67,68]. As a result, a major focus of pharmaceutical

research has been on the search for effective therapeutic approaches that target voltage-gated Na⁺ channels [33,36].

3. ROLES OF Na⁺ IN THE REGULATION OF Ca²⁺-MEDIATED NMDAR SIGNALING AND TOXICITY

3.1. Calcium Influx through Activated NMDARs is Regulated by Na⁺ Influx

Activated NMDARs are highly permeable to both Na⁺ and Ca²⁺ [20,22,23]. Prolonged increases of intracellular Ca²⁺ during NMDAR activation may act as a negative feedback mechanism controlling NMDAR activity [20,22,23]. In light of our findings demonstrating that: 1) intracellular Na⁺ up-regulates NMDAR channel gating and 2) multiple types of receptor/channels such as AM-PARs, voltage-gated Na⁺ channels, non-selective cation channels and remote NMDARs may regulate NMDAR activity through a Na⁺-dependent mechanism [27,28], we investigated how NMDARs are regulated when both Ca²⁺ and Na⁺ flow into neurons during the same time period through activated NMDARs [27,30]. Recordings were conducted in the cell-attached single-channel configuration. In this recording model, recorded surface NMDARs are isolated by a recording electrode from the bath environment and therefore cannot be directly stimulated by bath-applied agents. We recorded the activity of surface NMDARs before and after activation of remote NMDARs (outside the patch) induced by bath application of NMDA or L-aspartate [30]. To prevent toxic effects which may be induced by application of NMDA or L-aspartate, a standard extracellular solution in which NaCl and KCl were replaced by Na₂SO₄ and Cs₂SO₄, was utilized [30]. Consistent with previous findings [17,28,39, 69,70], no damage of neurons bathed with this standard solution was observed following NMDA or aspartate application. NMDAR single-channel activity was evoked with 10 µM NMDA and 3 µM glycine included in the standard extracellular solution filling the recording electrodes.

We found that bath application of NMDAR agonists may change NMDAR channel activity recorded in cell-attached patches in a concentration-dependent manner. While a significant increase in NMDAR channel gating occurred during L-aspartate (>100 μ M) application to neurons bathed with the standard extracellular solution, the activity of NMDARs was inhibited in neurons when Na⁺ influx was blocked by replacing extracellular Na⁺ with Cs⁺ or N-methyl-D-glutamine (NMDG) [28,30].

We measured the ratio of fluorescence at 346 nm versus 380nm for the Na^+ -sensitive dye, sodium-binding benzofuran isophthalate (SBFI), and the Ca^{2+} -sensitive

dye, Fura-2, in the soma region of neurons. When the Na⁺ gradient across the cell membrane was decreased by reducing extracellular Na^+ concentration ($[Na^+]_e$) to 20 mM and the Na⁺ ionophore, monensin (10 μ M) was included in the extracellular solution, basal $[Ca^{2+}]_i$ and [Na⁺]_i of neurons were approximately 84 nM and 16 mM, respectively. Under this condition bath application of L-aspartate increased $[Ca^{2+}]_i$ by 66 nM, decreased $[Na^{+}]_i$ by 5.8 mM and inhibited NMDAR activity [30]. On average, the overall channel open probability and mean open time were reduced to 64% and 77% of controls. The burst and cluster lengths were also significantly reduced. These inhibitory effects produced by the bath application of L-aspartate were prevented by either application of APV or removal of Ca²⁺ from extracellular solution, indicating that the activation of remote NMDARs may also down-regulate recorded NMDAR activity through Ca^{2+} influx [30]. Thus, it is demonstrated that NMDARs can be up- and down-regulated by influxes of Na⁺ and Ca^{2+} , respectively.

We then measured changes of $[Na^+]_i$ and $[Ca^{2+}]_i$ in neurons bathed with extracellular solution containing a [Na⁺] of 10, 20 or 145 mM before and during the activation of NMDARs induced by bath application of L-aspartate. We found that with an increase in $[Na^+]_e$, the activation NMDARs produced increases in [Na⁺]_i as expected, but also increased [Ca²⁺]_i. Excluding the effect of Ca²⁺ influx-induced Ca²⁺ release (CICR) from intracellular stores, the increase in $[Ca^{2+}]_i$ of neurons bathed with extracellular solution containing 145 mM Na⁺ was still significantly higher than that found in neurons bathed with extracellular solution containing 10 mM Na⁺ [28,30]. When $[Na^+]_{e}$ was reduced to 10 mM, the activation of NMDARs produced increases in $[Na]_i$ and $[Ca^{2+}]_i$ by around 0.8 mM and 35 nM, respectively. Under this condition, the activation of remote NMDARs inhibited NMDAR activity recorded in cell-attached patches [30]. When [Na⁺]_e was increased to 20 mM, NMDAR activation produced a 5 mM increase in [Na⁺]_i and a 50 nM increase in $[Ca^{2+}]_i$, but no change in the activity of recorded NMDARs [30]. Similarly, increasing $[K^+]_e$ by 30 mM in an extracellular solution containing 170 mM Na⁺ and 1 μ M TTX produced increases in [Na⁺]_i and [Ca²⁺]_i by around 7 mM and 48 nM, respectively, but again showed no change in the activity of NMDARs recorded in cell-attached patches either [28,30]. Thus, an increase in $[Na^+]_i$ of approximately 5 mM appeared to be a critical concentration for masking the inhibitory effects induced by Ca²⁺ influx on NMDARs in cultured hippocampal neurons [30]. Since a modest increase of $[Ca^{2+}]_i$ by approximately 35 nM inhibited NMDAR activity when [Na⁺]_e was reduced to 10 mM [30], it was possible that Na⁺ influx not only enhanced Ca²⁺ influx but also masked the inhibitory effects of Ca^{2+} .

To confirm this hypothesis, we recorded NMDAR

single-channel activity before and during the activation of remote NMDARs in cell-attached patches with pipettes filled with a Ca²⁺-free extracellular solution containing 200 mM Na⁺ from neurons that had been pre-treated with BAPTA-AM (10 µM for 4 hrs) and bathed with the same Ca²⁺-free extracellular solution, or with pipettes filled with extracellular solution containing 0.3 or 1.2 mM Ca²⁺ from neurons bathed with the extracellular solution containing the same amount of Ca²⁺, respectively. We found that the activation of remote NMDARs produced a similar up-regulation of NMDAR channel activity when local and bath $[Ca^{2+}]$ was set at 0, 0.3 and 1.2 mM, implying again that the effects of Ca^{2+} influx in the regulation of NMDARs by remote NMDARs are overcome by Na⁺ under normal condition [30]. Furthermore, removal of extracellular Ca2+ did not produce any effect on the up-regulation of NMDARs by remote NMDARs in neurons bathed with the standard extracellular solution containing 200 mM Na⁺ [28,30]. Thus, we conclude that Ca^{2+} influx through activated NMDARs is regulated by Na⁺ influx, and that the effect of Na⁺, which overcomes Ca²⁺-induced inhibition, provides an essential positive feedback mechanism enhancing both the NMDAR activity and the inward flow of Ca^{2+} .

3.2. Depletion of Extracellular Ca²⁺ Enhances Na⁺ Influx and Thereby Causes NMDAR-Mediated Toxicity

Based on the findings that glutamate concentration may increase in both humans [71,72] and animals after nervous system injury [73], and that application of NMDAR antagonists may protect neurons from excitotoxic injuries in both humans [74,75] and animal [39,74,76], it has been believed that NMDAR-mediated excitoxicity plays a ke- y role in the development of neuronal death associated with stroke/traumatic CNS injury. However, it remained unclear how NMDARs were recruited to cause neurotoxicity.

We examined the effects of extracellular Ca²⁺ depletion and reperfusion, which may occur in stroke patients, on cultured hippocampal neurons [27,77]. Neurons were bathed initially with an extracellular solution containing: 140 mM NaCl, 5 mM CsCl, 1.8 mM CaCl₂, 33 mM glucose, 25 mM HEPES; pH: 7.35; osmolarity: 310-320 mOsm. The reduction of $[Ca^{2+}]_e$ from 1.8 mM to 0.5 or 0 mM caused a significant increase in Caspase-3 activity and morphological changes in neurons such as swelling, beading, and/or process disintegration. Significantly less formazan was observed in 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) assays in which neurons were treated with the extracellular solution containing 0.5 or 0 mM Ca²⁺, indicating a change in mitochondrial function associated with neuronal injury [78-81]. Unexpectedly, application of NMDAR antagonists APV (100 µM) and MK801 (2 µM) significantly prevented the above mentioned changes in neurons only

when the drugs were applied concomitant to the reduction of $[Ca^{2+}]_e$ from 1.8 to 0 mM. No protective effects of the drugs could be found when they were applied during Ca^{2+} reperfusion or when $[Ca^{2+}]_e$ was reduced from 1.8 to 0.5 mM [77]. These findings suggest that the depletion of extracellular Ca^{2+} may evoke NMDAR-mediated neurotoxicity [77], and also raised the questions of how and when NMDAR activity is recruited to induce neuronal injury following the removal of extracellular Ca^{2+} .

To address this question, we recorded NMDAR single-channel activity before and during a depletion of extracellular Ca²⁺ from 1.8 to 1.3, 0.5 or 0 mM in cellattached patches from cultured hippocampal neurons. To prevent cell damage during the reduction of extracellular Ca^{2+} , the Cl⁻ in the standard extracellular solution was replaced by SO₄²⁻ [28,30,39,77]. Bath application of a low [Ca²⁺] solution to neurons caused a parallel shift of the current-voltage (I/V) relationship in NMDAR single-channels recorded in cell-attached patches, which indicates that there is a cell-depolarization, but no change in single-channel conductance [30,77]. In order to account for this, the holding potential was re-adjusted to maintain a 70 mV patch-potential from the reversal potential of recorded channels. We found that a depletion of extracellular Ca²⁺ from 1.8 to 1.3 or 0.5 mM did not induce any significant change in the activity of recorded channels until $[Ca^{2+}]_e$ was reduced from 1.8 to 0 mM [28,30,77]. The channel activity could be subsequently abolished with application of the NMDAR antagonist, MK801, confirming that a $[Ca^{2+}]_e$ reduction from 1.8 to 0 mM produces increases in NMDAR activity [28,30,77]. Since the concomitant blockade of NMDARs to the reduction of [Ca²⁺]_e from 1.8 to 0.5 mM may actually increase the number of injured neurons [77], the up-regulation of NMDARs appears to be essential in triggering of toxicity mediated by NMDARs and the application of NMDAR antagonists in the Ca²⁺ reperfusion model may be protective only when NMDARs are recruited.

To identify the mechanisms by which the removal of extracellular Ca^{2+} results in the up-regulation of NMD-ARs, we measured $[Ca^{2+}]_i$ and $[Na^+]_i$ in cultured hippocampal neurons before and during reductions of extracellular Ca^{2+} . A $[Ca^{2+}]_e$ reduction-dependent decrease in $[Ca^{2+}]_i$ and increase in $[Na^+]_i$ were observed [77]. A depletion of extracellular Ca^{2+} from 1.8 to 0 mM produced sufficient increases in $[Na^+]_i$ capable of enhancing NMDAR activity [28,30,77]. Furthermore, we found that the up-regulation of NMDAR activity induced by extracellular Ca^{2+} depletion was prevented by the blockade of Na⁺ influx [77].

Previous studies showed that the removal of extracellular Ca^{2+} to 0 mM may increase NMDAR single-channel conductance [20,23,82], and that reducing intracellular Ca^{2+} may reduce the Ca^{2+} -dependent inhibition of NMDARs and thereby enhance NMDAR channel activity [13-20,23]. Therefore, it is possible that NMDAR gating may be enhanced by the removal of extracellular Ca^{2+} through Na⁺ and/or Ca²⁺-dependent mechanisms.

The ensemble currents produced by the summation of consecutive super-clusters were compared before (1.8 mM) and after reducing $[Ca^{2+}]_e$ to 0 mM. We found that the removal of extracellular Ca^{2+} may significantly increase the decay time of ensemble currents and that this effect can be abolished by blocking Na⁺ influx. This suggests that the removal of extracellular Ca^{2+} may affect NMDAR-mediated whole-cell responses through the action of Na⁺ [77].

Large reductions in $[Ca^{2+}]_e$ have been found during instances of high neuronal activity [83-85], the development of seizures [86], hypoglycemic coma [87], and periods of hypoxia and ischemia [88,89]. Ca²⁺-depletion has also been reported to induce cell injury and death [90]. Thus, the Na⁺-dependent enhancement of NMDAR activity induced by depletion of extracellular Ca²⁺ may be an important mechanism underlying the development of neurotoxicity in the CNS.

3.3. Na⁺ Regulation of Ca²⁺ Homeostasis

Under resting conditions [Ca²⁺]_i in neurons is normally maintained at 10-100 nM, and is tightly regulated by both Ca^{2+} influx and efflux across the membrane. $[Ca^{2+}]_i$ can be increased by Ca^{2+} entry through Ca^{2+} channels (including ligand- and voltage-gated Ca^{2+} channels and non-selective cation channels) located on the plasma membrane and by CICR from the endoplasmic reticulum (ER) upon binding of inositol trisphosphate (IP3) to the inositol trisphosphate receptor (IP3R). Ca²⁺-mediated injury is usually acute and rapid [91]. Disturbances of Ca²⁺ homeostasis in the cytoplasm, ER, or mitochondria can be harmful to cells [3]. Since Ca^{2+} stores are closely connected within the cells and interact with each other, dysregulation of one compartment is usually followed by responses from the others. Together, they may overwhelm the cell's capacity to maintain overall homeostasis and kill the cell [3].

In the plasma membrane Ca^{2+} -ATPase and the Na⁺/ Ca²⁺ exchanger act to transport cytosolic Ca²⁺ to the extracellular space. The Na⁺/Ca²⁺ exchanger has a low affinity for Ca²⁺ but a high velocity; as such, it removes Ca²⁺ only when cytosolic concentrations are high. The Ca²⁺-ATPase, has a high affinity for Ca²⁺ and pumps out Ca²⁺ even at low cytosolic concentrations [3,92,93]. In resting cells, [Ca²⁺] in the mitochondrial matrix is around 100 nM. When cytosolic [Ca²⁺] rises, Ca²⁺ can enter the mitochondria through a uniporter and thereby regulate Ca²⁺ signals [94]. In mitochondria the Na⁺/Ca²⁺ exchanger extrudes Ca²⁺ [3,94,95]. However, the activity of the Na⁺/Ca²⁺ exchanger may be reversed on the influx of Na⁺ [96]. This reversal in Na⁺/Ca² exchange is observed under pathological conditions [3]. If the mitochondrial Na⁺/Ca²⁺ exchanger is overwhelmed by Ca²⁺ entry, the Ca²⁺ levels in the mitochondrial matrix may increase enough to trigger a mitochondrial permeability transition. The sustained transitions may cause mitochondrial depolarization, inhibition of ATP production, and cell death [97-99]. In the nucleus Ca²⁺ is involved in the gene transcription and DNA metabolism [100-102]. Unlike in the mitochondria, nuclear Ca²⁺ is found to be rapidly equilibrated with cytosolic Ca²⁺. This may occur by diffusion across nuclear pores [103] and/or Ca²⁺ channels in the nuclear envelope [104].

CICR during NMDAR activation has been reported [20,105,106]. We observed that when extracellular solution contained more Na⁺, NMDAR activation produced greater increases in both [Na⁺]_i and [Ca²⁺]_i [30]. Furthermore, in neurons bathed with extracellular solution containing 145 mM Na⁺, NMDAR activation-induced increases in $[Ca^{2+}]_i$ were significantly reduced from $100 \pm$ 30 nM (n = 5) to 62 ± 8 nM (n = 8) with thapsigargin (0.1 µM) treatment [30], which depletes intracellular stores of Ca^{2+} by blocking Ca^{2+} re-uptake. In the absence of thapsigargin, NMDAR activation only produced a 35 ± 8 nM (n=8) increase in $[Ca^{2+}]_i$ in neurons bathed with extracellular solution containing 10 mM Na⁺ [30]. The blockade of Ca²⁺ influx by removal of extracellular Ca²⁺ abolished the NMDAR activation-induced increase in $[Ca^{2+}]_i$, (data not shown) [2,107]. The increase in $[Ca^{2+}]_i$ induced by Ca²⁺ release from intracellular stores during NMDAR activation in neurons bathed with extracellular solution containing 10 mM Na⁺ was significantly reduced when compared with that in neurons bathed with extracellular solution containing 145 mM Na⁺ [30]. These data suggest that CICR from intracellular stores during NMDAR activation may be regulated by intracellular Na⁺. Stys and colleagues provided direct evidence showing that intra-axonal Ca^{2+} release during ischemia in rat optic nerves is mainly dependent on Na⁺ influx. This Na⁺ accumulation stimulates three distinct intra-axonal sources of Ca²⁺: 1) the mitochondrial Na⁺/Ca²⁺ exchanger driven in the Na^+ import/ Ca^{2+} export mode; 2) positive modulation of ryanodine receptors; and 3) promotion of IP3 generation by phospholipase C [108].

4. QUESTIONS AND FUTURE STUDIES

Na⁺ entry is a key factor that initiates fast action potentials and shapes sub-threshold electrical properties to thereby regulate neuronal excitability and neuronal discharge activity [109-113]. Present data have shown that: 1) intracellular Na⁺ up-regulates NMDARs; 2) via increasing intracellular Na⁺, multiple types of receptor/channels such as AMPA receptors, voltage-gated Na⁺ channels and non-selective cation channels, may regulate NMDAR activity; 3) Na⁺ influx may enhance Ca²⁺ influx, mask the Ca²⁺-dependent inhibition of NMDARs and significantly alter Ca²⁺ homeostasis.

Based on combined investigations of protein crystal structures in-vitro and functions in cells, Na⁺ binding motifs have been characterized in a number of proteins such as thrombin, Na⁺/K⁺-ATPase and various neurotransmitter transporters. Thrombin is a serine protease. the activity of which is regulated by Na⁺ binding. The sequence, CDRDGKYG, in the Na⁺ binding loop is highly conserved in thrombin from 11 different species [114]. Investigations into the crystal structure of a bacterial homologue of the Na⁺/Cl⁻ dependent transporters from Aquifex aeolicus revealed that there are two Na⁺ binding sites, named Na1 and Na2 [115]. Na⁺/K⁺-ATPase is found to have three Na⁺ biding sites. Na1 is formed entirely by the side chain oxygen atoms of residues on three helices in the transmembrane regions (TM) 5, 6 and 8. Na2 is formed almost "on" the TM4 helix with three main chain carbonyls plus four side chain oxygen atoms (Asp 811 and Asp 815 on TM6 and Glu 334 on TM4). The Na3 binding site is contiguous to Na1. The carbonyls of Gly 813 and Thr 814 (TM6), the hydroxyl of Tyr 778 (TM5), and the carboxyl of Glu 961 (TM9) contribute to the Na3 binding site [116,117].

To date there is no evidence of a similar amino acid sequence corresponding to a Na⁺ binding site, as seen in these Na⁺ binding proteins, present in NMDAR subunit proteins (Yu, unpublished data). Molecular mechanisms underlying the regulation of NMDARs and Ca²⁺ signaling by intracellular Na⁺ remain unclear. Investigations aiming to identify critical Na⁺ targeting site(s) in the regulation of NMDARs and Ca²⁺ homeostasis are essentially needed for understanding activity-dependent neuroplasticity in the CNS.

5. ACKNOWLEDGMENTS

This work was supported by grant NIH (1R01 NS053567) to X.-M.Y.

REFERENCES

- Berridge, M.J., Lipp, P. and Bootman, M.D. (2000) The versatility and universality of calcium signaling. *Nat Rev Mol Cell Biol*, 1, 11-21.
- [2] Clapham, D.E. (1995) Calcium signaling. *Cell*, **80**, 259-268.
- [3] Dong, Z., Saikumar, P., Weinberg, J.M. and Venkatachalam, M.A. (2006) Calcium in cell injury and death. *Ann. Rev Pathol.*, 1, 405-434.
- [4] Choi, D.W. (1995) Calcium: Still center-stage in hypoxic-ischemic neuronal death. *Trends. Neurosci.*, 18, 58-60.
- [5] Forder, J.P. and Tymianski, M. (2009) Postsynaptic mechanisms of excitotoxicity: Involvement of postsynaptic density proteins, radicals, and oxidant molecules. *Neuroscience*, **158**, 293-300.
- [6] Lipton, S.A. (2006) Paradigm shift in neuroprotection by

NMDA receptor blockade: Memantine and beyond. *Nat. Rev. Drug Discov.*, **5**, 160-170.

- [7] Mody, I. and MacDonald, J.F. (1995) NMDA receptor-dependent excitotoxicity: The role of intracellular Ca2+ release. *Trends. Pharmacol. Sci.*, 16, 356-359.
- [8] Soriano, F.X. and Hardingham, G.E. (2007) Compartmentalized NMDA receptor signaling to survival and death. J. Physiol., **584**, 381-387.
- [9] Tymianski, M. and Tator, C.H. (1996) Normal and abnormal calcium homeostasis in neurons: A basis for the pathophysiology of traumatic and ischemic central nervous system injury. *Neurosurgery*, 38, 1176-1195.
- [10] Zipfel, G.J., Babcock, D.J., Lee, J.M. and Choi, D.W. (2000) Neuronal apoptosis after CNS injury: The roles of glutamate and calcium. *J. Neurotrauma.*, **17**, 857-869.
- [11] Bredesen, D.E. (2000) Apoptosis: Overview and signal transduction pathways [In Process Citation]. J. Neurotrauma, 17, 801-810.
- [12] Fiskum, G. (2000) Mitochondrial participation in ischemic and traumatic neural cell death [In Process Citation]. J. Neurotrauma., 17, 843-855.
- [13] Krupp, J.J., Vissel, B., Thomas, C.G., Heinemann, S.F. and Westbrook, G.L. (1999) Interactions of calmodulin and alpha-actinin with the NR1 subunit modulate Ca2+dependent inactivation of NMDA receptors. *J. Neurosci.*, **19**, 1165-1178.
- [14] Ehlers, M.D., Zhang, S., Bernhardt, J.P. and Huganir, R.L. (1996) Inactivation of NMDA Receptors by direct interaction of calmodulin with the NR1 subunit. *Cell*, 84, 745-755.
- [15] Wechsler, A. and Teichberg, V.I. (1998) Brain spectrin binding to the NMDA receptor is regulated by phosphorylation, calcium and calmodulin. *EMBO J.*, **17**, 3931-3939.
- [16] Zhang, S., Ehlers, M.D., Bernhardt, J.P., Su, C.T. and Huganir, R.L. (1998) Calmodulin mediates calcium dependent inactivation of N-methyl-D-aspartate receptors. *Neuron*, **21**, 443-453.
- [17] Lieberman, D.N. and Mody, I. (1994) Regulation of NMDA channel function by endogenous Ca2+- dependent phosphatase. *Nature*, **369**, 235-239.
- [18] Mulkey, R.M., Endo, S., Shenolikar, S. and Malenka, R.C. (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature*, **369**, 486-488.
- [19] Tong, G, Shepherd, D. and Jahr, C.E. (1995) Synaptic desensitization of NMDA receptors by calcineurin. *Science*, **267**, 1510-1512.
- [20] Dingledine, R., Borges, K., Bowie, D. and Traynelis, S.F. (1999) The glutamate receptor ion channels. *Pharmacol. Rev.*, **51**, 7-61.
- [21] Kyrozis, A., Albuquerque, C., Gu, J. and MacDermott, A.B. (1996) Ca(2+)-dependent inactivation of NMDA receptors: Fast kinetics and high Ca2+ sensitivity in rat dorsal horn neurons. J. Physiol. (Lond), 495, 449-463.
- [22] Mayer, M.L. and Westbrook, G.L. (1987) The physiology of excitatory amino acids in the vertebrate central nervous system. *Prog. Neurobiol.*, 28, 197-276.
- [23] McBain, C.J. and Mayer, M.L. (1994) N-Methyl-D-aspartic acid receptor structure and function. *Physiol. Rev.*, 74, 723-760.
- [24] Nicholls, D. and Attwell, D. (1990) The release and uptake of excitatory amino acids. *Trends in Pharmacological Sciences*, **11**, 462-468.

- [25] Rose, C.R. (2002) Na+ signals at central synapses. *Neuroscientist.*, 8, 532-539.
- [26] Rose, C.R. and Konnerth, A. (2001) NMDA receptor-mediated Na+ signals in spines and dendrites. J. *Neurosci.*, 21, 4207-4214.
- [27] Yu, X.M. (2006) The role of intracellular sodium (Na+) in the regulation of NMDA receptor-mediated channel activity and toxicity. *Mol Neurobiol.*, **3**, 63-79.
- [28] Yu, X.M. and Salter, M.W. (1998) Gain control of NMDAreceptor currents by intracellular sodium. *Nature*, **396**, 469-474.
- [29] Yu, X.M. and Salter, M.W. (1999) Src, a molecular switch governing gain control of synaptic transmission mediated by N-methyl-D-aspartate receptors. *Proc. Natl. Acad. Sci.* USA, 96, 7697-7704.
- [30] Xin, W.K. *et al.* (2005) A functional interaction of sodium and calcium in the regulation of NMDA receptor activity by remote NMDA receptors. *J. Neurosci.*, 25, 139-148.
- [31] Ballard-Croft, C., Carlson, D., Maass, D.L. and Horton, J.W. (2004) Burn trauma alters calcium transporter protein expression in the heart. J. Appl. Physiol., 97, 1470-1476.
- [32] Banasiak, K.J., Burenkova, O. and Haddad, G.G. (2004) Activation of voltage-sensitive sodium channels during oxygen deprivation leads to apoptotic neuronal death. *Neuroscience*, **126**, 31-44.
- [33] Baptiste, D.C. and Fehlings, M.G. (2007) Update on the treatment of spinal cord injury. *Prog. Brain Res.*, 161, 217-233.
- [34] Bauer, R., Walter, B., Fritz, H. and Zwiener, U. (1999) Ontogenetic aspects of traumatic brain edema: Facts and suggestions. *Exp. Toxicol Pathol.*, **51**, 143-150.
- [35] Friedman, J.E. and Haddad, G.G. (1994) Anoxia induces an increase in intracellular sodium in rat central neurons in vitro. *Brain Res.*, 663, 329-334.
- [36] Schwartz, G. and Fehlings, M.G. (2002) Secondary injury mechanisms of spinal cord trauma: A novel therapeutic approach for the management of secondary pathophysiology with the sodium channel blocker riluzole. *Prog. Brain Res.*, **137**, 177-190.
- [37] Strichartz, G., Rando, T. and Wang, G.K. (1987) An integrated view of the molecular toxinology of sodium channel gating in excitable cells. *Ann. Rev. Neurosci.*, 10, 237-67.
- [38] Sheldon, C., Diarra, A., Cheng, Y.M. and Church, J. (2004) Sodium influx pathways during and after anoxia in rat hippocampal neurons. J. Neurosci., 24, 11057-11069.
- [39] Choi, D.W. (1993) NMDA receptors and AMPA/kainate receptors mediate parallel injury in cerebral cortical cultures subjected to oxygen-glucose deprivation. *Prog. Brain Res.*, 96, 137-43.
- [40] Blaustein, M.P., Fontana, G. and Rogowski, R.S. (1996) The Na(+)-Ca2+ exchanger in rat brain synaptosomes: Kinetics and regulation. Ann. N. Y. Acad. Sci., 779, 300-17.
- [41] Koch, R.A. and Barish, M.E. (1994) Perturbation of intracellular calcium and hydrogen ion regulation in cultured mouse hippocampal neurons by reduction of the sodium ion concentration gradient. J. Neurosci., 14, 2585-2593.
- [42] Baxter, K.A. and Church, J. (1996) Characterization of acid extrusion mechanisms in cultured fetal rat hippocampal neurones. J. Physiol. (Lond), 493, 457-470.

SciRes Copyright © 2010

- [43] Boonstra, J. *et al.* (1983) Ionic responses and growth stimulation induced by nerve growth factor and epidermal growth factor in rat pheochromocytoma (PC12) cells. *J. Cell Biol.*, **97**, 92-98.
- [44] Moolenaar, W.H., Defize, L.H. and de Laat, S.W. (1986) Ionic signaling by growth factor receptors. *J. Exp. Biol.*, 124, 359-73.
- [45] Moolenaar, W.H., Tsien, R.Y., van der Saag, P.T. and de Laat, S.W. (1983) Na+/H+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. *Nature*, **304**, 645-648.
- [46] Sin, W.C. *et al.* (2009) Regulation of early neurite morphogenesis by the Na+/H+ exchanger NHE1. J. *Neurosci.*, 29, 8946-8959.
- [47] Bortner, C.D. and Cidlowski, J.A. (2003) Uncoupling cell shrinkage from apoptosis reveals that Na+ influx is required for volume loss during programmed cell death. J. Biol Chem, 278, 39176-39184.
- [48] Werling, L.L., Brown, S.R., Puttfarcken, P. and Cox, B.M. (1986) Sodium regulation of agonist binding at opioid receptors. II. Effects of sodium replacement on opioid binding in guinea pig cortical membranes. *Mol Pharmacol*, **30**, 90-95.
- [49] Zhang, D. *et al.* (2009) Na, K-ATPase activity regulates AMPA receptor turnover through proteasome-mediated proteolysis. *J. Neurosci.*, **29**, 4498-4511.
- [50] Bhattacharjee, A. *et al.* (2003) Slick (Slo2.1), a rapidly-gating sodium-activated potassium channel inhibited by ATP. *J. Neurosci*, **23**, 11681-11691.
- [51] Bhattacharjee, A. and Kaczmarek, L.K. (2005) For K(+) channels, Na(+) is the new Ca(2+). *Trends Neurosci*, 28, 422-428.
- [52] Dryer, S.E. (2003) Molecular identification of the Na+activated K+ channel. *Neuron*, **37**, 727-728.
- [53] Niu, X.W. and Meech, R.W. (2000) Potassium inhibition of sodium-activated potassium (K(Na)) channels in guineapig ventricular myocytes. J. Physiol, 526(Pt 1), 81-90.
- [54] Yuan, A. *et al.* (2003) The sodium-activated potassium channel is encoded by a member of the Slogene family. *Neuron*, **37**, 765-773.
- [55] Liu, X. and Stan, L.L. (2004) Sodium-activated potassium conductance participates in the depolarizing afterpotential following a single action potential in rat hippocampal CA1 pyramidal cells. *Brain Res.*, **1023**, 185-192.
- [56] Agrawal, S.K. and Fehlings, M.G. (1996) Mechanisms of secondary injury to spinal cord axons in vitro: Role of Na+, Na(+)-K(+)-ATPase, the Na(+)-H+exchanger, and the Na(+)-Ca2+ exchanger. J. Neurosci., 16, 545-552.
- [57] Agrawal, S.K. and Fehlings, M.G. (1997) The effect of the sodium channel blocker QX-314 on recovery after acute spinal cord injury. *J. Neurotrauma.*, 14, 81-88.
- [58] Fehlings, M.G. and Agrawal, S. (1995) Role of sodium in the pathophysiology of secondary spinal cord injury. *Spine*, **20**, 2187-2191.
- [59] Hains, B.C., Saab, C.Y., Lo, A.C. and Waxman, S.G. (2004) Sodium channel blockade with phenytoin protects spinal cord axons, enhances axonal conduction, and improves functional motor recovery after contusion SCI. *Exp. Neurol.*, **188**, 365-377.
- [60] Teng, Y.D. and Wrathall, J.R. (1997) Local blockade of sodium channels by tetrodotoxin ameliorates tissue loss and long-term functional deficits resulting from experi-

mental spinal cord injury. J. Neurosci., 17, 4359-4366.

- [61] Cummins, T.R. and Waxman, S.G. (1997) Downregulation of tetrodotoxin-resistant sodium currents and upregulation of a rapidly repriming tetrodotoxin-sensitive sodium current in small spinal sensory neurons after nerve injury. J. Neurosci., 17, 3503-3514.
- [62] Appelgren, L., Janson, M., Nitescu, P. and Curelaru, I. (1996) Continuous intracisternal and high cervical intrathecal bupivacaine analgesia in refractory head and neck pain [see comments]. *Anesthesiology*, 84, 256-272.
- [63] Cox, J.J. *et al.* (2006) An SCN9A channelopathy causes congenital inability to experience pain. *Nature*, **444**, 894-898.
- [64] Dib-Hajj, S., Black, J.A., Cummins, T.R. and Waxman, S.G. (2002) NaN/Nav1.9: A sodium channel with unique properties. *Trends Neurosci.*, 25, 253-259.
- [65] Waxman, S.G. and Hains, B.C. (2006) Fire and phantoms after spinal cord injury: Na+ channels and central pain. *Trends Neurosci.*, 29, 207-215.
- [66] Rogawski, M.A. and Loscher, W. (2004) The neurobiology of antiepileptic drugs. *Nat. Rev. Neurosci.*, 5, 553-564.
- [67] Mentzer, R.M., Lasley, R.D., Jessel, A. and Karmazyn, M. (2003) Intracellular sodium hydrogen exchange inhibition and clinical myocardial protection. *Ann. Thorac. Surg.*, **75**, S700-S708.
- [68] Vornov, J.J., Thomas, A.G. and Jo, D. (1996) Protective effects of extracellular acidosis and blockade of sodium/hydrogen ion exchange during recovery from metabolic inhibition in neuronal tissue culture. *J. Neurochem.*, **67**, 2379-2389.
- [69] Kohr, G, De Koninck, Y. and Mody, I. (1993) Properties of NMDA receptor channels in neurons acutely isolated from epileptic (kindled) rats. *J. Neurosci.*, **13**, 3612-3627.
- [70] Yu, X.M., Askalan, R., Keil, G.J.I. and Salter, M.W. (1997) NMDA channel regulation by channel-associated protein tyrosine kinase src. *Science*, **275**, 674-678.
- [71] Baker, A.J., Moulton, R.J., MacMillan, V.H. and Shedden, P. M. (1993) Excitatory amino acids in cerebrospinal fluid following traumatic brain injury in humans. *J. Neurosurg.*, 79, 369-372.
- [72] Persson, L. and Hillered, L. (1992) Chemical monitoring of neurosurgical intensive care patients using intracerebral microdialysis. J. Neurosurg., 76, 72-80.
- [73] Benveniste, H., Drejer, J., Schousboe, A. and Diemer, N.H. (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.*, **43**, 1369-1374.
- [74] Chen, H.-S.V. and Lipton, S.A. (2005) Pharmacological implications of two distinct mechanisms of interaction of memantine with NMDA-gated channels. J. Pharmacol. Exp. Ther., 314, 961-971.
- [75] Lipton, S.A. (2004) Paradigm shifts in NMDA receptor antagonist drug development: Molecular mechanism of uncompetitive inhibition by memantine in the treatment of Alzheimer's disease and other neurologic disorders. J. Alzheimers. Dis., 6, S61-S74.
- [76] Lipton, P. (1999) Ischemic cell death in brain neurons. *Physiol Rev.*, **79**, 1431-1568.
- [77] Xin, W.K. *et al.* (2005) The removal of extracellular calcium: A novel mechanism underlying the recruitment of N-methyl-d-aspartate (NMDA) receptors in neurotoxicity. *Eur. J. Neurosci.*, **21**, 622-636.

- [78] Giardina, S.F. and Beart, P.M. (2002) Kainate receptormediated apoptosis in primary cultures of cerebellar granule cells is attenuated by mitogen-activated protein and cyclin-dependent kinase inhibitors. *Br. J. Pharmacol.*, **135**, 1733-1742.
- [79] Ohyashiki, T., Satoh, E., Okada, M., Takadera, T. and Sahara, M. (2002) Nerve growth factor protects against aluminum-mediated cell death. *Toxicology*, **176**, 195-207.
- [80] Sawyer, T.W. (1995) Practical applications of neuronal tissue culture in in vitro toxicology. *Clin. Exp. Pharmacol. Physiol.*, 22, 295-296.
- [81] Wang, X., Mori, T., Sumii, T. and Lo, E.H. (2002) Hemoglobin-induced cytotoxicity in rat cerebral cortical neurons: Caspase activation and oxidative stress. *Stroke*, 33, 1882-1888.
- [82] Westbrook, G.L., Krupp, J.J. and Vissel, B. (1997) Cytoskeletal interactions with glutamate receptors at central synapses. Soc. Gen. Physiol Ser., 52, 163-175.
- [83] Hardingham, N.R. *et al.* (2006) Extracellular calcium regulates postsynaptic efficacy through group 1 metabotropic glutamate receptors. *J. Neurosci.*, 26, 6337-6345.
- [84] Rusakov, D.A. and Fine, A. (2003) Extracellular Ca2+ depletion contributes to fast activity-dependent modulation of synaptic transmission in the brain. *Neuron*, **37**, 287-297.
- [85] Vassilev, P.M., Mitchel, J., Vassilev, M., Kanazirska, M. and Brown, E.M. (1997) Assessment of frequency-dependent alterations in the level of extracellular Ca2+ in the synaptic cleft. *Biophys. J.*, **72**, 2103-2116.
- [86] Heinemann, U. and Hamon, B. (1986) Calcium and epileptogenesis. *Exp. Brain Res.*, 65, 1-10.
- [87] Ekholm, A., Kristian, T. and Siesjo, B.K. (1995) Influence of hyperglycemia and of hypercapnia on cellular calcium transients during reversible brain ischemia. *Exp. Brain Res.*, **104**, 462-466.
- [88] Harris, R.J., Symon, L., Branston, N.M. and Bayhan, M. (1981) Changes in extracellular calcium activity in cerebral ischaemia. J. Cereb. Blood Flow Metab., 1, 203-209.
- [89] Nicholson, C., Bruggencate, G.T., Steinberg, R. and Stockle, H. (1977) Calcium modulation in brain extracellular microenvironment demonstrated with ion-selective micropipette. *Proc. Natl. Acad. Sci. USA*, 74, 1287-1290.
- [90] Smith, M.T., Thor, H. and Orrenius, S. (1981) Toxic injury to isolated hepatocytes is not dependent on extracellular calcium. *Science*, **213**, 1257-1259.
- [91] Trump, B.F. and Berezesky, I.K. (1995) Calcium-mediated cell injury and cell death. FASEB J., 9, 219-228.
- [92] Carafoli, E., Santella, L., Branca, D. and Brini, M. (2001) Generation, control, and processing of cellular calcium signals. *Crit Rev Biochem. Mol Biol.*, **36**, 107-260.
- [93] Berridge, M.J., Bootman, M.D. and Roderick, H.L. (2003) Calcium signaling: Dynamics, homeostasis and remodeling. *Nat Rev Mol Cell Biol*, 4, 517-529.
- [94] Duchen, M.R. (2000) Mitochondria and calcium: From cell signaling to cell death. J. Physiol., **529** (Pt 1), 57-68.
- [95] Gunter, T.E., Yule, D.I., Gunter, K.K., Eliseev, R.A. and Salter, J.D. (2004) Calcium and mitochondria. *FEBS Lett.*, 567, 96-102.
- [96] Hilgemann, D.W., Collins, A. and Matsuoka, S. (1992) Steady-state and dynamic properties of cardiac sodium-calcium exchange: Secondary modulation by cytoplasmic calcium and ATP. J. Gen. Physiol., 100, 933-961.
- [97] Bernardi, P. and Rasola, A. (2007) Calcium and cell death: The mitochondrial connection. *Subcell. Biochem.*, 45, 481-506.

- [98] Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. J. Biochem., 341 (Pt 2), 233-249.
- [99] Lemasters, J.J., Theruvath, T.P., Zhong, Z. and Nieminen, A.L. (2009) Mitochondrial calcium and the permeability transition in cell death. *Biochim. Biophys. Acta*, **1787**, 1395-1401.
- [100] Hardingham, G.E., Cruzalegui, F.H., Chawla, S. and Bading, H. (1998) Mechanisms controlling gene expression by nuclear calcium signals. *Cell Calcium*, 23, 131-134.
- [101] McKenzie, G.J. *et al.* (2005) Nuclear Ca2+ and CaM kinase IV specify hormonal- and Notch-responsiveness. *J. Neurochem.*, **93**, 171-185.
- [102] Papadia, S., Stevenson, P., Hardingham, N.R., Bading, H. and Hardingham, G.E. (2005) Nuclear Ca2+ and the camp response element-binding protein family mediate a late phase of activity-dependent neuroprotection. *J. Neurosci.*, 25, 4279-4287.
- [103] Allbritton, N.L., Oancea, E., Kuhn, M.A. and Meyer, T. (1994) Source of nuclear calcium signals. *Proc. Natl. Acad. Sci. USA*, **91**, 12458-12462.
- [104] Mazzanti, M., DeFelice, L.J., Cohen, J. and Malter, H. (1990) Ion channels in the nuclear envelope. *Nature*, 343, 764-767.
- [105] Sattler, R. and Tymianski, M. (2000) Molecular mechanisms of calcium-dependent excitotoxicity. J. Mol. Med., 78, 3-13.
- [106] Verkhratsky, A. (2002) The endoplasmic reticulum and neuronal calcium signaling. *Cell Calcium*, **32**, 393-404.
- [107] Verkhratsky, A.J. and Petersen, O.H. (1998) Neuronal calcium stores. *Cell Calcium*, 24, 333-343.
- [108] Nikolaeva, M.A., Mukherjee, B. and Stys, P.K. (2005) Na⁺-dependent sources of intra-axonal Ca²⁺ release in rat optic nerve during in vitro chemical ischemia. *J Neurosci*, 25, 9960-9967.
- [109] Cantrell, A.R. and Catterall, W.A. (2001) Neuromodulation of Na+ channels: An unexpected form of cellular plasticity. *Nat. Rev Neurosci.*, 2, 397-407.
- [110] Catterall, W.A., Goldin, A.L. and Waxman, S.G. (2003) International union of pharmacology. XXXIX. compendium of voltage-gated ion channels: Sodium channels. *Pharmacol Rev.*, 55, 575-578.
- [111] Goldin, A.L. (2001) Resurgence of sodium channel research. Ann. Rev. Physiol., 63, 871-894.
- [112] Hille, B. (1992) Ionic channels of excitable membranes. Sinauer, Sunderland.
- [113] Waxman, S.G., Dib-Hajj, S., Cummins, T.R. and Black, J.A. (2000) Sodium channels and their genes: Dynamic expression in the normal nervous system, dysregulation in disease states (1). *Brain Res.*, 886, 5-14.
- [114] Di Cera, E. et al. (1995) The Na [IMAGE] binding site of thrombin. J. Biol. Chem., 270, 22089-22092.
- [115] Yamashita, A., Singh, S.K., Kawate, T., Jin, Y. and Gouaux, E. (2005) Crystal structure of a bacterial homologue of Na+/Cl: Dependent neurotransmitter transporters. *Nature*, **437**, 215-223.
- [116] Li, C., Capendeguy, O., Geering, K. and Horisberger, J.D. (2005) A third Na+-binding site in the sodium pump. *Proc. Natl. Acad. Sci. USA*, **102**, 12706-12711.
- [117] Ogawa, H. and Toyoshima, C. (2002) Homology modeling of the cation binding sites of Na+K+-ATPase. *Proc. Natl. Acad. Sci. USA*, **99**, 15977-15982.

Translational research in acupuncture—teleacupuncture bridges science and practice

Gerhard Litscher

Research Unit of Biomedical Engineering in Anesthesia and Intensive Care Medicine and TCM Research Center Graz, Medical University Graz, Graz, Austria; <u>gerhard.litscher@medunigraz.at</u>

Received 15 October 2009; revised 1 December 2009; accepted 3 December 2009.

ABSTRACT

In March 2009, the first teleacupuncture between China and Austria was performed. This publication summarizes the first important results. 24hour electrocardiograms were registered in Beijing and analyzed in Graz. A heart rate variability monitor partly developed in Austria was used for recording. Data were transferred via internet over a distance of 7,650 km. For the spectral analysis of heart rate variability a new method, the so-called 'Fire of Life' illustration, was applied. The state of health of a 31-year-old patient before, during and after acupuncture treatment sessions was documented. Despite several limitations, transcontinental teleacupuncture opens up new possibilities in public health.

Keywords: Teleacupuncture; Heart Rate Variability (HRV); Electrocardiogram (ECG); Sustainable Health Research

1. INTRODUCTION

Telemedicine in general is defined as the 'delivery of health care and the exchange of health care information across distance' [1]. Telecommunication technology includes the assisted transmission of signals and biological data over a distance. In this context, telemedicine like telesurgery and also teleanesthesiology has become more interesting [2]. However, teleacupuncture has only been performed by our research group up to now [3]. The term 'teleacupuncture' was first mentioned and defined by our research group at the international symposium 'Modernization of Traditional Chinese Medicine' in May 2009 in Graz [4]. The first scientific descriptions by Litscher can be found in publications in a Korean [5], Austrian [6] and American [3] journals in the respective 2009 autumn issues.

The present paper summarizes the first important results obtained in China and Austria in the research area of teleacupuncture.

It was the aim of these biomedical pilot measurements to investigate whether teleacupuncture using computercontrolled HRV analysis can be performed over long distances (transcontinentally) via common internet connections.

2. METHODOLOGY

Teleacupuncture integrates mainly medical acupuncture and telecommunication. Telecommunication in this context means data transfer over a distance of 7,650 km via internet between Graz, Austria and Beijing, China (**Figure 1**).

In China, 24-hour electrocardiograms (ECGs) are registered and the data are transferred via internet to the Medical University of Graz immediately following the acupuncture treatment. In Graz, the analysis of the ECGs is performed. The acupuncturists in China are informed about the results of the analysis immediately (**Figure 2**).

The autonomic nervous system plays an important role in the current investigations. Computer-based heart rate and heart rate variability (HRV) measurements are the main parameters. The ECG was recorded with a sampling rate of 4096 Hz using a system partly developed in Austria (medilog AR12, Huntleigh Healthcare, Cardiff, UK; **Figure 3**).

Heart rate variability is measured as the percentage change in sequential chamber complexes (RR-intervals) in the ECG, which is controlled by the blood pressure control system, influenced by the hypothalamus and, in particular, controlled by the vagal cardiovascular centre in the lower brainstem. [7] In these pilot measurements, a new method of analysis, the so-called 'Fire of Life' diagram, was used. For the calculation of changes in spectral density the medilog Darwin HRV software (Huntleigh Healthcare, Cardiff, UK) including the method of Burg (autoregressive model) was applied.

3. RESULTS

Figure 4 shows exemplarily the first teleacupuncture



Figure 1. Teleacupuncture between the TCM Research Center Graz and the China Academy of Chinese Medical Sciences (CACMS) over a distance of 7,650 km.



Figure 2. Acupuncture and data recording is performed in China, the analysis at the Medical University of Graz (modified from [6]).



Figure 3. Bio-signal registration is performed in China using equipment from the TCM Research Center Graz (modified from [6]).

measurement in a 31-year-old female patient suffering from burn-out syndrome (Chinese diagnosis: kidney deficiency and blood stagnation).

The figure shows a 'Fire of Life'-analysis of the HRV and can be interpreted as an indicator of the state of health and the quality of sleep of the patient. Note the appearance of the three typical main spectral components (~ 0.3 Hz; ~ 0.11 Hz; < 0.05 Hz). The component at 0.3 Hz was significantly reduced. These different components represent biological rhythms that seem to be currently distinguishable among the following:

• Respiratory sinus arrhythmia (~ 0.3 Hz); centrally nervous respiratory impulses and interaction with pulmonary afferents;

• The so-called "10-second-rhythm" (~ 0.11 Hz); natural rhythm of cardiovascularly active neurons in the lower brainstem (circulatory center and its modulation by feedback with natural vasomotoric rhythms via baroreceptor feedback). Analogous blood pressure waves (blood pressure waves of third order) prove the connection;

• Longer wave HRV-rhythms (< 0.05 Hz); effects from the renin angiotensinsystem or temperature regulation as well as metabolic processes.

Figure 5 depicts the improvement of the state of health (sleep-wake-cycle) of the same 31-year-old patient from Beijing over a period of more than two months. At the beginning of the acupuncture treatment (comp. **Figure 4**) no distinct sleep-wake-cycle can be found. Already after four acupuncture sessions there is an obvious respiratory sinus arrhythmia during sleep (centre of **Figure 5B**; 0.2-0.3 Hz). After ten sessions this norm pattern has become stabilized (**Figure 5C**).

4. DISCUSSIONS

Heart rate and its variability are important parameters for the assessment of the autonomic nervous system and are indicators for 'neurocardial fitness' [7]. It has long been known that an extremely steady pulse represents a deadly risk. Using computer-based analysis in the time and frequency domain the influence of acupuncture treatments on HRV parameters was evaluated. HRV has its origin in the function of the vegetative nervous system. Therefore the state of health can be quantified [7].

Teleacupuncture may contribute to the omission of redundant research studies and a simplification of the diagnostic and therapeutic procedure, thereby not only cutting costs but also saving time [3-6].

The common research between Graz and Beijing shows one thing quite clearly: teleacupuncture bridges on the one hand Eastern and Western medicine and on the other hand science and practice. The next goal will be to translate the research results into arising practical possibilities available to all participants in different countries.

G. Litscher / HEALTH 2 (2010) 16-19



Figure 4. First teleacupuncture between Europe (Graz) and Asia (Beijing). Heart rate variability data of 24 hours are shown (modified from [5]).



Figure 5. Follow-up investigations during a total of ten acupuncture sessions in China. Note the appearance of an obvious sleep-wake-cycle already after the fourth acupuncture treatment (modified from [6]).

5. CONCLUSIONS

Apart from the research-related aspects, transcontinental teleacupuncture opens up new possibilities for interactive education and practical training in public health. This represents another important aspect in quality control and quality assurance in complementary medicine.

6. ACKNOWLEDGEMENTS

The investigations are part of the project "Bioengineering and clinical assessment of high-tech acupuncture: A Sino-Austrian research pilot study," supported by the Austrian Federal Ministries of Science and Research and of Health and the Eurasia Pacific Uninet. The author is grateful to Professor Weibo Zhang and Dr. Tao Huang, Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, Beijing, China; and Dr. Lu Wang and Mag. Ingrid Gaischek, both TCM Research Center Graz, Austria; and Jan Valentini (Graz and Beijing), for their valuable support of this project and for the excellent cooperation. The preliminary report is also part of the initiated 'OeNB Jubiläumsfonds' project 13463 and performed within the areas 'Sustainable Health Research' and 'Neuroscience' at the Medical University of Graz.

REFERENCES

[1] Wooton, R. and Craig, J. (eds.) (1999) Introduction to

telemedicine, Royal Society of Medicine Press, UK, 4.

- [2] Ihmsen, H., Naguib, K., Schneider, G., Schwilden, H., Schüttler, J. and Kochs, E. (2007) Teletherapeutic drug administration by long distance closed-loop control of propofol. *Br J Anaesth*, **98(2)**, 189-195.
- [3] Litscher, G. (2009) Teleacupuncture: A new approach for transcontinental long-distance cooperation between Graz, Austria and Beijing, China (7,650 km). *Medical Acupuncture*, **21(3)**, 223-224.
- [4] Litscher, G., Huang, T., Valentini, J., Zhang, W.B. and Wang, L. (2009) Transcontinental teleacupuncture by means of long distance (7,650 km) computer-based heart rate variability analysis: First results from pilot measurements. *Proceedings of the International Symposium* 'Modernization of Traditional Chinese Medicine', Graz, Austria, 39-40.
- [5] Litscher, G. (2009) Modernization of traditional acupuncture using multimodal computer-based high-tech methods: Recent results of blue laser and teleacupuncture from the Medical University of Graz. J Acupunct Meridian Stud, 2(3), 202-209.
- [6] Litscher, G. (2009) Transkontinentale Akupunkturforschung mittels Teleakupunktur. *promed komplementär*, 3, 8-11.
- [7] Litscher, G. (2007) Bioengineering assessment of acupuncture (part 7): Heart rate variability. *Crit Rev Biomed Eng*, **35**(**3-4**), 183-195.

Does conventional abdominal preoperative ultrasonography have a role in the detection of pancreatic insulinoma? A case report

L. Spadaro, O. Magliocco, G. Trovato, F. Purrello

Dpt di Medicina Interna e Medicina Specialistica, Università degli Studi di Catania, Ospedale Garibaldi, Italy; fpurrell@unict.it

Received 2 September 2009; revised 14 October 2009; accepted 16 October 2009.

ABSTRACT

Combinations of strategies as MRI, endoscopic sonography, selective arteriography, were the first choice for the detection of pancreatic insulinoma. In these proposal, therefore, abdominal was not in- cluded at all. Case presentation: a 78-vear-old no diabetic women was referred to us because fasting hypoglycemic symptoms. The clinical and laboratory findings suggested an insulinoma. Abdominal ultrasound showed a small solid mass in the head-istmus pancreatic tract. Conclusion: the solid mass was confirmed with a contrast-enhanced- computed tomography of the abdomen. A surgical enucleation of the tumor was achieved by laparoscopy and histological examination of the specimen established a diagnosis of insulinoma. This case reinforced the value of the conventional transabdomen ultrasound in addition to accuracy of anamneses and biochemical tests as the first step in the hospital clinical setting for management of pancreatic insulinoma, reserving as the second step more expensive and invasiveness techniques.

Keywords: Lunch Time Napping; Mental Work Efficiency

Insulinomas are rare endocrine tumors of the pancreas. Their incidence was estimated to be four cases per 1 million person-years [1]. In the earliest stages of the tumor's clinical history, they usually manifest because of hypersecretion of insulin, responsible for the typical hypoglycemic symptoms. The three criteria (Whipple's triad or Whipple's criteria) on which hypoglycemia due to pancreatic islet-cell disease (most commonly insulinoma) is diagnosed are: 1) symptoms known or likely to be caused by hypoglycemia, 2) low blood glucose levels associated with clinical signs, and 3) reversal of clinical signs with the administration of glucose. The optimal therapy is curative surgical removal of the tumor. Therefore, preoperative localization of the lesion is crucial for determining the proper surgical treatment. However, in several cases, insulinoma identification remains extremely difficult and there was a continuing debate on the optimal strategy for his localization. Some authors have proposed MR as the first choice for preoperative imaging [2,3], whereas recent reports suggested a combined imaging protocol that consists of both dual-phase thin-section multi-detector CT and endoscopic sonography [4,5]. Actually, conventional abdominal ultrasonography was not included in the imaging protocol due to his limited successful reports. We wish to present our own experience with abdominal conventional ultrasound in a case of insulinoma.

A non-diabetic 78-year-old woman was brought to our attention because of hypoglycaemic symptoms. The patient had a history of mild and transient sweating, anxiety and trembling recurring for about 2 years during her activities of ordinary life. These symptoms usually disappeared after food ingestion. Ten days before her referral to our hospital, she had a more severe episode with sweating, trembling, anxiety, palpitations, nausea, dizziness with tiredness and a severe headache crisis. She had been seen by her family physician. The practitioner had ordered general blood tests that had revealed low serum glucose level (2.2 mmol/L, 40 mg/dL). Therefore, he suggested the admission to the hospital, to determine the cause of hypoglycaemia.

She had a history of hypertension and dyslipidemia on pharmacological treatment. She didn't have any treatment or drugs that might have induced hypoglycaemia. On admission to our hospital, on examination, the patient was overweight; her height was 165 cm and body weight 94,5 Kg, BMI 34,74, CV 118 cm. Blood pressure was 140/80 mmHg and the pulse was 68. Neurological examination and an electrocardiogram were normal. Laboratory tests were performed: urinalysis, blood cell counts, and biochemical tests (e.g. the liver and kidney function tests) other than plasma glucose concentration were unremarkable.

On the second hospital day, we performed the supervised 72-Hour Fast. At the 12th hour, the patient showed pallor, sweating, trembling, tiredness, nervousness and confusion. The plasma glucose level was 42 mg/dl. Therefore, we stopped the test through correction of the low glucose level by food ingestion with rapid relieving of the symptoms ("Whipple's triad") (**Table 1**). Plasma insulin levels during the test were also shown in Table 1. These data supported the diagnostic suspect of insulinoma.

As a first attempt to locate it, an abdominal ultrasonography of the pancreas was performed with a 3.5 MHz probe mechanical real time sector scanner (Logiq 500). Patient was examined in a semierect position after fasting for 12 hours. Scans of the head, body, and tail of the pancreas were obtained in both transverse and longitudinal planes without respiratory maneuvers, by using the fluid-filled stomach as an acoustic window. The examination showed a 12,4 mm x 13,7 mm hypoechoic solid mass, with the echo texture finer in relation to surrounding pancreatic tissue, localized in the head-isthmus pancreatic tract, without dilatation of the Wirsung's duct. (Figure 1, left). A contrast-enhanced computed tomography of the abdomen was then performed. The patient was scanned in suspended respiration, following the oral administration of contrast material to visualize the small bowel. Pre-contrast abdominal scans CT, revealed the presence of a protrusion of the pancreatic contour in the head-isthmus pancreatic tract, but itself is non-specific. After the bolus injection of contrast material and rapid sequential scanning, a transient increase in contrast enhancement (tumor blush) in the surrounding pancreatic tissue was observed, confirming an area of increased density of 1,5 cm (Figure 1, right). One month later, a surgical enucleation of the tumor was achieved by

Table 1. 72-Hour fast (date onset of the fast was the last ingestion of calories, 12 hours before the first measurement; all of the non essential medications were stopped; no assumption of caffeine or tobaccos).

Time	Symptoms or signs	Glucose	Insulin	c-peptide
01:00	none	48	15	1,79
p.m.		mg/dl	μU/mL	nmol/L
03:00	none	59	11	1,29
p.m.		mg/dl	μU/mL	nmol/L
05:00	none	64	10	1,40
p.m.		mg/dl	µU/mL	nmol/L
11:00	none	62	4	0,89
p.m.		mg/dl	µU/mL	nmol/L
05:00	none	69	10	1,35
a.m		mg/dl	µU/mL	nmol/L
11:00	none	53	12	1,13
a.m.		mg/dl	µU/mL	nmol/L
12:00	Whipple's triad:	42	7	1,04
a.m.	end of the fast	mg/dl	µU/mL	nmol/L

laparoscopy, which was performed initially to confirm by direct view the presence of the small nodule on the surface of the pancreatic; its enucleation was then carried out. Histological examination of the specimen established a diagnosis of an endocrine tumour of the pancreas compatible with an insulinoma, confirmed by the presence of insulin secretory granules. The patient's clinical symptoms completely disappeared.

Endocrine tumors of the pancreas originate from multipotential stem cells that have retained the capacity to proliferate and differentiate themselves in the various cellular lines that make up this group of neoplasms. Insulinomas represent the most frequently found functioning endocrine tumors of the pancreas and, in most cases, are benign (85%-99%), single (93%-98%) and less than



Figure 1. Imaging studies of the pancreatic lesion. Ultrasound (left) and CT (right) technique show the characteristic mass of the pancreas (arrows).

2.5 cm in diameter [6,7]. Age of onset is usually 40-60 years, ranging from 6 weeks to 70 years. No sex difference has been reported [8]. In our case, the patient was a 78-year-old female.

Insulinoma become clinically evident because of signs and symptoms linked to hypoglycemia due to the hypersecretion of insulin. The symptoms of hypoglycemia arise from the autonomic nervous system and from an insufficient supply of glucose to the brain (neuroglycopenia). During acute insulin-induced hypoglycemia in healthy persons, symptoms have been recognized at plasma glucose levels of approximately 60 mg per deciliter as measured in arterialized venous blood, and impairment of brain function has occurred at approximately 50 mg per deciliter [9-10-11]. In our patient, the first report of low plasma glucose levels (40 mg/dL) was associated with typical hypoglycemic symptoms, sweating, trembling, anxiety, palpitations, nausea, dizziness with tiredness and headache crisis. Moreover, her past history was positive for sporadic and vague symptoms, recurring for about 2 years, but no plasma glucose levels was measured before. It has been reported that the time interval from the onset of symptoms to diagnosis ranges from 10 days to 30 years (median 2 years), and that the hypoglycemic episodes may happen at irregular intervals with a varying duration [8].

The symptoms intermittency and the multifaceted characters delay the diagnosis, although all signs and symptoms are usually reverted rapidly by oral or parenteral glucose administration. Obesity or weight gain is present in 25% of patients, justified, at least in part, by the need for frequent feeding. Accordingly, our patient was overweight, and she reported a weight gain (over 20 kg) in the past two years. Because of the lack of specificity of the symptoms, it is necessary to measure a low glucose level at the time that the spontaneous symptoms occur and that the symptoms are relieved through correction of the low glucose level ("Whipple's triad") before concluding that the patient have a hypoglycemic disorder. The supervised 72-hour fast is the classic diagnostic test for hypoglycemia. In our case, the supervised 72-hour fast confirmed a hypoglycemic disorder. In addition, the appearance of the Whipple's triad associated with plasma glucose level ≤ 45 mg/dl, insulin level \geq 6 µ/mL and c-peptide level \geq 0.2 mmol/L suggested the diagnosis of insulinoma [12].

When the diagnosis of pancreatic insulinoma has been established clinically and biochemically, the precise localization of the tumor is crucial, to facilitate the surgical resection and to prevent a blind partial or subtotal pancreatectomy [13]. Insulinomas are usually small and difficult to detect. The main problem in detecting a pancreatic insulinoma lies in the organ's anatomic structure and the tumor's small dimensions at clinical appearance. Most of the tumors are in the body-tail area of the pancreas and are less than 2 cm in diameter [14]. Many procedures have been proposed for their localization. These imaging techniques vary in accuracy, invasiveness, operator dependence, and cost. Recent protocols propose the combination of advanced imaging techniques as well as MRI, endoscopic sonography, and CT as the first choice for preoperative detection of insulinomas [2,4]. In these proposals, therefore, abdominal ultrasonography is not included at all. However, ultrasonography is a noninvasive and relatively inexpensive technique, and actually it is easily available everywhere. In the case report we discuss here, we performed abdominal ultrasonography as the imaging technique of first choice because it was immediately available in our unit. In our patient, this technique correctly localized a pancreatic solid mass as a well-defined, hypoechoic lesion, without calcifications or necrosis. This finding was then confirmed by CT examination.

Early studies of preoperative ultrasound localization primarily used static scanners, yielding disappointing results. More recently, a higher sensitivity (approximately 60-80%) has been reported using real-time US equipment [7,15,16]. This level of accuracy was highly dependent on meticulous attention to scanning technique [6]. Ultrasonographic detection of insulinomas was difficult because of the frequent location at the body-tail area of the pancreas and the small sizes of the tumours in generally obese patients. In our patient insulinoma was small. < 1.5 cm, and was localized in head-isthmus pancreatis tract. It is important to emphasize the improvement in ultrasonographic technology in terms of spatial resolution with increased diagnostic sensitivity and accuracy. In addition, ultrasonography technique enabled us to view continuous images of the lesion from several angles, with relative ease compared to other imaging technique.

In the current case, after correct diagnosis and localization of pancreatic insulinoma, surgical resection was performed successfully and without complication. Actually, after one year, the patient was free from symptoms.

In conclusion, the present case supported the value of the conventional abdomen ultrasonography as the first step in the preoperative localization of pancreatic insulinomas, reserving as the second step more expensive and invasiveness techniques.

REFERENCES

- Service, F.J., McMahon, M.M., O'Brien, P.C., *et al.* (1991) Functioning insulinoma: incidence, recurrence, and long term survival of patients: a 60-years study. *Mayo Clin Proc*, 66, 711-719.
- [2] Virally, M.L., Guillausseau, P.J. (1999) Hypoglycemia in adults. *Diabetes e Metabolism*, 25, 477-490.

- [3] Catalano, C., Pavone, P., Laghi, A., *et al.* (1999) Localization of pancreatic insulinomas with MR imaging at 0.5 T. *Acta Radiol*, **39**, 644-648.
- [4] Moore, N.R., Rogers, C.E., Britton, B.J. (1995) Magnetic resonance imaging of endocrine tumors of the pancreas. *Br J Radiol*, 68, 341-347.
- [5] Gouya, H., Vignaux, O., Augui, J., Dousset, B., Palazzo, L., Louvel, A., Chaussade, S., Legmann, P. (2003) CT, endoscopic sonography, and a combined protocol for preoperative evaluation of pancreatic insulinomas. *AJR*, **181**, 987-992.
- [6] Schumacher, B., Lubke, H.J., Frieling, T., Strohmeyer, G., Starke, A.A. (1996) Prospective study on the detection of insulinomas by endoscopic ultrasonography. *Endoscopy*, 28, 273-276.
- [7] Liu, T.H., Tseng, H.C., Zhu, Y., Zhong, S.X., Chen, J., Cui, Q.C. (1985) Insulinoma: an immunohistochemical and morphologic analysis of 95 cases. *Cancer*, 56, 1420-1429.
- [8] Stefanini, P., Carboni, M., Patrassi, N., Basoli, A. (1974) Beta islet-cell tumors of the pancreas: result of a study on 1067 cases. *Surgery*, **75**, 597-609.
- [9] Villary, M.L., Guillausseau, P.J. (1999) Review: Hypoglycemia in adults. *Diabetes & Metabolism*, 25, 477-490.
- [10] Schwartz, N.S., Clutter, W.E., Shah, S.D., Cryer, P.E. (1987) Glycemic thresholds for activation of glucose counterregulatory systems are highter than the threshold for symptoms. *J Clin Invest*, **79**, 777-781.
- [11] Mitrakou, A., Ryan, C., Veneman, T., et al. (1991) Hier-

rarchy of glycemic thresholds counterregulatory hormone secretion, symptoms, and cerebral dysfunction. *Am J Physiol*, **260**, E67-E74.

- [12] Cyper, P.E. (1993) Glucose counterregulation: the physiological mechanisms that prevent or correct hypoglycaemia. In: Frier, B.M., Fisher, B.M., (eds.), *Hypoglycaemia and Diabetes: Clinical and Physiological Aspects*. Edward Arnold, London, 34-55.
- [13] Service, F.J. (1995) Review: Hypoglycemic disorders.. NEJM, 332, 1144-1152.
- [14] Placzkowski, K.A., Vella, A., Thompson, G.B., Grant, C.S., Reading, C.C., Charboneau, J.W., Andrews, J.C., Lloyd, R.V., Service, F.J. (2009) Secular trends in the presentation an management of functioning insulinoma at the majp clinic, 1987-2007. J Clin Endocrinol Metab.
- [15] D'Onofrio, M., Mansueto, G., Vasori, S., Falconi, M., Procacci, C. (2003) Contrast-enhanced ultrasonographic detection of small pancreatic insulinoma. *J Ultrasound Med*, 22, 413- 417.
- [16] Angeli, E., vanzulli, A., Castrucci, M., Venturini, M., Sironi, S., Zerbi, A., Di Carlo, V., Del Maschio, A. (1997) Value of abdominal sonography and MR imaging at 0.5 T in preoperative detection of pancreatic insulinoma: a comparison with dynamic CT and angiography. *Abdom Imaging*, 22(3), 295-303.
- [17] Galiber, A.K., Reading, C.C., Charboneau, J.W., Sheedy, P.F., James, E.M., Gorman, B., Grant, C.S., van Heerden, J.A., Telander, R.L. (1998) Localization of pancreatic insulinoma: comparison of pre-and intraoperative US with CT and angiography. *Radiology*, **166(2)**, 405-8.

Applicability of the P19CL6 cells as a model of cardiomyocytes – a transcriptome analysis

Iraj Khodadadi^{1, 2}, Nick J. Plant², Vassilis Mersinias³, Alfred E. Thumser^{2*}

¹Department of Biochemistry and Nutrition, Hamedan University of Medical Sciences, Hamedan, Iran ²Division of Biochemical Sciences, University of Surrey, Guildford, United Kingdom

³B.S.R.C. "Alexander Fleming", Varkiza, Greece; <u>a.thumser@surrey.ac.uk</u>

Received 15 November 2009; revised 4 December 2009; accepted 7 December 2009.

ABSTRACT

The P19CL6 cell-line, a clone of the P19 mouse embryonal carcinoma cell-line, has been extensively used as a model for cardiomyocytes as these cells can be differentiated into a cardiomyocyte phenotype upon incubation with dimethyl sulfoxide. Uniquely, these cells can be observed to "beat" when monitored by microscopy. We started investigating the response of P19CL6 cells to fatty acids, but highly variable results lead us to investigate the phenotype of the P19CL6 cells in more depth. In this study we demonstrated that the P19CL6 cells are responsive to adrenaline, but loose the "beating" phenotype after 16 passages in culture. Analysis of specific mRNA transcripts indicated that the P19CL6 cells express both cardiac- and skeletal muscle-specific genes, while global analysis of microarray data showed clear differences between the P19CL6 cells and cardiac tissue of embryonic or adult origin. In conclusion, our observations suggest caution in the use of the P19CL6 cells as a model of cardiomyocytes unless rigorous validation for the intended analysis has been undertaken.

Keywords: Gene expression; Cardiomyocyte; P19CL6 Cell-line

1. INTRODUCTION

There is a requirement for the development of realistic cell culture models both for basic research and the development of novel therapeutic agents. However, for several tissues, including heart, no individual cell line has been successfully validated for these purposes; such a failure is usually the result of the loss of one or more specific phenotypic features associated with the target tissue in the cell line. One approach to mitigate these issues has been the utilisation of chemically-stimulated differentiation of stem cells, with the hope that these cell lines will have a more realistic phenotype than cell lines derived from fully differentiated tissue. Pluripotent embryonic carcinoma cells have been reported as successful in vitro models of cardiac differentiation; for example, the P19 mouse embryonal carcinoma cell-line has been reported to differentiate into an embryonic cardiac-muscle phenotype in vitro [1] upon the addition of dimethyl sulfoxide (DMSO) [2-5]. Differentiated P19 cells have been reported to retain the ability to spontaneously contract and shown to express transcripts in a temporal manner during culture, suggestive of a cardiac-muscle phenotype [5-7], and as such these cells have therefore been extensively used to study cardiac cell physiology [1,2,5,6,8], although with the caveat that these cells are embryonic instead. However, in addition to these cardiacmuscle-specific properties, P19 cells also display pluripotent properties and can be differentiated into cells displaying either a skeletal muscle or neural phenotype [1, 3-5]. There has thus been some concern about the homogeneity of DMSO-differentiated P19 cultures, with a heterogeneous cell population following differentiation significantly reducing the utility of these cells as a cardiac-muscle-specific model: There has thus been much interest in identifying subclones of P19 cells that more robustly differentiate into cardiomyocytes. The P19CL6 cell-line, a sub-clone of P19 embryonal cells, has been reported to efficiently differentiate into beating cardiomyocytes upon exposure to DMSO under adherent culture conditions [9] and has been widely used as an in vitro model of cardiovascular cells [1,2,10-16].

It is clear that the P19CL6 cell line has potential as a model system for the study of cardiomyocyte development and differentiation, and indeed they are currently used as such. However, full characterisation and validation is required before they can be used for this purpose with full confidence. A review of the literature, focusing on P19CL6 and P19 cell culture conditions, shows that two separate methods are commonly used, namely adherent and non-adherent culture conditions [1,5,9,10,17]. In addition, vitamins and hormones such as adrenaline have been shown to act as potent inducers of P19 cell differentiation into cardiomyocytes in addition to the aforementioned DMSO [1,5,7,9]. In this study we have characterized the P19CL6 cells in more detail under different culture conditions, focusing in particular on utilising microarray methodologies to compare the P19CL6 transcriptome against native cardiac-muscle and skeletal-muscle transcriptomes. These investigations represent the first robust examination of both P19CL6 transcriptome and cardiac phenotype, and demonstrate that the P19CL6 cell-line displays only a limited cardiomyocyte phenotype that is dependent on passage conditions. As such we would advise caution in the use of this cell line as a 'complete' in vitro model of cardiac-muscle cells.

2. MATERIALS AND METHODS

2.1. Materials

Cell culture media and reagents were obtained from Invitrogen Corporation (Paisley, U.K.) and Sigma-Aldrich Company Ltd. (Poole, U.K.). Materials and kits for RNA extraction, cDNA synthesis and RT-PCR were supplied by Promega Corporation (Southampton, U.K.), Amersham Biosciences (Chalfont St. Giles, U.K.) and Qiagen Ltd. (Crawley, U.K.). Corning Life Sciences (Schiphol-Rijk, Netherlands) supplied the ProntoPlus microarray kit. Ambion Ltd. (Huntingdon, U.K.) supplied mouse heart and embryonic total-RNA, whereas mouse skeletal muscle and embryonic heart total-RNA were purchased from Panomics Inc. (Redwood City, U.S.A.) and Zyagen (San Diego, U.S.A.), respectively.

2.2. Cell culture

P19CL6 cells were purchased at passage 9 from Riken Cell Bank (Ibaraki, Japan) in growing flask and cultured in medium containing α -MEM (minimal essential media) supplemented with 10% FBS (foetal bovine serum) and 1% penicillin-streptomycin (10,000 Units/ml and 10 mg/ml, respectively) [9]. To differentiate P19CL6 cells into cardiomyocytes, cells were plated in 6-well culture plates (10 cm^2) at a density of 2×10^4 cells/cm² in standard medium containing 1% DMSO [9]. Cells were cultured for 15 days at 37°C and 5% CO₂ with medium refreshed every second day. For culturing P19CL6 cells under non-adherent conditions, cells were stimulated to form aggregates by incubation in bacterial petri dishes $(1 \times 10^{\circ})$ cells/dish; 78 cm²), containing a thin layer of 0.5% agar, for 4 days with standard media containing 1% DMSO, before transfer to regular cell culture flasks for the remainder on the incubation period [5]. Cell aggregates were collected by centrifugation and replated into culture flasks for 15 days at 37°C and 5% CO₂ in the presence of 1% DMSO. The H9C2 (2-1) cell-line, a murine cell-line

that expresses a skeletal muscle phenotype, was obtained from the European Collection of Cell Cultures (ECACC; Salisbury, U.K.) and cultured under the same adherent conditions as the P19CL6 cells.

2.3. Determination of mRNA Levels by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and cDNA Microarrays

Total RNA was isolated from cells with Trizol reagent, as per manufacturer's instructions (Invitrogen Corporation, Paisley, U.K.). Whole heart and skeletal muscle tissues (upper leg muscle) were dissected from 10 week old male CD1 wild-type mice (+/+), homogenised in Trizol reagent using an Ultra-Turrax T8 homogeniser, and total RNA isolated as per manufacturer's instructions. Gene-specific forward and reverse primers used in the one-step RT-PCR or nested PCR reactions are shown in **Table 1**. In samples that did not show detectable cDNA levels after an initial RT-PCR amplification the PCR products were reamplified by nested PCR [18]. PCR products were separated by electrophoresis on 2% agarose gels containing ethidium bromide, and the identity of PCR products verified by sequencing.

Microarray experiments were designed as dual-hybridisation optimal interwoven loops (http://exgen.ma.umist.ac.uk) [19-21]. cDNA was synthesised from purified total- RNA and labelled by a direct labelling method in the presence of Oligo dT, nucleotide mixture, Cy3-/Cy5- dCTP dyes, and SuperScript-II Reverse Transcriptase, based on Human Genome Mapping Project protocols (http://www.hgmp.mrc.ac.uk/). Purified Cy3- and Cy5- labelled cDNA samples (40 pmol each) were mixed in pairs (total volume 40 µl), according to the experimental plan, and hybridised to microarrays by incubation in hybridization chambers for 20 hours at 50°C, before post-hybridization washing and drying. Microarray images were acquired using an Affymetrix 428 scanner (Affymetrix, Santa Clara, USA) and analysed with BlueFuse (BlueGnome Ltd., Cambridge, U.K.), including quantification of pixel intensities of the spots and excluding background intensity and artefact areas on the arrays. The data was filtered by eliminating low quality array spots, as determined by BlueFuse's spot uniformity and circularity measurements (spot uniformity and circularity >0.5 in at least 50% of the arrays), and normalised by intensity-dependent per spot and per chip (LOWESS) normalisation with GeneSpring-7 data analysis software (Agilent Technologies UK Ltd, Stockport, UK).

After Lowess normalisation and filtering of microarray data linear modelling (LLAMA; Live Linear Analysis of MicroArray) was performed (<u>http://exgen.ma.umist.ac.uk</u>) to convert data from the loop experiment into a linear model and generate differential gene expression estimates



Figure 1. Determination of the effect of (a) passage number and (b) adrenaline on the beating characteristic in P19CL6 cells cultured under adherent conditions. (a) P19CL6 cells (passage 12) were cultured with 0-20 µM adrenaline and pulse rate was recorded on day 15 of incubation. Linear regression analysis showed a correlation of pulse rate with adrenaline concentration (y = 1.1x + 21.8, $\overline{R}^2 = 0.541$, P < 0.001) and one-way ANOVA confirmed a significant effect of adrenaline on pulse rate in P19CL6 cells (P<0.001). Cells were cultured in three biological repeats and beating was recorded in at least six localized areas in the well. a, b, and c represent groups with a significant difference in average pulse rate. (b) P19CL6 cells from different passage numbers (12-16) were cultured in the presence of DMSO and pulse rate (beats/min) was recorded on day 15 of incubation. Linear regression analysis shows a significant effect of passage number on pulse rate (y = -2.58x + 63.3, R² = 0.486, P < 0.001) and two-way ANOVA confirmed significance difference between the different passage numbers of P19CL6 cells (P < 0.01).

between contrast pairs, i.e. Cy3/Cy5 ratios [20,21,33]. The

P19CL6 samples consisted of three biological repeats, with each sample analyzed on at least three microarray slides (technical repeats), and these were reduced to a single sample during the LLAMA analysis. The data reduction generated by the LLAMA analysis was further evaluated by PCA (principal component analysis) and PLS (partial least squares regression) (Simca-P+, Umetrics, New Jersey, U.S.A.). The PCA analysis generated correlations between the different samples. In order to simplify the interpretation of the PLS analysis, the P19CL6 sample was compared to skeletal muscle, adult and embryonic heart samples, with the H9C2 (2-1) sample removed. The PLS analysis was used to produce a variable importance (VIP) list and all genes with a VIP > 1, *i.e.* having a significant impact on the difference between the P19CL6 and other samples, were further analysed with the DAVID software suite (http://david.abcc.ncifcrf.gov/home.jsp) for functional annotation clustering according to their biological functions [24].

3. RESULTS

In a series of separate cultures of P19CL6 cells it was noted that the cultures did not consistently display a "beating" phenotype, a characteristic that has previously been used as evidence of the cardiomyocyte properties of this P19 sub-clone [9].

3.1. P19CL6 Cells Exhibit a Cardiac-Muscle-Like 'Beating' Phenotype under Specified Culture Conditions

A characteristic of the P19CL6 cells is that they display beating in localised nodes following differentiation in the presence of DMSO [9]. The cardio-stimulatory chemical adrenaline elicited a dose-dependent linear increase in the observed pulse rate with statistically significant increases observed between 0-20 uM adrenaline (Figure 1a). However, observation of P19CL6 cells over time in culture (passages 12-22) demonstrated that beating was consistently only observed between passages 12-16, and quantification of this beating showed a significant inverse correlation of the pulse-rate with passage number (Figure 1b). Microscopic analysis of DMSO-differentiated P19-CL6 cells demonstrated mono-nuclear cells with no evidence of cell fusion; these characteristics were distinct from the morphological characteristics of skeletal muscle cells, as typified by the H9C2 (2-1) murine cell-line (Figure 2), and added further weight to the assertion that differentiated P19CL6 cells exhibit a cardiac-specific muscle phenotype [5,10,34]. The microscopic analysis and response to adrenaline provided an indication of a cardiac-type phenotype, although the loss of beating with passage number suggested that the phenotype may not be robust with regards to culture duration.



Figure 2. Microscopic examination of P19CL6 and H9C2(2-1) cells. Cells were cultured on coverslips under adherent conditions in the presence of DMSO and collected on day 15 of incubation. Cells were fixed using paraformaldehyde and observed by light microscopy under $40 \times$ magnification. (a) P19CL6 cells; (b) H9C2(2-1) cells.

3.2. The P19CL6 Cell-Line Expresses both Cardiac- and Skeletal Muscle-Specific Transcripts

To further examine the reported cardiac properties of the P19CL6 cell-line, mRNA transcript levels of α-MHC, β-MHC, MyoD and myogenin were determined by RT-PCR followed by nested PCR. The expression of these gene products are characteristic of cardiac- (a-MHC, β -MHC) and skeletal muscle (MyoD, myogenin) tissues [35-38]. Figure 3a shows that P19CL6 cells expressed significant levels of α -MHC, β -MHC, MyoD and myogenin transcripts, inconsistent with a cardiac only phenotype: To examine if the unexpected expression of skeletal muscle markers in P19CL6 cells was caused by the culture conditions we also examined P19CL6 under non-adherent culturing conditions; under these conditions we once again observed both cardiac- and skeletal muscle-specific markers (Figure 3b), suggestive of a mixed cardiac/skeletal-muscle transcriptome in P19CL6 cells regardless of culture conditons. The identity of the α -MHC, β-MHC, MyoD and myogenin transcripts was confirmed by sequencing of the nested PCR products (data not shown). The marker transcript specificity was confirmed using both the H9C2 (2-1) cell-line, which expresses a skeletal muscle phenotype, and only expressed MyoD and myogenin transcripts and mouse cardiac tissue, which only expressed the aforementioned transcript markers of cardiac phenotype, α -MHC and β -MHC (Figure 3c). These data were thus consistent with P19CL6 cells exhibiting a mixed cardiac/skeletal muscle phenotype, and therefore a global transcriptome analysis was under-taken to examine this hypothesis, comparing P19CL6 cells with the H9C2 (2-1) cell-line and mouse cardiac and skeletal muscle tissue.

3.3. Characterisation of the P19CL6 Cell-Line by Microarray Analysis

Transcriptomes from three independent P19CL6 cultures were compared by microarray analysis to the transcript-



Figure 3 Expression of α-MHC, β-MHC, MyoD and myogenin, as detected by nested-PCR, in (a) P19CL6 and H9C2(2-1) cells, cultured under adherent conditions, (b) P19CL6 cells cultured under non-adherent conditions, upon exposure to 1% DMSO, and (c) mouse heart tissue. Cells were cultured under adherent or non-adherent conditions for 15 days in the presence of 1% DMSO, as described under Methods. Heart tissue was dissected from 10 weeks old CD1 wild type (+/+) male mice. Total RNA was isolated and mRNA levels determined by RT-PCR followed nested-PCR. Theoretical sizes of PCR products are 275 bp or 413 bp (GAPDH), 302 bp (α-MHC), 410 bp (β-MHC), 392 bp (MyoD) and 337 bp (myogenin), as indicated by the arrows. For Figure 3c the theoretical sizes of PCR products were: 556 bp (GAPDH); 491 bp (α-MHC); 587 bp (β-MHC); 513 bp (MyoD); and 608 bp (Myogenin), as indicated by arrows. Data shown of one experiment that is representative of three separate biological experiments.

tomes of mouse cardiac and skeletal muscle tissue, H9C2 (2-1) cells, and a reference sample, which was a mixture of cDNA from all the samples in equal proportions. An interwoven loop design and data reduction by linear modelling [21-23] was utilized to compare all samples to the reference sample (Figure 4). The transcriptomes of all samples were initially analysed by PCA and showed a low correlation between the P19CL6 cells and other samples (Table 2, Figure 5). Only the embryonic heart sample did not differ significantly from the P19CL6 cells (P>0.05), although even this comparison showed a very low correlation of 0.007 (Table 2). Subsequent PLS analysis, excluding the H9C2 (2-1) samples in order to simplify interpretation, showed that principal component 1 (PC1) accounted for 52% of the total variance and clearly separated the P19CL6 sample from the heart and muscle tissue (Figure 5). The loading factors for PC1 showing a variable importance (VIP) of >1.0 represent those transcripts driving the separation of P19CL6 cells from the other samples; these were put into a biological context by ascertaining Gene Ontology (GO) identifiers that were significantly over-represented in the identified transcript level changes, using the DAVID bioinformatics suite [24]. Such over-representation is often indicative of a significant biological effect in the pathway(s) associated with the GO identifiers. Several annotation clusters with an enrichment score of greater than 1.0, i.e. showing significant enrichment, were identified (502 separate genes) and the five main Biological Processes that were identified are shown in Table 3. A more detailed analysis of the genes identified within the "Regulation of cellular processes" cluster showed that 57% (total number 146 separate gene products) of the identified mRNA levels were up-regulated in P19CL6 cells compared to embryonic heart tissue, whereas the remainder were downregulated (data not shown). The latter tissue was the primary focus for comparison as P19-derived cardiomyocytes are embryonic in nature and have previously been used as a model system for the embryonic heart [1,6,8]. However, in the original P19CL6 paper this cell-line was proposed to be a good model system for adult heart [9].

4. DISCUSSION

The P19CL6 cell-line, a derivative of P19 embryonal carcinoma cells, is widely used as an *in vitro* model of cardiovascular cells [1,2,10,16], and has been shown to differentiate into a beating phenotype that is reminiscent of cardiomyocytes upon exposure to DMSO [9]. Data presented here confirm that differentiated P19CL6 cells do exhibit some markers of a cardiac phenotype in the P19CL6 cells: a beating phenotype that is positively responsive to adrenaline; expression of transcript markers of cardiac phenotype (α -MHC, β -MHC); microscopic



Figure 4. Representation of dual-hybridisation optimal interwoven loop design for microarray experiments. Loop designs showing three, four, and seven samples (nodes) that were compared in three different experiments. The samples analysed in each experiment were (a) P19CL6 cells, mouse embryonic heart and mouse adult heart tissue (b) P19CL6 cells, H9C2 (2-1) cells, mouse adult heart and mouse skeletal muscle tissue (c) P19CL6 cells (two different biological samples), H9C2 (2-1) cells, mouse skeletal muscle tissue, mouse adult heart tissue, mouse skeletal muscle tissue and a reference sample, the latter containing cDNA from all the samples. The samples at the start of the arrows were labelled with Cy3 and the target samples with Cy5.



Figure 5. Analysis of transcriptomic expression data (microarrays) by PLS analysis. Gene expression data from microarray analysis was reduced by linear regression, using the LLAMA algorithm [19-21]. The data was further analysed by PCA and subsequently PLS to demonstrate discrimination between the samples (\mathbb{R}^2X for principal components 1 and 2 is 0.52 and 0.33, respectively) and generate a list of variable importance that could be used in the DAVID analysis.

analysis showing mono-nuclear cells with no evidence of cell fusion [1,5,8,25]. However, closer examination of these features raises some concerns, and is suggestive of an unstable, mixed, cardiac/skeletal muscle phenotype. We demonstrated that the pulse-rate for beating was negatively correlated with the passage number of the cells, with no beating observed after passage 16, suggesting

Table 1. Sequences of oligonucleotide primers used for PCR.

Oligo name	Sequences	%GC	T _m °C	Application	Product size
GAPDH-F	ATT CAA CGG CAC AGT CAA GGC	52	59.8	RT-PCR	556 hn
GAPDH-R	CAC ATT GGG GGT AGG AAC AC	55	59.4		ee o op
GAPDH-F	AGC TIG ICA TCA ACG GGA AGC CTA AGC AGT	52	59.8	nested PCR	275 bp
GAPDH-R	TGG TGG TGC AG	55	59.4		
GAPDH-F	CAC GGC AAA TTC AGT CTT CTG	52	59.8	nested PCR	413 bp
GAPDH-R	GGT GGC AGT GAT ATG GAG CAG	52	59.8		
α-MHC-F	ACC ATC AAG GAC TTG TGT ATT	52	59.8	RT-PCR	491 bp
α-MHC-R	GGC CAC AGC GAG AAG AGT GAG	52	59.8		
α-MHC-F	CGG CGC ATC AA CTG CTG GAG	55	59.4	nested PCR	302 bp
α-MHC-R	AGG TTA TTC CTC CTT CAA CCA	52	59.8		
β-MHC-F	CCA CAT GTT CGT G TCA CCC CTG	50	60.3	RT-PCR	587 bp
β-MHC-R	GAG ACT TTG TCT GAG GGC ATT	52	59.8		
β-MHC-F	GAG TGG ACA TTC CTT TCT TTG	52	59.8	nested PCR	412 bp
β-MHC-R	CCT TGC CTT TGC C AGT GAA TGA	50	60.3		
MyoD-F	GGC CTT CGA GAC CTG GGT TCC	52	59.8	RT-PCR	513 bp
MyoD-R	CTG TTC TGT GT TAC CCA AGG	55	59.4		
MyoD-F	TGG AGA TCC TG GTG GTG CAT	55	59.4	nested PCR	392 bp
MyoD-R	CTG CCA AAA GC AGC TGT ATG	55	59.4		
MyoG-F	AGA CAT CCC CCT ACG ATG GAC	52	59.8	RT-PCR	608 bp
MyoG-R	GTA AGG GAG TG ACC AGG AGC	55	59.4		
MyoG-F	CCC ACT TCT AT GCG CAG GAT	55	59.4	nested PCR	337 bp
MyoG-R	CTC CAC TTT AG	55	59.4		

F and R indicate forward and reverse primers, respectively. T_m : annealing temperature (°C); bp: base pair.

Table 2. Correlation matrix of mRNA expression levels in P19CL6 and H9C2 (2-1) cells, and skeletal muscle, adult heart and embryonic heart tissue samples, as analyzed by microarray analysis.

Samples	H9C2 (2-1)	Skeletal muscle	Adult heart	Embryonic heart
P19CL6	-0.058*	-0.154**	-0.210**	0.007
H9C2 (2-1)		0.031	0.175**	-0.023
Skeletal muscle			0.299**	-0.024
Adult heart				0.373**

Microarray data was reduced by linear analysis using LLAMA and the data analyzed by PCA, with the data fitted to 5 components (explaining 100% of the cumulative variance). P<0.05; **P<0.001.

that the P19CL6 cells are subject to phenotypic drift with time in culture, and hence may not represent a stable cardiomyocyte phenotype. In addition, while P19CL6 cells expressed the aforementioned transcript markers of cardiac phenotype (a-MHC, \beta-MHC), they also expressed transcript markers for a skeletal muscle phenotype (MyoD and myogenin), again suggestive of a mixed cardiac/skeletal muscle phenotype. Whereas examination of single markers can give an indication of the potential phenotype for a cell-line they are not necessarily indicative of a fully functioning biological system. For example, liver cell-lines such as HepG2 have been validated for use in drug screening as hosts for both reporter genes and marker transcripts [26,27]; however, in-depth analysis demonstrates that this validation is simplified, with HepG2 cells unable to support some genome-based transcriptional activation and for the transcriptome to be markedly affected by culture conditions [28,29]. Therefore whereas low complexity measurements may be suitable for validation of cell-lines for use in individual assays, approaches such as transcriptome/proteome analysis are more appropriate to fully characterize celllines [30]; anchoring this data to known phenotypic markers then allows an accurate assessment of the appropriateness of any cell-line to the in vivo system they are supposed to be modelling [31]. Transcriptome analysis of P19CL6 cells demonstrated significant differences in the transcript profile between these cells and other samples, including importantly both embryonic and adult cardiac cells. Gene Ontology over-representation analysis suggests that these transcripts are linked to biological pathways associated with cellular metabolism, an interesting observation since it is generally accepted that cardiac muscle cells have specific metabolic processes that differentiate embryonic and adult cardiomyocytes, and also skeletal and cardiac muscle cells.

In conclusion, we have both undertaken physiological and transcriptome analysis of P19CL6 cells, assessing their suitability as models of cardiomyocytes for *in vitro* experimentation. Our data suggests that whereas the P19CL6 cell-line has some phenotypic similarities to cardiomyocytes (*e.g.* the ability to pulse) there exist significant differences between these cells and the *in vivo* situation. Our observations clearly demonstrate that the P19CL6 cell line does not maintain a robust cardiamyocyte phenotype as shown by the loss of cell beating with time in culture. In addition, transcriptome analysis clearly shows that even freshly differentiated cells do not exhibit a clear cardiac or muscle transcript profile, further questioning the utility of P19CL6 as a model system for the study of cardiomyocyte physiology.

5. ACKNOWLEDGMENTS

Technical advice and guidance on cDNA microarray work from Dr Giselda Bucca and Prof. Colin Smith, and Dr George Kass with microscopy, in the Faculty of Health and Medical Sciences, University of Surrey are gratefully acknowledged. Mr Ben Routley and Dr Mark Muldoon (The School of Mathematics, University of Manchester) provided valuable assistance in the use and interpretation of Live Linear Analysis of MicroArray (LLAMA) for analysis of microarray data. Mr Peter Kentish helped with the animal tissue studies. Financial support for this study was provided by the Iranian Ministry of Health and Medical Sciences and the Hamedan University of Medical Sciences.

REFERENCES

- [1] Van der Heyden, M.A. and Defize, L.H. (2003) Twenty one years of P19 cells: what an embryonal carcinoma cell line taught us about cardiomyocyte differentiation. *Cardiovascular Research*, **58**, 292-302.
- [2] Anisimov, S.V., Tarasov, K.V., Riordon, D., Wobus, A.M., and Boheler, K.R. (2002) SAGE identification of differentiation responsive genes in P19 embryonic cells induced to form cardiomyocytes in vitro. *Mechanisms of Development*, **117**, 25-74.
- [3] McBurney, M.W., Jones-Villeneuve, E.M., Edwards, M.K. and Anderson, P.J. (1982) Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature*, **299**, 165-167
- McBurney, M.W. (1993) P19 embryonal carcinoma cells. *International Journal of Developmental Biology*, 37, 135-140.
- [5] Skerjanc, I.S. (1999) Cardiac and skeletal muscle development in P19 embryonal carcinoma cells. *Trends Cardiovascular Medicine*, 9, 139-143.
- [6] Skerjanc, I.S., Petropoulos, H., Ridgeway, A.G. and Wilton, S. (1998) Myocyte enhancer factor 2C and Nkx2-5 up-regulate each other's expression and initiate cardiomyogenesis in P19 cells. *Journal of Biological Chemistry*, 273, 34904-34910.
- [7] Wobus, A.M., Kleppisch, T., Maltsev, V. and Hescheler, J. (1994) Cardiomyocyte like cells differentiated in vitro from embryonic carcinoma cells P19 are characterized by functional expression of adrenoceptors and Ca²⁺ channels. *In Vitro Cellular Development and Biology*, **30A**, 425-434.
- [8] Rudnicki, M.A., Jackowski, G., Saggin, L. and McBurney, M.W. (1990) Actin and myosin expression during development of cardiac muscle from cultured embryonal car-

cinoma cells. Developmental Biology, 138, 348-358.

- [9] Habara-Ohkubo, A. (1996) Differentiation of beating cardiac muscle cells from a derivative of P19 embryonal carcinoma cells. *Cell Structure and Function*, 21, 101-110.
- [10] Van der Heyden, M.A., van Kempen, M.J., Tsuji, Y., Rook, M.B., Jongsma, H.J. and Opthof, T. (2003) P19 embryonal carcinoma cells: a suitable model system for cardiac electrophysiological differentiation at the molecular and functional level. *Cardiovascular Research*, 58, 410-422.
- [11] Eaton, S., Chatziandreou, I., Krywawych, S., Pen, S., Clayton, P.T. and Hussain, K. (2003) Short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency associated with hyperinsulinism: a novel glucose-fatty acid cycle? *Biochemical Society Transactions*, **31**, 1137-1139.
- [12] Monzen, K., Hiroi, Y., Kudoh, S., Akazawa, H., Oka, T., Takimoto, E., Hayashi, D., Hosoda, T., Kawabata, M., Miyazono, K., Ishii, S., Yazaki, Y., Nagai, R. and Komuro, I. (2001) Smads, TAK1, and their common target ATF-2 play a critical role in cardiomyocyte differentiation. *Journal of Cell Biology*, **153**, 687-698.
- [13] Paquin, J., Danalache, B.A., Jankowski, M., McCann, S.M. and Gutkowska, J. (2002) Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes. *Proceedings of the National Academy of Sciences USA*, 99, 9550-9555.
- [14] Peng, C.F., Wei, Y., Levsky, J.M., McDonald, T.V., Childs, G. and Kitsis, R.N. (2002) Microarray analysis of global changes in gene expression during cardiac myocyte differentiation. *Physioogical Genomics*, 9, 145-155.
- [15] Ridgeway, A.G., Wilton, S. and Skerjanc, I.S. (2000). Myocyte enhancer factor 2C and myogenin up-regulate each other's expression and induce the development of skeletal muscle in P19 cells. *Journal of Biological Chemistry*, 275, 41-46.
- [16] Young, D.A., Gavrilov, S., Pennington, C.J., Nuttall, R.K., Edwards, D.R., Kitsis, R.N. and Clark, I.M. (2004) Expression of metalloproteinases and inhibitors in the differentiation of P19CL6 cells into cardiac myocytes. *Biochemical and Biophysical Research Communications*, **322**, 759-765.
- [17] Morley, P. and Whitfield, J.F. (1993) The differentiation inducer, dimethyl sulfoxide, transiently increases the intracellular calcium ion concentration in various cell types. *Journal of Cellular Physiology*, **156**, 219-225.
- [18] Newton, C.A. and Graham, A. (1997) PCR. BIOS Scientific Publishers Ltd, Oxford.
- [19] Goldsmith, Z.G. and Dhanasekaran, N. (2004) The microrevolution: applications and impacts of microarray technology on molecular biology and medicine. *International Journal of Molecular Medicine*, **13**, 483-495.
- [20] Vinciotti, V., Khanin, R., D'Alimonte, D., Liu, X., Cattini, N., Hotchkiss, G., Bucca, G., de Jesus, O., Rasaiyaah, J., Smith, C.P., Kellam, P. and Wit, E. (2005) An experimental evaluation of a loop versus a reference design for two-channel microarrays. *Bioinformatics*, 21, 492-501.
- [21] Wit, E. and McClure, J. (2004) Statistics for microarrays: design, analysis, and inference. *John Wiley & Sons Ltd.*. Chichester, United Kingdom.
- [22] Dobbin, K. and Simon, R. (2002) Comparison of microarray designs for class comparison and class discovery. *Bioinformatics*, 18, 1438-1445.

- [23] Simon, R., Radmacher, M.D. and Dobbin, K. (2002) Design of studies using DNA microarrays. *Genetic Epidemiology*, 23, 21-36.
- [24] Dennis, G., Sherman, B.T., Hosack, D.A., Yang, J., Gao, W., Lane, H.C. and Lempicki, R.A. (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biology*, **4**, 3.
- [25] Rudnicki, M.A., Sawtell, N.M., Reuhl, K.R., Berg, R., Craig, J.C., Jardine, K., Lessard, J.L. and McBurney, M.W. (1990) Smooth muscle actin expression during P19 embryonal carcinoma differentiation in cell culture. *Journal* of Cellular Physiology, **142**, 89-98.
- [26] El-Sankary, W., Gibson, G.G., Ayrton, A. and Plant, N.J. (2001) Use of a reporter gene assay to predict and rank the potency and efficacy of CYP3A4 inducers. *Drug Metabolism & Disposition*, **29**, 1-6.
- [27] Morgan, K.T., Ni, H., Brown, H.R., Yoon, L., Qualls, C.W., Crosby, L.M., Reynolds, R., Gaskill, B., Anderson, S.P., Kepler, T.B., Brainard, T., Liv, N., Easton, M., Merrill, C., Creech, D., Sprenger, D., Conner, G., Johnson, P.R., Fox, T., Sartor, M., Richard, E., Kuruvilla, S., Casey, W. and Benavides, G. (2002) Application of cDNA mi croarray technology to in vitro toxicology and the selection of genes for a real-time RT-PCR based screen for oxidative stress in Hep G2 cells. *Toxicologic Pathology*, **30**, 435-451.
- [28] Morgan, K.T., Casey, W., Easton, M., Creech, D., Ni, H., Yoon, L., Anderson, S., Qualls, C.W., Crosby, L.M., MacPherson, A., Bloomfield, P. and Elston, T.C. (2003) Frequent sampling reveals dynamic responses by the transcriptome to routine media replacement in HepG2 cells. *Toxicologic Pathology*, **31**, 448-461.
- [29] Phillips, A., Hood, S.R., Gibson, G.G. and Plant, N.J. (2005) Impact of transcription factor profile and chromatin conformation on human hepatocyte CYP3A gene ex-

ABBREVIATIONS

ANOVA: Analysis of variance;

DMSO: Dimethyl sulphoxide;

RT-PCR: Reverse transcriptase polymerase chain reaction;

pression. Drug Metabolism and Disposition, 33, 233-242.
[30] Plant, N. (2004) Strategies for using in vitro screens in drug metabolism. Drug Discovery Today, 9, 328-336.

- [31] uo, W., Fan, W., Xie, H., Jing, L., Ricicki, E., Vouros, P., Zhao, L.P. and Zarbl, H. (2005) Phenotypic anchoring of global gene expression profiles induced by N-hydroxy-4 acetylaminobiphenyl and benzo[a]pyrene diol epoxide reveals correlations between expression profiles and mechanism of toxicity. *Chemical Research in Toxicology*, 18, 619-629.
- [32] [Laderas, T. and McWeeney, S. (2007) Consensus framework for exploring microarray data using multiple clustering methods. *OMICS*, **11**, 116-128
- [33] Smyth, G.K. (2005) Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray Experiments. *Statistical Applications in Genetics* and Molecular Biology, 3, 1-26.
- [34] Kimes, B.W. and Brandt, B.L. (1976) Properties of a clonal muscle cell line from rat heart. *Experimental Cell Research*, 98, 367-381.
- [35] Buckingham, M. (2001) Skeletal muscle formation in vertebrates. *Current Opinion in Genetics & Development*, 11, 440-448.
- [36] Gulick, J., Subramaniam, A., Neumann, J. and Robbins, J. (1991) Isolation and characterization of the mouse cardiac myosin heavy chain genes. *Journal of Biological Chemistry*, **266**, 9180-9185.
- [37] Metzger, J.M., Lin, W.I., Johnston, R.A., Westfall, M.V. and Samuelson, L.C. (1995) Myosin heavy chain expression in contracting myocytes isolated during embryonic stem cell cardiogenesis. *Circulation Research*, 76, 710-719.
- [38] Sabourin, L.A. and Rudnicki, M.A. (2000) The molecular regulation of myogenesis. *Clinical genetics*, **57**, 16-25.

PCA: Principle components analysis;

PLS: Partial least squares regression;

LLAMA: Live Linear Analysis of MicroArray.

Development of a high-throughput cell based 384-well influenza A quantification assay for interpandemic and highly pathogenic avian strains

Melicia R. Gainey, Ann M. Wasko, Jennifer N. Garver, David J. Guistino, Eric M. Vela*, John E. Bigger

Battelle Biomedical Research Center West Jefferson, OH 43162, USA; velae@battelle.org

Received 25 November 2009; revised 1 December 2009; accepted 2 December 2009.

ABSTRACT

Influenza remains a world wide health threat. thus the need for a high-throughput and robust assay to quantify both seasonal and avian influenza A strains. Therefore, a 384-well plate format was developed for the median tissue culture infectious dose assay (TCID₅₀) utilizing the detection of nucleoprotein by an in situ enzyme linked immunosorbent assay (ELISA) which was optimized for sensitivity in this assay. Highly pathogenic avian influenza, A/Vietnam/ 1203/04 (H5N1), and interpandemic strains, A/ New Caledonia/20/99 (H1N1) and A/Brisbane/ 10/07 (H3N2), were guantified using this highthroughput assay. Each 384-well plate can be used to analyze ten viral samples in quadruplicate, eight dilutions per sample, including all necessary assay controls. The results obtained from 384-well plates were comparable to traditional 96-well plates and also demonstrate repeatability, intermediate precision, and assay linearity. Further, the use of 384-well plates increased the throughput of sample analysis and the precision and accuracy of the resulting titer.

Keywords: Avian Influenza; ELISA;

High-Throughput Assay; Interpandemic Influenza A; $TCID_{50}$

1. INTRODUCTION

Interpandemic, or seasonal, influenza takes a toll on public health resources, economic productivity, and causes up to 500,000 deaths annually world-wide [1]. Highly pathogenic avian influenza (HPAI) strains cause heavy economic losses in countries dependent upon poultry revenues and have caused more than 250 deaths globally [2]. In addition, HPAI strains could potentially give rise to a pandemic. Given the economic and health implications of these viral strains, many new vaccines and therapeutics are being developed to counter this threat. In order to determine efficacy of these novel products, it is necessary to quantify antibody responses and influenza A viruses in various matrices, ideally in a high-throughput format. Currently, several high throughput assays to detect influenza virus exist which utilize reverse transcription polymerase chain reaction (RT-PCR) alone [3], and coupled with flow cytometery [4] or utilize a latex turbidimetric immunoassay [5]. There are also a few high-throughput screening methods to determine putative antiviral compounds [6] and influenza antagonists [7]. Although these methods are able to analyze many samples for the presence of influenza or screen potential therapeutics, these assays do not quantify influenza virus. For high-throughput quantification, a reverse-phased high performance liquid chromatography has been used to enumerate the amount of hemagglutinin in several types of viral stocks [8], but does not provide an infectious titer. A branched DNA technology can be used for quantification of influenza A viruses, but this assay was developed for use as an antiviral assay [9] and not for viral quantitation. Median tissue culture infectious dose assays (TCID₅₀ assay) utilizing Alamar blue in 96-well plates have also been described [10]. Analysis by this assay provides an infectious titer by indirect means; determining the metabolism of uninfected healthy cells instead of detecting the presence of viral infection. To date, 384-well plate assays have been described for the detection of influenza via RT-PCR [11] and for the screening of antivirals utilizing Flash plate technology [12] or cell based luminescence assays [13]. Thus, 384-well plates have not been harnessed to provide assessment of infectious titers of influenza viruses.

As no world wide standard exists for the quantification of influenza viruses, the World Health Organization's (WHO) Manual on Animal Influenza Diagnosis and Surveillance [14] was chosen as the basis for this work. The manual recommends that viral samples be analyzed
in quadruplicate by the TCID₅₀ assay in a 96-well microplate format. These assays may be interpreted by the visual observation of cytopathic effect (CPE) or the detection of influenza nucleoprotein by an in situ ELISA. To increase the assay throughput and efficiency, a 384-well microplate assay with an ELISA readout was developed based on recommended WHO manual TCID₅₀ procedures. Titration of HPAI and interpandemic influenza A viruses in a 384-well microplate was compared to the traditional 96-well format. Based on preliminary qualification data, the 384-well format measures accurate and precise titers with less variability and higher assay linearity than the 96-well plates, suggesting this method provides more reliable titer data. Further, the use of 384-well plates reduces the time and effort required for analysis to 24 minutes per sample, a reduction of 80%. This assay format has also been adapted for micro-neutralization assays and is amenable for use with manual or robotic liquid handlers.

2. MATERIALS AND METHODS

2.1. Metabolic Assay

384-well plates were seeded with MDCK cells at 2.5 x 10^5 or 3.0 x 10^5 cells/mL and incubated overnight at 37°C and 5.0% CO₂. A 5% (w/v) Tetrazolium salt, 3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution was prepared in 1X phosphate buffered saline (PBS), added to each well and incubated 2 hr at 37°C. The reduced MTT was released from the cells by adding Solubilization Buffer (50% dimethyl formamide solution containing 20% w/v sodium dodecyl sulfate) and incubating for 2 hr at 37°C. The plates were read at 550 nm with a 690 nm reference. All subsequent experiments utilized a seeding density of 3.0 x 10^5 cells/mL due to lower variability, which was analyzed by calculating the coefficient of variance according to the location on the plate.

2.2. Viruses and Cells

Each viral strain was received from the Centers for Disease Control (Atlanta, GA) and propagated in 8-10 day old specific-pathogen free chicken embryos (Charles River Franklin, CT) at 37°C for 24 hr for A/ Vietnam/ 1203/04, 37°C for 72 hr for A/New Caledonia/20/99, and 35°C for 48 hr for A/Brisbane/10/07. The harvested allantoic fluid was stored at \leq -70°C. Madin-Darby canine kidney (MDCK) cells (Salisbury lineage, Sigma St. Louis, MO), were maintained in complete Eagle's minimal essential media (EMEM) containing 1% Penicillin- Streptomycin and 10% fetal bovine serum. MDCK plates seeded at 70% confluency were purchased from Diagnostic Hybrids, Inc. (Athens, OH).

2.3. Viral Quantification

Viral stocks were quantified by inoculating serial dilutions in quintuplicate on 96-well plates or quadruplicate on 384-well plates onto confluent MDCK monolayers. TPCK-treated trypsin at 2.0 μ g/mL (US Biological Swampscott, MA) was added to the inoculation media for the interpandemic strains. Each plate contained cell culture control (CC) wells containing inoculation media alone. The inoculated plates were incubated at 37°C and 5.0% CO₂ in a humidified incubator for 20 ± 1 hr prior to fixation.

2.4. In Situ Influenza A Nucleoprotein ELISA

The ELISA was performed as previously described [14] with the following noted changes. Plates were fixed by the addition of 1 1/2 volumes of 80% cold acetone in water directly to the inoculum and incubated at room temperature (RT) for 30 min. All subsequent incubations were increased from the recommended RT to 37°C in a humidified incubator to increase the sensitivity of the assay. For the primary incubation, an equal mixture of mouse anti-influenza A nucleoprotein monoclonal antibody clone MAB8257 and MAB8258 (Chemicon International; Bellirica, MA) was used. The ABTS Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories, prepared according to manufacturer's instructions) substrate and stop solution was used according to the manufacturer's instructions. The plates were read at 405 nm with a 490 nm reference. The positive threshold was calculated as the average optical density (OD) of the CC wells plus two standard deviations. Each sample well was scored as positive for infection if the OD was above the positive threshold and negative for infection if the OD was less than or equal to the positive threshold. The median infectious dose for all samples was calculated using the Spearman Kärber formula [15].

3. RESULTS

3.1. Optimization of the 384-Well Plate

To assess the suitability of 384-well plates for a cellbased infectivity assay, MDCK cells were seeded at two concentrations and the metabolic activity was determined by reduction of MTT. The metabolism of cells seeded in the outer wells was identical to the cells seeded in the inner wells (see **Table 1**). The variability was analyzed by calculating the coefficient of variance (CV) by location on the plate and found to be less than 13% for inner and outer wells as well as the entire plate. Therefore, 384-well plates were found suitable for this assay due to the low variability demonstrated between inner and outer wells. Further, seeding the plate at 3 x 10⁵ cells/mL provided less variability (CV < 8%, see **Table 1**).

Table 1. 384-well plate variability measured by metabolism. 384-well plates were seeded with two concentrations of MDCK cells and metabolism was determined by the reduction of MTT. The delta OD (difference between OD at 550 nm and 690 nm) was averaged for the outer and inner wells in comparison to the entire plate. The standard deviation (SD) and coefficient of variance (CV) were determined for each location.

		2.5 x 10 ⁵ cells/mL		$3.0 \ge 10^5$ cells/mL			
	Outer Wells	Inner Wells	Entire Plate	Outer Wells	Inner Wells	Entire Plate	
Average ΔOD	0.28	0.29	0.29	0.35	0.35	0.35	
SD	0.04	0.03	0.03	0.03	0.03	0.03	
CV	13%	11%	11%	8%	7%	8%	

Table 2. *In situ* nucleoprotein ELISA threshold calculation. Changes made to the WHO animal influenza diagnosis and surveillance ELISA threshold obtained titers comparable to visual determination of CPE.

CPE Readout	WHO ELISA	ELISA (AVG CC + 2SD)
5.7	5.0	5.6
Different plate	Same plate analyzed wi	ith different thresholds

The in situ nucleoprotein ELISA was adapted for this plate format and optimized for sensitivity, efficiency, and safety (see Materials and Methods 2.4). Removing the inoculum and washing the monolayer prior to fixation was not desirable for the 384-well plate due to the involvement of multiple pipetting steps and manipulation of potentially infectious materials. Thus, the critical step for making this assay feasible and safe is the addition of the fixative directly to the inoculated wells. Tests demonstrated no adverse effects to the reported titer or increase in the background of the assay by changing the fixation step (data not shown). To further optimize the ELISA, the incubation temperature was raised which reduced the concentration of conjugate required thus increasing the sensitivity of the assay. The use of the ABTS microwell peroxidase substrate system improved the environmental impact of this assay by eliminating the waste generated from the o-phenylenediamine dihydrochloride (OPD) substrate, which is both toxic to humans and dangerous to aquatic systems according to the material safety data sheet. Use of the ABTS substrate increased the positive signal without significantly increasing the background of negative wells (data not shown). Unlike OPD, the ABTS substrate does not change color upon the addition of the stop solution thus eliminating concerns about the timing of stopping the reaction. Finally, the positive threshold was defined as the average of optical density (OD) of the CC wells plus two standard deviations which provides titers consistent with side by side CPE readout experiments (Table 2).

Samples and controls are diluted in a standard 96-tube microtiter box (see Figure 1A). Inoculation of the 384-well plate is efficient and simple when utilizing a 24-channel pipette (see Figure 1B). The content of each microtiter tube is inoculated into four wells on the 384-well plate. This process can be adapted easily to

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1-1	S2-1	S3-1	S4-1	S5-1	S6-1	S7-1	S8-1	S9-1	S10-1	PC-1	NC-1
В	S1-2	S2-2	S3-2	S4-2	S5-2	S6-2	S7-2	S8-2	S9-2	S10-2	PC-2	NC-2
С	S1-3	S2-3	S3-3	S4-3	S5-3	S6-3	S7-3	S8-3	S9-3	S10-3	PC-3	NC-3
D	S1-4	S2-4	S3-4	S4-4	S5-4	S6-4	S7-4	S8-4	S9-4	S10-4	PC-4	NC-4
Е	S1-5	S2-5	S3-5	S4-5	S5-5	S6-5	S7-5	S8-5	S9-5	S10-5	PC-5	CC
F	S1-6	S2-6	S3-6	S4-6	S5-6	S6-6	S7-6	S8-6	S9-6	S10-6	PC-6	CC
G	S1-7	S2-7	S3-7	S4-7	S5-7	S6-7	S7-7	S8-7	S9-7	S10-7	PC-7	00
Н	S1-8	S2-8	S3-8	S4-8	S5-8	S6-8	S7-8	S8-8	S9-8	S10-8	PC-8	CC



(a) TITER-BOX. Samples and controls are diluted in a standard 96-tube microtiter box. Each color represents a different viral sample or control, thus ten samples (S1 through S10) with a positive control virus (PC), sample matrix matched negative control (NC), and cell culture control (CC) are prepared. Eight dilutions of each sample and PC are prepared with four dilutions for the NC.

(b) 384-WELL PLATE: Each tube is inoculated into four wells in the 384-well plate. The number in each well represents the sample/control dilution inoculated into that location

Figure 1. 384-well plate set up and layout.

	Repeatability (Intra-Assay Variability) ¹		Intermediat (Inter-Assay	Expected Titer ³	
=	384-well	96-well	384-well	96-well	
		A/Vietnam/1	203/04 (H5N1)		
GEOMEAN	7.6	7.8	7.6	7.9	
SD	0.2	0.2	0.3	0.4	7.6
CV	3%	3%	4%	5%	
		A/New Caledo	nia/20/99 (H1N1)		
GEOMEAN	4.7	4.5	4.9	5.3	
SD	0.0	0.1	0.3	0.8	5.1
CV	0%	2%	6%	15%	
		A/Brisbane/	/10/07 (H3N2)		
GEOMEAN	6.3	5.7	6.3	5.5	
SD	0.1	0.2	0.2	0.3	6.1
CV	2%	4%	3%	5%	

Table 3. Intra-assay and inter-assay variability data for $TCID_{50}$ assays. The repeatability (intra-assay variability) and intermediate precision (inter-assay variability) of each plate format was assessed for three strains of influenza A.

1) Value reported is the geometric mean of the titer of three iterations by one operator on one testing day

2) Value reported is the geometric mean of the titer of three iterations by two operators on two testing days

3) The expected titer was based on prior titer certification on 96-well plates by two operators over three testing days using nine randomly selected aliquots of the stock

robotic or manual liquid handler capabilities and has been used in our facility.

3.2. Assay Acceptability

To compare the new 384-well plate with the traditional 96-well plate, the repeatability (intra-assay variability) and intermediate precision (inter-assay variability) were evaluated (see **Table 3**) for three influenza strains (H1N1, H3N2, and H5N1). Use of three different influenza viruses demonstrated the robustness of the assay for quantification of any influenza A strain. The expected titer of each strain was established using 96-well plates prior to the experiment. To establish repeatability and precision, the mean titer obtained must be within 0.5 log of the expected titer.

The repeatability was assessed by three iterations of the same viral lot by one operator on a single day of testing. The geometric mean and standard deviation were calculated for each plate type and viral strain (see **Table 3**). For the 384-well plates, all mean titers were within 0.4 log of the expected titer and were deemed repeatable for all three strains. Further, the standard deviations were less than or equal to 0.2 log TCID₅₀/mL which demonstrates low variability regardless of the influenza strain quantified on the 384-well plate. The 96-well plates produced titers within 0.6 log of the expected titer, indicating less repeatability with this plate format.

The intermediate precision for each assay format and viral strain was assessed by three iterations of the same viral lot by two operators on two testing days. The geometric mean and standard deviation of the resulting 12 data points were calculated (see Table 3). All of the mean titers obtained with 384-well plates were within 0.2 log of the expected titer, thus establishing the intermediate precision of the 384-well plate. The 96-well plate demonstrated less precision with all mean titers falling within 0.6 log of the expected titer. In addition, the standard deviations for the 384-well plates were calculated to be 0.3 log TCID₅₀/mL or less, compared with 0.8 log TCID₅₀/mL or less for 96-well plates, indicating lower variability between assays, operators, and testing days for the highthroughput plate format. All strains performed as expected in comparison to titers established using 96-well plates, thus verifying the robustness of the 384-well plate. The linearity of an assay measures the ability to obtain results proportional to the concentration of the sample. For each assay format, the linearity was assessed by preparing four serial dilutions of the viral stock. Each dilution was then quantified in each plate type. The predicted titer for each dilution was calculated based on the dilution from the certified stock titer. The observed titer was plotted against the predicted titer and a linear regression analysis was performed (see Figure 2). For the purposes of this study, an $r^2 \ge to 0.85$ was considered linear. Although the 96-well plate for both the A/ Vietnam/1203/04 and A/New Caledonia/20/99 strains demonstrated linearity, $r^2 > 0.96$, the 384-well plate for both strains demonstrated greater linearity, $r^2 > 0.99$ which suggests the use of a 384-well plate format provides more reliable data. However, both 96-well and 384-well plates are expected to produce results proportional to the concentration of the sample quantified.



Four serial dilutions of each strain were prepared and then quantified in each assay format. The observed log titer was compared with the predicted titer for each dilution. The linear regression for each format was determined. An $r^2 > 0.85$ was considered linear for each assay format.

Figure 2. Assay linearity for influenza A (H5N1 and H1N1).

3.3. Advantage of High-Throughput 384-Well Plate

To compare efficiency of the new 384-well plate format with the standard 96-well plate, the average time required to analyze one sample in a TCID₅₀ assay from seeding the plate to analysis of the data was determined. The average time for the 96-well assay (plates seeded in house) was 2 hr per sample from start to finish. For the most efficient 384-well assay, commercially available seeded plates were used, removing the need for cell culture maintenance. An additional advantage of these pre-seeded plates is that more assays can be conducted each week without the need of multiple cell lines being maintained for harvest. The average time for the analysis of one sample on a pre-seeded 384-well plate was 0.4 hr. Thus, using purchased 384-well plate increases efficiency of the TCID₅₀ assay by 80%, thereby reducing cost significantly.

4. DISCUSSION

HPAI infections in animals cause economic losses in countries dependent on poultry revenues [16]. Further,

HPAI and interpandemic (seasonal) influenza infections in humans burden public health resources [17]. A myriad of vaccines and therapeutics are under development; however, assays that test influenza virus titers and screen the efficacy of vaccines and therapeutics cannot be used to quantify influenza virus and those that do measure infectious virus, do so by indirect methods [9, 10]. The TCID₅₀ assay directly quantifies infectious influenza A viruses by utilizing an in situ nucleoprotein ELISA that detects the influenza A nucleoprotein in infected monolayers [14]. Therefore, a 384-well plate TCID₅₀ assay with an ELISA readout was developed and tested for robustness, accuracy, precision, and reliability. The time and effort required for sample testing was also determined. For this study, two plate formats were examined: a traditional 96-well plate and a high throughput 384-well plate. To demonstrate the robustness of the assays, three influenza A strains were quantified: A/ Vietnam/1203/04 (H5N1), A/New Caledonia/20/99 (H1N1), and A/ Brisbane/10/07 (H3N2). All viral strains performed as expected without modification of the procedure, demonstrating the robustness of the new 384-well plate format when compared with the traditional 96-well plate. Addi-

tionally, each assay format was tested for intra- and inter-assay variability to ascertain the precision of the assay and for linearity to verify the overall performance of the plate format. Both 96-well and 384-well plate formats tested were found to be repeatable, precise, and linear. The 384-well plate appears to be more accurate, precise and linear than the traditional 96-well plate and demonstrated less overall variability. Further, the efficiency afforded by using purchased pre-seeded 384-well plates was substantial. The increase in efficiency greatly lowers the cost and time required to obtain an infectious titer for a variety of viral samples without sacrificing precision. The 384-well plate format with ELISA readout offers a high-throughput, more efficient alternative for the determination of infectious influenza A titers in compliance with WHO recommendations for the assay. The 384-well plate has also been adapted for automation via liquid handling, considerably increasing the throughput of sample analysis in order to determine the efficacy of novel vaccines and therapeutics.

5. ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr. Hank Lockman and Mr. Aaron Martin for their assistance in the production of this manuscript. In addition, the authors would like to thank Drs. Herb Bresler, Catherine Smith, and Carol Sabourin for providing their support and technical assistance. This work was funded through the Battelle Independent Research and Development Program.

REFERENCES

- [1] (2009) Influenza statistics. Roche.
- [2] (2009) WHO confirmed human cases of avian influenza a (H5N1). World Health Organization Department Communicable Disease Surveillance and Response.
- [3] Tewari, D., Zellers, C., Acland, H. and Pedersen, J.C. (2007) Automated extraction of avian influenza virus for rapid detection using real-time RT-PCR. *Journal of Clinical Virology*, **40**, 142-145.
- [4] Lee, W.-M., Grindle, K., Pappas, T., Marshall, D.J., Moser, M.J., Beaty, E.L., Shult, P.A., Prudent, J.R. and Gern, J.E. (2007) High-throughput, sensitive, and accurate multiplex PCR-microsphere flow cytometry system for large-scale comprehensive detection of respiratory viruses. *Journal of Clinical Microbiology*, **45**, 2626-2634.
- [5] Nemoto, H., Komoriya, T. and Kohno, H. (2007) Development of latex turbidimetric immunoassay for rapid and sensitive detection of influenza virus. *Rinsho Biseibutshu Jinsoku Shindan Kenkyukai Shi.*, 18, 117-126.
- [6] Severson, W. E., McDowell, M., Ananthan, S., Chung, D.H., Rasmussen, L., Sosa, M.I., White, E.L., Noah, J.

and Jonsson, C.B. (10-1-2008) High-throughput screening of a 100,000-compound library for inhibitors of influenza a virus (H3N2). *Journal of Biomolecular Screening*, **13**, 879-887.

- [7] Wang, S.Y., Su, C.Y., Lin, M., Huang, S.Y., Huang, W. I., Wang, C.C., Wu, Y.T., Cheng, T. J., Yu, H.M., Ren, C.T., Wu, C.Y., Wong, C.H. and Cheng, Y.S. (2-11-2009) HA-pseudotyped retroviral vectors for influenza antagonist screening. Journal of Biomolecular Screening, 1087-0571, 0833-0786.
- [8] Kapteyn, J.C., Porre, A.M., de Rond, E.J.P., Hessels, W.B., Tijms, M.A., Kessen, H., Slotboom, A.M.E., Oerlemans, M.A., Smit, D., van der Linden, J., Schoen, P. and Thus, J.L.G. (2-25-2009) HPLC-based quantification of haemagglutinin in the production of egg- and MDCK cell- derived influenza virus seasonal and pandemic vaccines. *Vaccine*, **27**, 1468-1477.
- [9] Wagaman, P.C., Leong, M.A. and Simmen, K.A. (2002) Development of a novel influenza a antiviral assay. *Journal of Virological Methods*, **105**, 105-114.
- [10] Mo, C., Yamagata, R., Pan, A., Reddy, J., Hazari, N. and Duke, G. (2008) Development of a high-throughput Alamar blue assay for the determination of influenza virus infectious dose, serum antivirus neutralization titer and virus ca/ts phenotype. *Journal of Virological Methods*, 150, 63-69.
- [11] Aguero, M., San Miguel, E., Sanchez, A., Gomez-Tejedor, C. and Jimenez-Clavero, M. (2007) A fully automated procedure for the high-throughput detection of avian influenza virus by real-time reverse transcriptionpolymerase chain reaction. *Avian Diseases*, **51**, 235-241.
- [12] Maroto, M., Fernandez, Y., Ortin, J., Pelaez, F. and Cabello, M.A. (8-1-2008) Development of an HTS assay for the search of anti-influenza agents targeting the interaction of viral RNA with the NS1 protein. *Journal of Biomolecular Screening*, **13**, 581-590.
- [13] Noah, J.W., Severson, W.D., Noah, L., Rasmussen, L., White, E.L. and Jonsson, C.B. (2007) A cell-based luminescence assay is effective for high-throughput screening of potential influenza antivirals. *Antiviral Research*, **73**, 50-59.
- [14] Cox, N., Webster, R. and Stohr, K. (2002) WHO manual on animal influenza diagnosis and surveillance. World Health Organization Department Communicable Disease Surveillance and Response.
- [15] Hamilton, M.A., Russo, R.C. and Thurston, R.V. (1977) Trimmed spearman-karber method for estimated median lethal concentration in toxicity bioassays. *Environmental Science & Technology*, **11**, 714-719.
- [16] (2007) Food and Agriculture Organization of the United Nations (FAO) and World Organization for Animal Health (OIE). The Global Strategy for Prevention and Control of H5N1 Highly Pathogenic Avian Influenza1-39.
- [17] (2009) Financial impact of the Flu, http://www. flufacts.com/impact/financial.aspx.

Gait spectral index (GSI): a new quantification method for assessing human gait

Rodolphe Héliot¹, Christine Azevedo-Coste², Laszlo Schwirtlich³, Bernard Espiau⁴

¹Inria, Montbonnot, France; <u>rodolphe.heliot@gmail.com</u>

²Demar Inria/Lirmm, Montpellier, France; <u>azevedo@lirmm.fr</u>

³Institute for Rehabilitation "Dr Miroslav Zotovic", Belgrade, Serbia; laslo@bitsyu.net

⁴Inria, Montbonnot, France; <u>espiau@inrialpes.fr</u>

Received 25 November 2009; revised 7 December 2009; accepted 12 December 2009.

ABSTRACT

This paper introduces a simple, quantitative assessment tool to follow up the recovery of gait. Today, micro-electro-mechanical systems (MEMS) technology provides with small, simple, low-power consuming and easy to don and doff sensors. In our approach we have selected an accelerometer and introduced a new quantity that characterizes the gait pattern in the frequency domain, we term it Gait Spectral Index (GSI). GSI allows assessing gait quality and closely relates to the speed and cadence of gait (dynamics). We have tested the GSI approach to quantify the quality of the gait of healthy young and elderly, and poststroke hemiplegic individuals. We investigated the repeatability and coherence of GSI in healthy individuals (young and elderly) and contrasted this to the post-stroke hemiplegic individuals. We found that high correlation of the GSI with conventional gait parameters. This suggests that GSI, which needs only data from one accelerometer, could be an objective quantitative measure of the quality of the walking thereby a simple yet reliable measure of the recovery of function during neuronrehabilitation.

Keywords: Gait Evaluation; Walk Training; Accelerometer; Spectral Analysis

1. INTRODUCTION

Clinical methods for gait evaluation consist in standardized functional tests (Barthel Index, Rivermead Mobility Index (Bohannon *et al.* 1987), Functional Ambulation Category (FAC)) are qualitative; thereby, somewhat subjective because they depend on individual observation skills of the rater. We developed a simple instrument that after appropriate processing provides objective quantitative measure of the gait performance. This new instrument is of great importance for clinical use for assessing the gait pattern in all, including elderly and individuals with disabilities (Sekine *et al.*, 2002). The heart of the instrument is the accelerometer that is being mounted on the leg, does not need calibration and provides data that is highly reproducible.

The development of this instrument follows the fast growth of the filed of Micro-electro-mechanical systems (MEMS). The MEMS sensors have been applied for the assessment of physical activities using body-mounted systems (Jasiewicz et al., 2006; Luinge and Veltink, 2004; Pappas et al., 2002). MEMS based accelerometers provide a good representation of movement dynamics (Jasiewicz et al., 2006; Hester et al., 2006; Brandes et al., 2005; Luinge and Veltink, 2004). The typical application of multiple accelerometers is for reconstruction of the kinematics of the gait (Wagenaar et al., 1992; Bussmann et al., 2000). Reconstruction of gait kinematics is difficult since the integration of data captured by accelerometers results with drift and substantial errors. The alternative is to use the acceleration data directly as a measure of the quality of gait. Acceleration patterns of trunk have extensively been employed since they have been shown to comprise low intra-variability; thereby suitable for detection of phases of the gait cycle (Ziljstra et al., 2003; Brandes et al., 2006). The reproducibility of trunk accelerations declined as gait speed decreases which is the case when analyzing the gait of elderly and even more of subjects with gait disabilities (Saremi et al., 2006).

Acceleration can be analyzed in the frequency domain. The fundamental frequency is equivalent to the stride frequency, and the harmonics amplitudes vary depending on the gait pattern. It is important to notice that the position of the accelerometer on a body segment does not affect the frequency content of the signal. One of the best confirmations was presented by Waarsing *et al.* (1996) showing that the power contained in the peaks is inversely proportional to stability performance.

Here, we show that only one accelerometer can be used

as a good source of data for gait characterization. One of the features of this gait assessment system is that it does not need to be calibrated to each individual, and provides with easy donning and doffing. The major novelty that allows the use of this simple hardware is the Gait Spectral Index (GSI). The GSI is the new measure that characterizes the gait pattern in the frequency domain. We hypothesized that the GSI is highly correlated with the conventional gait parameters. Based on proved hypothesis we performed the experiments in which we compared the GSI determined from analysis of the gait of healthy young and elderly subjects with the GSI determined for the gait of individuals of hemiplegia.

2. METHODS

2.1. Subjects

Six healthy young **(HY)** (age 31. 8 years \pm 7. 2), and ten healthy elderly **(HE)** (age 67 years \pm 6. 9) individuals participated in the study to confirm the high correlation of the GSI and conventional gait parameters. 19 post-stroke hemiplegic individuals (SP) (age 58. 6 years \pm 10.18) also participated in the study in order to analyze the differences between healthy and pathological gait. Basic tada on study the subjects is summarized in **Tables 1-4**.

The HY group was formed from volunteers from our laboratory in LIRMM, France. Two subjects, out of ten, in the HE group were recruited in an elderly cultural association while the other eight from a local waling club.

Hemiplegic individuals were recruited from the inpatient population of the Institute for Rehabilitation "Dr Miroslav Zotovic" in Belgrade. The hemiplegic gait abilities were assessed by using the Functional Ambulation Category scale (Holden et al, 1984). The FAC scale has five grades: 1 - person needs to be physically supported for any ambulation (the worst), and 5 - the person can walk independently anywhere (the best). All hemiplegic individuals could walk with their usual walking aid (13 patients used cane or tripod, 4 patients had also an ankle

Table 2. Healthy elderly subject group (HE) gait description.

or foot orthosis) as shown in Tables 3 and 4.

The (SP) group was divided into two sub-groups: (SP1) includes the eight individuals with a 5 FAC rank and (SP2) with the 11 remaining individuals.

This study was approved by the local ethics Committee, and all study participants signed the informed consent.

2.2. Equipment

We used a uniaxial-accelerometer (ADXL-203, Analog Devices) positioned on the shank close to the ankle. The accelerometer axis was directed along the shank with the positive direction pointing upwards (**Figure 1**). The alignment was performed visually, except from this constraint, no specific care was needed for the positioning of the accelerometer. In the case of hemiplegic individuals the accelerometer was positioned on the non-paretic leg. In order to determine the correlation with the conventional gait parameters subjects were also equipped with two 3-contact point insoles used to detect gait phases, gait cadence and assess the averaged stride length when associated to chronometric recordings. The sampling rate was 100 samples per second based on known low frequency content of the signal.

2.3. Protocol

Subjects were asked to walk 10 meters at their normal self-paced speed. Depending on the individual ability to

Table 1. Healthy young subject group (HY) gait description.

#	Age	Cadence
6	30	0.90
1	46	0.96
2	28	0.78
3	32	1.05
5	26	0.99
4	29	0.84
Mean	31.8	0.92
Std	7.2	0.1

#	Age	Walking speed	Cadence	Stride length
5	73	1.39	1.02	1.36
1	67	1.49	0.93	1.61
8	65	1.35	0.97	1.39
7	63	1.24	0.92	1.36
6	61	1.19	0.9	1.31
2	63	1.06	0.92	1.53
3	60	1.23	0.91	1.36
4	62	1.22	0.86	1.4
10	79	1.36	1.05	1.29
9	77	1.24	1.09	1.14
Mean	67	1.27	0.96	1.38
Std	6.87	0.12	0.07	0.13

#	Age	FAC	Walking aid	Walking speed	Cadence	Stride length	SI
4	54	4	Cane	0.3	0.44	0.68	15.47
3	69	4	Cane	0.38	0.56	0.68	27.61
6	71	3	Tripod	0.13	0.29	0.46	27.47
13	53	4	AFO+tripod+physio	0.1	0.22	0.48	16.99
5	67	1	AFO+tripod	0.12	0.32	0.4	9.83
16	77	3	Tripod	0.17	0.33	0.51	17.06
17	59	2	Tripod+physio	0.11	0.2	0.58	22.18
8	47	3	Tripod	0.29	0.39	0.75	66.57
11	46	3	Cane	0.07	0.33	0.24	3.71
7	77	4	AFO+tripod	0.2	0.54	0.72	6.54
9	58	4	Tripod	0.17	0.28	0.6	11.65
Mean	61.64	3.18		0.19	0.35	0.55	20.46
Std	11.20	0.98		0.10	0.12	0.15	17.15

Table 3. Stroke patient group 1 (SP1) gait description.

Table 4. Stroke patient group 2 (SP2) gait description.

#	GSI
6	1.57
1	1.46
2	1.42
3	1.40
5	1.28
4	0.92
Mean	1.34
Std	0.23

walk for extended period of time, the data between four and eight trials were collected for each subject.

2.4. Symmetry Index

The symmetry index (SI) can be calculated for swing and stance phases using the following formula (Robinson *et al.*, 1987):

SI[%]=200(
$$T_{nonparetic}$$
- $T_{paretic}$)/($T_{paretic}$ + $T_{nonparetic}$) (2)

 $T_{paretic}$ and $T_{nonparetic}$ are the durations of stance or swing phases for paretic and non paretic legs. SI can be positive or negative and the perfect symmetry index is SI=0.

2.5. Spectral Analysis and Gait Spectral Index

The recorded acceleration was transformed using the Fast Fourier transform (FFT) to obtain the frequency spectrum of the signal. There was no averaging; the spectrums were computed over the whole trial duration (10 meters). The gait spectral index (GSI) was defined as a ratio between the power of the second harmonic component with respect the power of the fundamental component.



Figure 1. Protocol description.

3. Results

In Tables 1-4 we reported the gait characteristics (walking speed, cadence, stride length, symmetry index) measured for each of the four groups.

Figure 2 shows the spectrums corresponding to one individual of each population. Spectrums from a given subject are highly reproducible from a trial to another and spectrums for each of the groups are significant in terms of harmonic repartitions (Tables 7 and 8). The computed GSI for the three groups are reported in tables 5 to 8. The aim of healthy group individuals was to compute GSI, therefore we did not analyze all the gait characteristics for these two populations.

3.1. Patients

In patient group GSI index varies from 0.52 to 1.69 (tables 7 and 8) with a low intra variation (average standard deviation 0.16). The spectrum reproducibility is good as stated by the low intra variability associated to fundamental and 2nd harmonic amplitudes (tables 7 and 8). For each individual, we have also estimated the correlation between each trial spectrum and the average spectrum for this given individual. The good correlation (r=0.78) validates the hypothesis of the spectrum reproducibility. There is a moderate correlation between GSI and FAC rank (r= 0.66). In figure 3 we plotted the GSI values obtained for the two patient groups in function of FAC rank. In table 7, the patients are classified in terms of GSI index values. The higher GSI values correspond to best walkers in FAC quotation (level 5-group SP1), they vary from 1.22 to 1.69. The mean value is 1.21. In SP2 group, GSI varies from 0.52 to 0.89 (table 8). For lower levels, the GSI does not seem to allow to discriminate between the different levels. The mean value is 0.71.

There is a good correlation between GSI and gait speed (r=0.81) and gait cadence (r=0.74). Correlation between GSI and stride length is moderate (r=0.66). Correlation with symmetry index is not significant. The mean value of symmetry index is 21%.

It is interesting to notice that FAC rank and symmetry index are not correlated (r=0.03). FAC rank is correlated with gait speed (r=0.7), gait cadence (r=0.72) and stride length (r=0.66).

3.2. Elderly

In elderly subject group GSI index varies from 0.4 to 1.3 (table 5). The mean value is 1.02. In healthy elderly group, only stride length appeared to be significant in terms of correlation with GSI (r=0.72). The two older subjects (77 and 79) have the lower GSI scores.

3.3. Young

In young subject group GSI index varies from 0.92 to 1.57 (table 6). The mean value is 1.34.

4. Discussion

The results presented document that the spectrum of the shank acceleration is highly reproducible from trial to trial in the same subject and can be considered as an individual signature pattern. We noticed the presence of strong high frequencies components in the spectrums of healthy and patient best walkers. High frequencies components are likely present due to the richer dynamics of healthy gait compared to the gait of hemiplegic and poor walkers.

The proposed GSI ratio compares fundamental amplitude with second harmonic amplitude; it correlates with gait speed which is classically considered as an indirect measurement of gait quality. It is important to notice that to be valuable speed should be evaluated through distances long enough to assess adaptation and gait efficiency.

The low correlation between FAC score and GSI should be counterbalanced by the fact that the higher GSI values correspond to best classification in FAC. But. FAC classes are not varying linearly with gait quality and the number of patients in each of the categories is not similar enough to conclude.

The GSI scores in elderly population are higher compared to our expectations. We suggest that this is due to the fact that the population included in the study was mainly recruited in a gait club. Hence, they are not representative for typical aged walkers with limited sensory-motor capacity.

Nor GSI, nor FAC are correlated with symmetry of gait in patients. This should be an improvement of GSI in future.

5. CONCLUSIONS

The spectral analysis of the shank acceleration, in the direction aligned with the shank can characterize the gait. Two main observations can be made: 1) dealing with a given subject: high reproducibility of spectrums from trial to trial, and 2) presence of strong high frequencies components in the spectrums of healthy and patients who are good walkers.

We found out that placing one accelerometer on the "healthy" leg of post-stroke hemiplegic patient allows assessing the effects of gait reeducation. The instrumentation and processing that we described are simple and they comprise light and non expensive setup, including wireless communication of sensory output to computer.

At this point the GSI index is mainly correlated with gait speed and gait cadence and better than what FAC rank. This is an interesting result as our approach is technically less constraining than classical methods to



Figure 1. Hemiparetic stroke GSI.



Figure 2. Accelerometer signals spectrums examples for all 4 groups. The values on the vertical axis are normalized relatively to the amplitude of the fundamental amplitude. The value of the 2nd harmonic therefore corresponds to GSI.

Table 5. Healthy young subject group (HY) mean value of the GSI index over different trials for each subject for comparison purposes.

#	GSI
6	1.57
1	1.46
2	1.42
3	1.40
5	1.28
4	0.92
Mean	1.34
Std	0.23

assess the gait speed and cadence (chronometer, insoles).

The GSI could be applied not only for the individuals who participated in this study, but in many other types of pathological gait. The GSI needs then to be improved and adapted to meet the specific pathologies and give object**Table 6.** Healthy elderly subject group (HE) mean value of the GSI index over different trials for each subject for comparison purposes.

#	GSI
5	1.3
1	1.29
8	1.2
7	1.19
6	1.12
2	1.11
3	0.98
4	0.88
10	0.78
9	0.4
Mean	1.025
Std	0.28

tive measures of the improvement.

#	GSI GSI		Fundamental amplitude		2 nd harmonic amplitude		Spectrum correlation
	mean	std	mean	std	mean	std	
2	1.69	0.10	0.85	0.07	1.46	0.20	0.79
1	1.36	0.20	0.70	0.25	0.93	.027	0.81
20	1.31	0.22	0.82	0.16	1.09	0.27	0.79
15	1.22	0.21	0.94	0.05	1.15	0.19	0.51
14	1.16	0.09	0.89	0.13	1.03	0.21	0.77
19	1.02	$\hat{0}.\hat{0}\hat{9}$	0.93	0.08	0.96	0.14	0.67
10	1.01	0.09	0.89	0.08	0.90	0.11	0.79
18	0.95	0.14	0.93	0.03	0.89	0.16	0.79
Mean	1.22	0.14	0.87	0.11	1.05	0.19	0.74
Std	0.24	0.05	0.08	0.07	0.18	0.05	0.10

Table 7. Stroke patient group 1 (SP1) GSI indexes and spectral analysis results.

Table 8. Stroke patient group 2 (SP2) GSI indexes and spectral analysis results.

#	GSI GSI		Fundamental amplitude		2 nd harmonic amplitude		Spectrum correlation
	mean	std	mean		mean	std	
4	0.89	0.15	0.90	0.06	0.80	0.16	0.80
3	0.87	0.10	0.92	0.05	0.81	0.13	0.85
6	0.75	0.25	0.71	0.31	0.47	0.14	0.92
13	0.75	0.45	0.61	0.42	0.35	0.15	0.63
5	0.75	0.12	0.85	0.07	0.64	0.14	0.89
16	0.72	0.13	0.91	0.07	0.66	0.10	0.94
17	0.69	0.24	0.53	0.40	0.32	0.14	0.49
8	0.68	0.19	0.75	0.19	0.51	0.16	0.80
11	0.63	0.12	0.64	0.37	0.37	0.18	0.65
7	0.59	0.14	0.75	0.25	0.43	0.14	0.95
9	0.52	0.04	0.88	0.11	0.46	0.07	0.96
Mean	0.71	0.17	0.77	0.21	0.53	0.14	0.81
Std	0.11	0.10	0.13	0.14	0.17	0.03	0.15



Figure 3. Hemiparetic stroke GSI.

In the future, we propose to improve GSI in order to include gait symmetry parameter. It would also be possible to give different weights to different gait quality criteria within GSI.



Figure 4. GSI / subject ages.

The low intra variability of this spectral analysis could easily be employed to assess the gait improvement of each individual patient. A low GSI score could alert the therapist on possible pathological problems.

REFERENCES

- [1] Bohannon, R.W., Smith, M.B. (1987) Interrater reliability of modified Ashworth scale of muscle spasticity. *Phys Ther*; **67**, 206-7.
- [2] Bonnet, S. and Heliot, R. (2007) A magnetometer-based approach for studying human movements to appear. *IEEE Trans. Biomedical Engineering*.
- [3] Brandes, M., Zijlstra, W., Heikens, S.R., Lummel, R. Van and Rosenbaum, D. (2005) Accelerometry based assessment of gait parameters in children. *Gait & Posture*, 24, 482-486.
- [4] Bussmann, J.B. and Damen, L. and Stam, H.J. (2000) Analysis and decomposition of signals obtained by thigh-fixed uni-axial accelerometry during normal gait. *Med Biol Eng Comput*, **38**, 632-638.
- [5] Hester, T., Hughes, R., Sherrill, D., Knorr, B., Akay, M., Stein J., and Bonato, P. (2006) Using wearable sensors to measure motor abilities following stroke. *International Workshop on Wearable and Implantable Body Sensor Networks*, Massachusetts.
- [6] Holden, M.K., Gill, K.M., Magliozzi, M.R. and *et al.* (1984) Clinical gait assessment in the neurologically impaired, reliability and meaningfulness. *Physical Therapy*, **64**, 35-40.
- [7] Jasiewicz, J.M., Allum, J. H.J., Middleton, J. W., Barriskill, A., Condie, P., Purcell, B. and Che Tin Li, R.C.T. (2006) Gait event detection using linear accelerometers or angular velocity transducers in able-bodied and spinal-cord injured individuals. *Gait & Posture*, 24, 502-509.
- [8] Lee, J.A. and Cho, S.H. and Lee, J.W. and Lee, K.H. and. Yang, H.K. (2007) Wearable Accelerometer System for Measuring the Temporal Parameters of Gait. *Engineering in Medicine and Biology Society EMBS*, 483-486.
- [9] Luinge, H. and Veltink, P. (2004) Inclination measurement of human movement using a 3d accelerometer with autocalibration. *IEEE Trans. Neural Sys. and Rehabil. Eng.*, **12**, 112-121.

- [10] Montoye, H.J., Washburn, R., Servais, S., Ertl, A., Webster, J.G. and Nagle, F.J. (1983) Estimation of energy expenditure by a portable accelerometer. *Medi. Sci. Sport Exercise*, **15**, 403-407.
- [11] Pappas, I.P.I., Keller, T. and Mangold, S. A reliable, gyroscope based gait phase detection sensor embedded in a shoe insole. (2002) In *Proceedings of IEEE Sensors 002*, *First IEEE International Conference on Sensors*, 2, 1085-1088.
- [12] Robinson, R.O., Herzog, W., Bigg, B.M. (1987) Use of platform variables to quantify the effects of chiropractors manipulation on gait symmetry. *J Manipulative Physiol Ther.* 10, 172-176.
- [13] Roth, E.J. Merbitz, C., Mroczek, K., Dugan, and Suh, W.W. (1997) Hemiplegic gait. relationships between gait speed and other temporal parameters. *Am J Phys Med Rehabil.*, 76(2), 128-133.
- [14] Saremi, K., Marehbian, J., Yan, X., Regnaux, J.P., Elashoff, R., Bussel, B. and Dobkin, B.H. (2006) Reliability and validity of bilateral thigh and foot accelerometric measures in healthy and hemiparetic subjects. *The American Society of Neurorehabilitation*, 20(2).
- [15] Sekine, M., Tamura, Akay, M.T., Fujimoto, T., Togawa, and Fukui, Y. (2002) Discrimination of gait patterns using wavelet-based fractal analysis. *IEEE transactions on Neural Systems and Rehabilitation Engineering*, **10-3**, 188-196.
- [16] Waarsing, J.H., Mayagoitia, R.E. and Veltink, P.H (1996) Quantifying the stability of gait using accelerometers. In 18th Annual International Conference of the IEEE Engineering in Medicine and Biology Society, Amsterdam. utilisation d'accéléros pour quantifier la marche.
- [17] Wagenaar, R.C. and Beek, W.J. (1992) Hemiplegic gait, a kinematic analysis using gait speed as a basis. J Biomech.25, 1007-1115.
- [18] Ziljstra, W. and Hof, A.L. (2003) Assessment of spatio-temporal gait parameters from trunk accelerations during human gait. *Gait & Posture*, 4, 212-221.

The effect of high fat food on erythrocyte phospholipids, fatty acids composition and glutathione redox-system of rats with alimentary dyslipidemia

Tatyana P. Novgorodtseva¹, Yulia K. Karaman¹, Natalia V. Zhukova²

¹Institute of Medical Climatology and Rehabilitative Treatment SB RAMS, Vladivostok, Russia; <u>karaman@inbox.ru</u> ²Institute of marine biology of name A.V. Zhirmunskogo FEB RAS, Vladivostok, Russia

Received 6 November 2009; revised 7 December 2009; accepted 9 December 2009.

ABSTRACT

To evaluate the effects of high fat food consisted of tallow (19% of total diets) and cholesterol (2%) on modification of erythrocyte phospholipids, fatty acids composition and glutathione redoxsystem of male Wistar rats with alimentary dyslipidemia. The results demonstrated that after 30 and 180 days of high-fat feed erythrocyte phosphatidylinositol and phosphatidylcholine levels were reduced, phosphatidylserine were increased. Only on the 90 days of the experiment phosphatidylinositol level increased. In all growups the erythrocyte 18:0 saturated fatty acids and 20:4n6, 22:4n6 polyunsaturated fatty acids (PUFA) were increased. Deficit of n3 PUFA-20:5n3 and 22:6n3 after 90 and 180 days high fat feed promoted compensatory synthesis from 18:1n9 on 20:3n9. Erythrocyte maleic dialdehyde increased, glutathione level decreased in all groups of rats after fed with high-fat feed. Glutathione reductase and glutathione peroxidase activity decreased in erythrocytes after 30 and 180 days of high-fat feed. In conclusion: high-fat diet during 30-90 days started adaptive answer in lipids of membrane and glutathione redox-system. Important mechanism of adaptation of a cellular membrane to high-fat diet is increase major, structuring a membrane phosphatidylethanolamine and minor, most metabolic significant fractions phospholipids (phosphatidylinositol), keeps homeostasis of 18:2n6 and 22:6n3, 20:3n9 compensatory synthesis, decrease in activity of processes lipid peroxidation, activation of enzymes of redox-system alutathione. But prolonging the high-fat feeding (180 days and more) formed failure compensatory processes (dysadaptation). It is a risk factor of developmening atherosclerosis, diabetes, steatogepatitis and other diseases.

Keywords: Fatty Acids; Phospholipids; Adaptation; Dyslipidemia; GSH; Glutathione Reductase; Glutathione Peroxidase

1. INTRODUCTION

Clinical, experimental and epidemiological researches have proved that the high-fat feeding is a risk factor of development of atherosclerosis, diabetes, steatogepatitis and other diseases [1-4]. Insufficient or superfluous consumption of separate components of food (deficiency of fiber, surplus of fat, carbohydrates etc.) at the initial stages forms the cascade of the stressful reactions directed on activation of mechanisms of adaptation in an organism.

In cellular processes of adaptation one of leading roles is occupied with a plasma membrane and forming it phospholipids (PL) and fatty acid composition [5,6]. The fatty acid composition of phospholipids affects the physicochemical properties of the membrane and thus influences conformation and function of membrane-bound proteins, such as receptors, ion channels, and transporters and also influences cell function by serving as precursors for prostaglandins and other signaling molecules and modulating gene expression through the activation of transcription factors [7-9]. Thanks to ability PL transform each other and also to be redistributed in a membrane between an internal and external layer realize important adaptable mechanism supporting the structural organization and functional properties of a plasma membrane [10] is carried out. The lipid peroxidation initiate modification membrane PL and it fatty acid composition. The important role in realization antioxidant protection from lipid peroxidation is played by glutathione redox-system which regulation of NF $\kappa\beta$ (nuclear factor- $\kappa\beta$), ARE (antioxidant response element) [11-14]. The urgent fast adaptive answer of glutathione redox-system, consisting of activation of enzymes glutathione reductase, glu-

Table 1. Daily allowance of rats (g/kg	g of animal weight).
---	----------------------

Ingredients	Experimental diet	General vivarium diet
Tallow	42.5	5
Cholesterol	4.3	-
Sunflower oil	5	5
Grain mixture	50	50
Bread	20	20
Grits	13	13
Beef	20	20
Skim cheese	8	8
Carrot	33	33
Greens	33	33

Table 2. Effect of high-fat diet on serum lipids.

		Days of fed high-fat diet		
Parameter	Control group, n=10	30 days (group 1), n=10	90 days (group 2), n=10	180 days (group 3), n=10
TC, mmol/l	1.57±0.04	3.34±0.04***	***1.68±0.08	2.04±0.17*
TG, mmol/l	1.12 ± 0.04	1.95±0.06***	***0.51±0.05***	1.17 ± 0.08
HDL-C, mmol/l	0.67 ± 0.04	0.26±0.02***	***0.50±0.08	***0.5±0.15
IA	1.43±0.15	11.87±1.55***	***2.46±0.35*	***7.4±0.8***

in Table 2, 3, 4 (*) left - statistic significance of differences in to control group; right - group 1;

* p < 0.05; ** - p < 0.01; *** - p < 0.001.

tathione peroxidase and synthesis GSH prevents oxidation phospholipids and membrane infringement. Data on the specific features of membrane lipids behavior and adaptive answer by high-fat diet are scarce. Identification of adaptive response to the nutrition factor through the investigation of membrane forming lipids and glutathione redox-system will help to understand mechanism of cellular adaptation and dysadaptation, how predictor of pathogenesis many dietary-induced diseases.

The aim of the current study was to evaluate the effects of high fat food on serum lipid, erythrocyte phospholipids, fatty acids and oxidative stress in rats with experimental dyslipidemia and establish the possible adaptation mechanism by high-fat diet.

2. METHODS

2.1. Animals and Diets

Subject to the research were 40 pubescent white male Wistar rats with initial weight 173 ± 5.6 g. Alimentary dyslipidemia was induced in rats by high-caloric diet (**Table 1**) [15].

The animals were divided into 4 groups, 10 rats in each: normal, control group, fed with basic feed; and groups comprising animals kept on experimental high-fat diet (30 days – group 1; 90 days – group 2; and 180 days – group 3). Experimental high-fat diet consisted on 19% tallow and 2% cholesterol. Euthanasia was performed by in conformity with the requirements of Directive 86/609 EEC on the protection of animals used in scientific research [16].

2.2. Material

Venous blood samples of rats were drawn after decapitation.

2.2.1. Blood Serum Lipid Investigation

Lipid spectrum of blood serum was investigated by Labsistems biochemical analyzer FP-901 (Finland). Parameters to be determined included level of total cholesterol (TC), triglyceride (TG), and high density lipoproteins (HDL). Index of atherogenesis (IA) was calculated by formula: (TG - HDL)/HDL.

2.2.2. Erythrocyte Phospholipids and Fatty Acid Investigation

The erythrocytes were washed three times with 0.9% NaCl solution. Lipids were extracted from erythrocytes by Bligh and Dyer method [17]. Bidirectional micro thin layer chromatography on glass plates 6x6 with silica gel and plaster suspension was used to separate polar lipids. Quantitative analysis of certain phospholipids (PL) classes after thin layer chromatography was made according to V.E. Vaskovsky et al method [18,19]. Content of each component was represented as percentage of total PL. FA methyl ethers were received by Carreau and Duback method [20], analyzed on Shimadzu GC17A gas-liquid chromatographer equipped with flame ioniza-

tion detector and capillary column (0.25 mm x 30 m) with implanted phase Supelcowax 10. Temperature of column and detector was 210°C, temperature of evaporator was 240°C. Media gas was helium. Gas discharge ratio in the evaporator was 1:30 at 1.8 atm. Z-Chrom station was used for calculating area of chromatographic peaks and processing the results. FA methyl ethers were identified by retention time using standards and carbon numbers [21]. Results were represented in relative percentage of total FA.

2.2.3. Determination of Srythrocyte Pro-Oxidant and Anti-Oxidant

Erythrocyte maleic dialdehyde (MDA) was estimated spectrophotometrically using thiobarbituric acid assay [22]. GSH was measured according to the Ellman method [23]. Glutathione reductase activity was measured according to the method described [24], glutathione per-oxidase activity – [25].

2.2.4. Statistic Methods

All data were analyzed by ANOVA using computer program Statistika 6.1 (series 1203C for Windows). Data present as means \pm SEM. (M). Differences between means were assessed by Student's significance test.

3. RESULTS AND DISCUSSION

Analysis result of serum lipids indexes of rats in different stages are shown in **Table 2.** Serum TC, TG levels, index of atherogenesis increased (p < 0.001) of rats in the group 1. Serum HDL-C levels reduced (p < 0.001). After 90 days of the experiment, serum TG, TC levels of rats in the group 2 decreased compared with of rats in group1. At the end experiment, no significant difference was present between TG levels of group 3 and control group, neither between HDL-C levels. Compared with the result of group 2, the TG, TC levels were increased; the index of atherogenesis of group 3 was lower than that of group 1 and group 2.

3.1. Erythrocyte Phospholipids

Separation of erythrocyte PL of rats identified six components including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), phosphatidylinositol (PI) and phosphatidic acid. Phosphatidic acid was found as traces (**Table 3**).

Erythrocyte phospholipids fraction of phosphatidylinositol and phosphatidylcholine were reduced in the group 1 (p < 0.001). Levels of phosphatidylserine and phosphatidylethanolamine were increased in erythrocyte membrane. Onset of the deficit PI and PC which form outer layer of membrane, indicate activation of specific phospholipases and intensification of lipid peroxidation processes contributing to plasma membrane destruction [26,27]. A quick-response was increase in PE level accumulating in inner layer of membrane lipid fraction, where most of the active centers of membrane bonded enzymes are located. Research results prove that on the 30 day of high-fat diet membrane forming lipids promptly responded to the stress to prevent deep structural and functional damage to cell.

Changes in erythrocyte membrane at PS, PC and PE level in rats of group 2 was of the same direction as in rats of group 1. Decrease PC lever in outer layer of erythrocyte membranes was compensated by SM maintenance within the range pertinent to control group animals. Such condition can be described as a compensatory response of cell to long-term exposure to stress alimentary factors. Moreover, due to the high saturation of PE the cholesterol is not hardly building into the inner monolayer. This helps to preserve hydrophilic surrounding of cell membrane integral proteins and, therefore, their function [28].

Depletion of erythrocyte PC which forms outer shell of cell lipid matrix was evidenced at a longer exposure to high-caloric diet (180 days) as well. PC reduction in group 3 was accompanied by reliable increase in PS and SM level as compared to control group, meaning that erythrocyte cell was structurally and functionally inadequate. Due to high saturation with SM, the clusters forming phospholipids in a membrane receive large quantity of cholesterol, and this results in lower permeability of cell membrane and interference in active metabolic processes [28]. Thus, on the 180 day of the experiment cell membrane became unable to resist the continuous flow of alimentary stress factors, and the stage of cell compensatory protection depletion occurred that had been formed by the 90 day of high-caloric diet.

3.2. Erythrocytes Fatty Acids

Qualitative composition of erythrocytes FA in rats was represented by components with carbon chain length from $C_{10 to} C_{24}$, both even and odd, of normal and isomeric structure, saturated, monounsaturated and polyunsaturated (**Table 3**). Certain FA with content below 0.1% were not included into Table 3. These mostly include saturated FA with normal structure, (10:0, 19:0, 20:0, 22:0), some monounsaturated (14:1, 18:1n5, 20:1, 22:1), 18:2n5/9 and 20:3n3 FA.

Analysis of the qualitative composition of FA of erythrocyte lipids showed that rats with dyslipidemia have considerable changes in FA composition as compared to control group. Myristic (14:0), stearic (18:0) FA share was observed to increase in group 1. Unsaturated FA demonstrated minor increase of monoenoic oleic FA (18:1n9, p<0.01) level, reliable accumulation of n6 FA – 20:4n6, 22:4n6, 22:5n6 and decrease in essentially li group 1 is related to the specific features of experimentalnoleic FA (18:2n6). Share of n3 polyunsaturated fatty acids (PUFA) slightly reduced due to the identified de

48	

		Days of fed high-fat diet		
Phospholipids and fatty acids components	Control group, – n=10	30 days (group 1), n=10	90 days (group 2), n=10	180 days (group 3), n=10
		Phospholipids, %		
PS	6.80±0.85	13.00±0.67***	11.1±0.5***	*13.01±1.04***
PI	3.90±0.00	1.14±0.17***	***4.69±0.19*	1.66±0.29***
SM	14.42±0.97	15.77±2.09	14.5±0.39	***17.8±0.52***
PC	55.88±1.14	42.38±0.96***	47.7±0.66**	46.4±2.37**
PE	21.50±0.75	28.82±0.99***	*22.01±0.34	**20.4±1.05
		Fatty acids, %		
12:0	0.25±0.02	tr	0.10±0.01***	***0.40±0.07***
14:0	0.52±0.04	$0.83 \pm 0.08*$	**0.5±0.06	***1.01±0.12***
15:0	0.74±0.11	0.58 ± 0.07	0.43±0.02**	*0.56±0.04
15:1	0.24 ± 0.08	0.18 ± 0.02	tr	**0.30±0.05
16:0	23.72±0.84	24.38±0.57	**28.6±0.56***	27.6±1.4***
16:1n7	1.90±0.25	1.83±0.12	***0.83±0.05***	2.04±0.21
17:0	0.94±0.11	0.65±0.10*	0.7±0.01**	0.58 ± 0.05
17:1	0.96±0.16	0.32±0.02***	***0.18±0.03*	0.26±0.04
18:0	10.1±0.5	14.68±0.48**	17.3±0.57***	13.9±2.77***
18:1n9	7.66±0.57	11.45±1.22**	10.5±0.31	11.2±1.38
18:1n7	3.90±0.25	2.95±0.38	3±0.08	2.59±0.13**
18:2n6	14.02±0.64	9.78±0.23***	*12.6±0.4	**11.3±0.97**
18:3n3	1.45±0.32	tr	tr	0.3±0.08***
20:2n6	0.27 ± 0.02	0.43±0.03	**0.78±0.06***	***0.29±0.02**
20:3n9	0.35±0.12	tr	0.17±0.03	***0.95±0.09***
20:3n6	0.6±0.13	0.88 ± 0.09	*1.23±0.06**	0.9±0.08**
20:4n6	6.78±1.72	21.27±1.92***	*16.3±0.5***	**16.3±0.94***
20:4n3	0.17±0.03	0.38±0.02***	tr	0.32±0.08***
20:5n3	0.9±0.23	0.73±0.29	$0.58 \pm 0.05*$	0.68±0.05**
21:5n3	0.67 ± 0.09	tr	0.3±0.01***	**0.78±0.18
22:4n6	0.58±0.07	1.58±0.09**	***0.85±0.05**	***0.66±0.08
22:5n6	0.23±0.15	$0.55 \pm 0.05*$	***0.17±0.03	***0.53±0.13***
22:5n3	1.32±0.16	1.62±0.27	1.68±0.09**	1.15±0.27
22:6n3	5.53±0.92	3.93±0.71	*2.22±0.08**	*2.85±0.53***
Total n6	36.4±1.1	34.32±1.86	31.9±0.26**	*27.1±1.93***
Total n3	8.77±0.22	6.47±1.19	4.55±0.1**	*3.75±0.68***
UI	183.5±20.8	167.92±3.14*	*140±1.35*	***118±11.3***

Table 3. Effect of high-fat diet on phospholipids and fatty acids percentage in the erythrocyte of rats.

UI -- unsaturation index (sum of products of double bond and FA relative percentage in each FA); tr -- less 0.1 %.

crease in eicosapentaenoic (20:5) and docosahexaenoic (22:6) FA. FA saturation index in rats of group 1 was low. The reduction of general erythrocyte lipids unsaturation revealed by the experiment is mostly conditioned by decrease in relative level of essential linoleic FA and redistribution between n6 and n3 family acids [29,30]. Metabolism of n6 FA is known to originate from linoleate consumed with food. Low linoleate content in rats of diet,

poor in PUFA. Further metabolic transformations of essential FA resulted in accumulation of arachidonic (20:4n6) FA, which is a predecessor of synthesis of anti-inflammatory leukotriens and thromboxanes with their strong aggregation and vasoconstriction properties [31, 32]. Growing share of 22:4n6 and 22:5n6 in erythrocyte lipids can be deemed a compensatory response to the deficiency of docosahexaenoic FA.

		Days of fed high-fat diet		
Parameter	Control group, n=10	30 days (group 1), n=10	90 days (group 2), n=10	30 days (group 1), n=10
MDA, mkmol/g Hb	4,6±0,3	5,3±0,3*	***8,1±0,2**	9,1±0,2***
GSH, nmol/g Hb	5,3±0,2	3,5±0,3***	**4,1±0,1*	**2,0±0,2***
Glutatione reductase, mkmol NADPH/mgHb/min	75,1±1,5	68,0±1,5**	*73,3±1,6	**50,1±2,4***
Glutathione peroxidase, mkmol GSH /mgHb/min	44,5±0,8	32,5±1,3**	**40,1±2,2	***21,4±1,2***

Table 4. Effect of high-fat diet on parameter of erythrocytes pro-oxidant and anti-oxidant.

In group 2 unsaturated fatty acids (12:0, p<0.001) increase in comparison to the control group. On the contrary, rats of group 3 demonstrated growth of 12:0 level (p<0.001). Accumulation of saturated 14:0, 16:0 FA in erythrocyte lipids group 3 was more evident than in rats of group 2. Modification of n6 composition in PUFA was distinguished by reducing 18:2n6 (p<0.01) share in comparison to the control group, reliable figures being obtained from rats of group 3; and growth of 20:2n6 (p<0.001), 20:3n6 (p<0.01) and 22:4n6 (p<0.01) content, more considerable in rat of group 2. Increase in 22:5n6 (p<0.001) share was noticed for rats of group 3 only. Rats subject to long-term high-caloric diet demonstrated deficit of n3 PUFA – 20:5n3 (p<0.01) and 22:6n3 (p<0.001) FA. 20:3n9 compensatory synthesis from 18:1n9 FA was a natural consequence of n3 deficit in rats with prolonged dyslipidemia. The change in FA composition profile, as revealed by the experiment indicates higher risk of cardiovascular pathology occurrence [30].

3.3. Erythrocytes Pro-Oxidant and Anti-Oxidant

MDA erythrocytes content increased in all groups of rats with dyslipidemia in comparison to the control group (**Table 4**). GSH level decreased in all groups after fed with high-fat feed in comparison to the control group (p < 0.001). GSH differences of rats in group 1 and group 2 had no statistical significance. Glutathione reductase activity decreased in erythrocytes in group 1 (p < 0.01) and group 3 (p < 0.001) in comparison to the control group (p < 0.001). Glutathione reductase activity in group 2 was higher then in group 1 (p < 0.05) and no statistical significance in comparison to the control group. Glutathione peroxidase activity decreased in 1 group and 3 group of rats with dyslipidemia. Glutathione reductase activity in group 2 had no statistical significance the control group.

4. CONCLUSIONS

Thus, generalizing results of researches it is possible to conclude that the important mechanism of adaptation of a cellular membrane to high-fat diet loading is increase major, structuring a membrane PL (PE) and minor, most metabolic significant fractions PL (PI), keeps homeostasis of 18:2n6 and 22:6n3 FA, 20:3n9 compensatory synthesis, decrease in activity of processes lipid peroxidation at the expense of activation of enzymes of glutathione redox-system and glutathione synthesis. Active formation and realization by a cage of the adaptable answer at high-fat diet loading occurs during the period from 30 till 90 days. Apparently, at the expense of exhaustion compensatory mechanisms in glutathione system and intensifications of processes lipid peroxidation for 180 days of fatty loading occur more essential deep reorganizations of lipids at biological membrane. This is one of the important mechanisms of pathogenesis heart diseases, metabolic syndrome. The received results of research expand knowledge of mechanisms of adaptation and dysadaptation cages at stressful loadings and can be a basis for diagnostics and treatment of a cellular pathology.

REFERENCES

- Jacobsen, M.U., Overvard, K., Dyerberg, J. *et al.* (2004) Dietary Fat and Risk of Coronary Heart Disease: Possible Effect Modification by Gender and Age. *Am. J. Epidemiol*, **160(2)**, 141-149.
- [2] Susan, M., Castracane, V., Mantzoros, S. (2004) Energy homeostasis, obesity and eating disorders: Recent Advances in Endocrinology. J. Nutr, 134, 295-298.
- [3] Wymann, M.P., Schneiter, R. (2008) Lipid signaling in disease. *Nature*, 9, 162-176.
- [4] Denke, M.A. (2005) Diet, lifestyle and nonstatin trials: review of time to benefit. *Am J Cardiol*, **96**, 3-10.
- [5] Hulbert, A.J., Turner, N., Storlien, L.H. *et al.* (2005) Dietary fats and membrane function: implications for metabolism and disease. *Biol. Rev. Camb. Philos. Soc*, 80(1), 155-169.
- [6] Beth, L., Marlies, K.O., Carlson, S.E. (2007) Diet (n-3) Polyunsaturated Fatty Acid Content and Parity Affect Liver and Erythrocyte Phospholipid Fatty Acid Composition in Female Rats. J. Nutr, 137, 2425-2430.
- [7] Beth, L., Marlies, K.O., Susan, E. Carlson. (2007) Diet (n-3) Polyunsaturated Fatty Acid Content and Parity Affect Liver and Erythrocyte Phospholipid Fatty Acid Composition in Female Rats. J. Nutr, 137, 2425-2430.
- [8] Berra, B., Mortontano, G., Adouni, L. *et al.* (1998) Serum lipids and lipid composition of red blood cell membranes

after diet with sunflower oil normal or high content of oleic acid. *Riv. Ital. Sostanze Grasse*, **75**, 127-132.

- [9] Jula, A., Marniemi, J., Ronnemaa, T. *et al.* (2005) Effects of diet and simvastatin on fatty acid composition in hypercholesterolemic men. *Arterioscler Thromb Vasc Biol*, 25, 1952-1959.
- [10] Poppitt, S.D., Kilmartin, P., Butler, P., Keogh, G.F. (2005) Assessment of erythrocyte phospholipid fatty acid composition as a biomarker for dietary MUFA, PUFA or saturated fatty acid intake in a controlled cross-over intervention trial. *Lipids Health Dis*, **4**, 30.
- [11] Poole, L.B., Karplus, P.A., Claiborn, A. (2004) Protein sulfenic asids in redox signaling. *Annu. Rev. Pharmacol. Toxicol*, 44, 325-347.
- [12] Iton, K., Ishii, T., Wakabayashi, N., Yamomoto, M. (1999) Regulatory mechanisms of cellular response to oxidative stress. *Free radic. Res*, **31**, 319-324.
- [13] Bea, F., Hudson, F.N., Chait, A. *et al.* (2003) Induction of glutathione synthesis in macrophages by oxidized lowdensity lipoproteins is mediated by consensus antioxidant response elements. *Circ. Res*, **92**, 386-393.
- [14] Format, H.J., Fukuto, J.M., Torres, M. (2004) Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act second messengers, Am. J. Physiol. *Cell Physiol*, **287**, 246-256.
- [15] Fan, J.G., Zhong, L., Xu, Z.J. (2003) Effect of low-cal orie diet on steatohepatitis in rats with obesity and hyperlipidemia. World J. Of Gastroenterology, 9(9), 2045-2049.
- [16] European Convention for the Protection of Vertebrate Animals used for exsperimental and other scientific purposes. *Strasburg: Council of Europe*, (1986), 51.
- [17] Bligh, E.G., Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol*, 37(8), 911-917.
- [18] Svetashev, V.I., Vaskovsky, V.E. (1972) A simplified technique for thinlayer chromatography of lipids. *J. Chromatogr.*; 67:376-378.
- [19] Vaskovsky, V.E., Kostetsky, E.X., Vasendin, J.M. (1975) A universal reagent for phospholipids analysis. J. Chromatogr, 111, 129-141.
- [20] Carreau, J.P., Duback, J. P. (1978) Adaptation of a macroscale method to the microscale for fatty acid methyl

transesterification of biological lipid extract. J. Chromatogr, **151(3)**, 84-390.

- [21] Stransky, K., Jursik, T., Vitek, A., Skorepa, J. (1992) An improved method of characterizing fatty acids by equivalent chain length values, J. High. *Res, Chromatogr*, 15, 730-740.
- [22] Yagi, K. (1987) Lipid peroxides and human diseases. *Chem Phys Lipids*, 45, 337-351.
- [23] Ellman, G.L. (1959) Tissue sulfhydryl group. Arch. Biochem. Biophys, 82: 70-77.
- [24] Ramos-Martines I.L., Torres A.M. (1985) Glutatione reductase of mantle tissue from sea mussel medulis 1. Purification and characterization two seasonal enzymatic forms. *Biochem. Physiol*, **80**(213), 355-360.
- [25] Mills, G.C. (1959) The purification and properties of glutathione peroxidase of erythrocytes. *J. Biol. Chem*, 234 (3), 502-506.
- [26] McIntyre, T.M., Zimmerman, G.A., Prescott, S.M. (1999) Biologically Active Oxidized Phospholipids. *J Biol Chem*, 274(36), 25189-25192.
- [27] Steenbergen, R., Nanowski, T.S., Nelson, R. *et al.* (2006) Phospholipid homeostasis in phosphatidylserine synthase-2-deficient mice. *Bioch. Et Biophys. Acta.* **1761(3)**, 313-323.
- [28] Hulbert, A.J., Turner, N., Storlien, L.H. *et al.* (2005) Dietary fats and membrane function: implications for metabolism and disease. *Biol. Rev. Camb. Philos. Soc*, 80(1), 155-169.
- [29] Simopoulos, A.P. (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*, 56, 365-79.
- [30] Harris, W.S., Poston, W.C., Haddock, C.K. (2007) Tissue n-3 and n-6 fatty acids and risk for coronary heart disease events. *Atherosclerosis*, **193**, 1-10.
- [31] Zhou L., Nilsson A. (2001) Sources of eicosanoid precursor fatty acid pools in tissues. J. Lipid Res, 42, 1521-1542.
- [32] Novgorodtseva, T.P., Karaman, Yu.K., Antoniuk, M.V., Zhukova, N.V. (2009) The role of free and esterified fatty acids in pathogenesis of metabolic syndrome. *Klin Med*, 87(5), 33-7.

Bioengineering functional copolymers. XII. Interaction of boron-containing and PEO branched derivatives of poly(MA-*alt*-**MVE) with HeLa cells**

Mustafa Türk¹, Zakir M. O. Rzayev^{2,*}, Gülcihan Kurucu²

¹Department of Biology, Faculty of Science and Art, Kırıkkale University, Yahsihan, 71450 Kirikkale, Turkey ²Department of Chemical Engineering, Faculty Engineering, Hacettepe University, Beytepe, 06800 Ankara, Turkey; Correspondence author: <u>zmo@hacettepe.edu.tr</u>

Received 20 October 2009; revised 20 November 2009; accepted 11 November 2009.

ABSTRACT

Novel boron-containing bioengineering copolymer and its α-hydoxy-ω-methoxy-poly(ethylene oxide (PEO) macrobranched derivatives were synthesized by (1) partially amidolysis of poly(maleic anhydride-alt-methyl vinyl ether) with ethanolamine ester of diphenylboronic acid and (2) esterification of synthesized B-containing copolymers with PEO. They had a combination of hydrophilic/hydrophobic linkages, free carboxylic groups, positive charges and an ionized organoboron linkage as antitumor sites, along with an ability to interact with HeLa cells. The structure, composition and properties (cytotoxicity and antitumor activity) of synthesized copolymers were investigated. In vitro cytotoxicity results, obtained by the fluore scence microscopy measurements indicate that unlike the virgin copolymer, boron-containing and PEO macrobranched derivatives exhibit higher antitumor activity. It was found that organoboron copolymer exhibits the most apoptotic and necrotic effects against HeLa cells whereas a minor effect relative to cancer cells was observed on L929 Fibroblast cells.

Keywords: Synthesis; Organoboron Copolymers; Structure-Property Relations; Cytotoxicity; Antitumor Activity; Apoptotic Effect

1. INTRODUCTION

The bioengineering functional polymers exhibit the characteristics of 1) alternating and random copolymers of maleic anhydride (MA) and 2) poly(ethylene oxide) (PEO), as well as 3) PEO grafted functional macro-molecules. They are of great interest for many researchers

due to their nontoxic, cell-compatible, biodegradable, stimuli-responsive properties, and therefore, a wide range of biomedical and bioengineering applications exist as drug or enzyme carriers and biomacromolecular conjugates both in diagnostics and chemotherapy as effective antitumor agents [1-8]. It is known that these copolymers can be regarded as pre-activated polymers due to the presence of anhydride moieties susceptible to the reaction with a primary amine of a biomolecule [9]. The alternating copolymers of maleic anhydride (MA) with methyl vinyl ether (MVE) or divinyl ether (DVE) were utilized in various applications in diagnostics [10,11] and in chemotherapy as effective antitumor agents [8]. Poly (MA-alt-DVE), known as pyran copolymer is one of the well known bioengineering polymers having a wide range of biological activity. It processes antitumor, antiviral, antibacterial and antifungal activities, induces interferon formation, and acts as an anticoagulant and anti-inflammatory agent [8,12-17]. Hirano et al. [18,19] reported that the poly (MA-alt-DVE) conjugated with bovine erythrocyte superoxide dismutase (SOD) is resistant against the proteolytic enzymes in serum, and shows a prolonged half-life in vivo. They established an increase in half-life after intravenous injection, as well as its decreased immunogenicity [19]. It was demonstrated that the copolymer-SOD conjugate shows anti-inflammatory effect against rat re-expansion pulmonary edema at the first step of leukocyte adhesion [15]. Maeda [20] discussed the development and therapeutic potential of prototype macromolecular drugs for use in cancer chemotherapy an artificial bioconjugate of neocarzinostatin (NCS) and poly(maleic acid-alt-styrene) copolymer. The biological response-modifiying effects, the mechanism of a tumor "enhanced permeability and retention" effect and the tumor-targeting mechanism of NCS-copolymer conjugate were also discussed. According to the author, a principal advantage in the use of this bioconjugate is the potential for a reduction or elimination of toxicity.

The copolymers of fumaric, citraconic and itaconic

acid and their derivatives as isostructural analogues of MA, as well as copolymers of some *N*-substituted maleimides can also included to class of bioengineering polymer systems. Cam *et al.* [21] evaluated the *in vitro* cytotoxicities of glycinylmaleimide (GMI) copolymers using K-562 human leukemia cells and HeLa cells. They also evaluated the *in vitro* antitumor activities of copolymers against mice bearing sarcome 180. Monomeric GMI and its copolymers showed higher antitumor activity than well known 5-fluorouracil (5-FU) at any dosage tested.

One the other hand, growing interest and much effort have been also focused on the synthesis of organoboron low molecular-weight functional compounds, biopolymer and drugs with boron ligands and evaluation of their suitability for the bioengineering applications. Aromatic boronic acid and its functional derivatives, and some functionalized carboranes have become an very important class organic compounds, which are utilized in a variety of biological and medical applications, such as carbohydrate recognition [22], neutron capture therapy for cancer treatment as effective tumour-targeting agents [23,24], especially for brain tumours [25,26], and protease enzyme inhibition [27]. Kataoka et al. [28-32] synthesized a novel water-soluble polymer with lectin-like function by introducing phenylboronates, as sugar -recognizing moieties, into the side-chain of poly (N,N-dimethylacrylamide) [28,29]. According to the authors, at physiological pH medium, phenyl-boronates form an appreciably stable complex with sialic acid (Neu5Ac), a chacteristic anionic carbohydrate on the surface of the plasma membranes [30,31]. Authors suggested that boronate-containing polymer may be an effective immune-adjuvant for the indu ction of lymphokine-activatd killer (LAK) cell [31]. They also demonstrated that the copolymers of 3-acry- lamidophenylboronic acid and dimethylacrylamide with different compositions coated onto solid substrates support function as synthetic mitogens for mouse lymphocytes [32].

However, a wide range of functional polymer synthesis techniques can be utilized for the design of more effective synthetic routes to prepare new B-containing bioengineering polymers, especially copolymerization of organoboron monomer and chemical modification of biocompatible polymers with organoboron reactive compounds and monomers. Several researchers synthesized some bioengineering copolymers containing phenylboronic acid linkages by radical copolymerization and chemical modification methods, which are exhibit glugose-, RNA- and DNA-sensitive behavior [33-36]. Recently, we report the synthesis and chracterization of organoboron copolymers by complex-radical copolymerization of *p*-vinylphenylboronic acid with *N*-isopropylacrylamide (NIPA), maleic and citraconic anhydrides, maleimide and chemical modification of poly(NIPA-

rand-MA)s with organoboron amine, as well as synthesis of supramacromolecular poly(ethylene imine) macrocomplexes and PEO long branched derivatives of organoboron copolymers having stimuli-responsive and high HeLa cell transfection behavior [37-40].

The objective of this work is to develop novel boroncontaining functional copolymers with antitumor activity. In the present article, results of synthesis and characterization of a new generation of biocompatible boron-containing functional macromolecules having a combination of hydrophilic and hydrophobic segments, free carboxvlic groups, positive charges and an ionized linkage as antitumor sities, along with an ability to conjugate with cancer cells were described and discussed. These organoboron copolymers were synthesized by 1) partially amidolysis of bioengineering alternating copolymer of maleic anhydride (MA) and methyl vinyl ether (MVE) with ethanolamine ester of diphenylboronic acid (EAPB) and 2) chemical modification (esterification) of synthesized organoboron copolymer with a-hydoxy-a-methoxy-poly (ethylene oxide (PEO). Special attention was paid to the role of structural effects, especially to the influence of organoboron linkage, for the interaction of organoboron functional copolymers with HeLa cells and to the evaluation of citotoxisity and antitumour activity by using a combination of various methods such as statistical, hematoxylen/eosin staining, apoptotic and necrotic cell indexes, and M30 immunostaining analyses.

2. MATERIALS AND METHODS

2.1. Materials

Ethanolamine ester of diphenyl boronic acid (EAPB) (Sigma-Aldrich, Germany) was purified by recrystallization from anhydrous ethanol: m.p. 193.5°C (by DSC). ¹H NMR spectra (δ , ppm) in CHCl₃-d₁: CH₂-O 1.49, CH₂-NH₂ 2.96, and 7.38-7.40 (1H), 7.19-7.24 (2H) and 7.13-7.16 (2H) for protons of *p*-, *o*-and *m*-positions in benzene ring, respectively. Poly(maleic anhydride-*alt*-methyl vinyl ether), poly(MA-*alt*-MVE) (C1) (Sigma-Aldrich, Germany): M_n 80,000 g.mol⁻¹, T_g 148°C (by DSC); ¹H NMR spectra (δ , ppm) in DMSO-d₆: CH₂ 1.23, CH-O 2.11, O-CH₃ 2.08 and CH-CH 3.38. α -Hydoxy- ω -methoxy-poly(ethylene oxide) (Fluka; PEO, M_n 2000 g.mol⁻¹): ¹H NMR spectra (δ , ppm) in CHCl₃-d₁: CH₂-O 3.75-3.45, OH end group 2.61 and O-CH₃ end group 2.16.

Human cervix epithelioid carcinoma cell line (HeLa) was obtained from the tissue culture collection of the SAP Institute (Turkey). Cell culture flasks and other plastic material were purchased from Corning (USA). The growth medium, which is Dulbecco Modified Medium (DMEM) without L-glutamine supplemented fetal calf serum (FCS), and Trypsin-EDTA were purchased from Biological Industries (Israel). M30 CytoDEATH antibody (Roche).

2.2. Synthesis

Boron-contaning copolymer (C1-B) was synthesized by the partially amidolysis of succinic anhydride units of alternating copolymer C1 with EAPB, containing a primary amine group, in the 1,4-dioxane solution at 40°C for 3 h under nitrogen atmosphere at molar ratio of C1:EAPB = 2:1. Approbriate quantities of C1 and EAPB, solvent were placed in a standard Pyrex-glass tube and flushed with dried nitrogen gas for at leeast 3 min, then placed in a carousel type microreactor with a thermostated heater and magnetic mixer. The resulting copolymer C1-B was isolated from reaction mixture by precipitating with diethyl ether. Purification of copolymers was done by dissoving in dioxane and reprecipitating in diethyl ether, extraction with hexane and draying under vacuum at 50°C until constant weight.

PEO macrobranched copolymer (C1-B-PEO) was synthesized by the esterification of anhydride units of partially amidolysed C1-B copolymer with PEO, containing an end hydoxyl group, in the same conditions using in our previous publications [5,37].

2.3. Characterization

FTIR spectra of the organoboron copolymers (KBr pellet) were recorded with FT-IR Nicolet 510 spectrometer in the 4000-400 cm⁻¹ range, where 30 scans were taken at 4 cm⁻¹ resolution. ¹H { 13 C} NMR spectra were performed on a JEOL 6X-400 (400 MHz) spectrometer with DMSO-*d*₆ as a solvent at 25°C.

The differential scanning calorimetry (DSC) analysis was performed on a Shimadzu calorimeter (Japan) at a heating rate of 5°C/min, under nitrogen atmosphere. The X-ray diffraction (XRD) patterns were obtained from a Rigaku D-Max 2200 powder diffractometer. The XRD diffractograms were measured at 2 θ , in the range 1-50°, using a Cu-K_a incident beam ($\lambda = 1.5406$ Å), monochromated by a Ni-filter. The scanning speed was 1 °/min, and the voltage and current of the X-ray tubes were 40 kV and 30 mA, respectively.

The number of living and dead cells were counted with a haemacytometer (C.A. Hausse & Son Phluila, USA) at X200 magnification. The number of apoptotic and necrotic cells were determined by Fluorescence Inverted Microscope (Olympus IX70, Japan). The cell images were also recorded using the both above mentions microscopes. Statistical analyses were performed using Student's *t*-test for unpaired data and *P* values of less than 0.05 were considered significant. Data are presented as means \pm SEM (standard errors of the mean).

2.3. Cytotoxicity

For cytotoxicity experiments, HeLa cells and L929 Fibroblast cells respectively. (25x10³ cells per well) were placed in DMEM by using 24-well plates. Different amounts of copolymers (C1, C1-B and C1-B-PEO)

(about 50-500 μ g.mL⁻¹ in aqueous solutions) were put into wells containing cells, respectively. The plates were kept in the CO₂ incubator (37 °C in 5% CO₂) for 2-24 h; the medium was replaced with fresh medium, and incubated at the same conditions for 24 h. Following of this incubation, HeLa cells and L929 Fibroblast cells were harvested with trypsin-EDTA, and then were dyed with trypan blue [41]. The viable cells were counted with a haemacytometer (C.A. Hausse & Son Phluila, USA), using light microscope.

2.4. Hematoxylen/Eosin Staining

HeLa cells and L929 Fibroblast cells $(25 \times 10^3 \text{ cells per well})$ were placed in DMEM by using 24-well plates. After treating with different amount functional copolymers (C1, C1-B and C1-B-PEO) (about 50-500 µg.mL⁻¹ in aqueous solutions) for 2-24 hours period, the medium was removed, the cells washed with distilled water and fixed in ethanol, and stained with Hematoxylen/Eosin. After staining, the cells were observed by light microscopy. By this way, cellular and nuclear morphology have been shown in cultured cells stained with Hematoxylen/Eosin.

2.5. Analysis of Apoptotic and Necrotic Cells

Double staining were performed to quantify the number of apoptotic cells in culture on basis of scoring of apoptotic cell nuclei. HeLa cells and L929 Fibroblast cells $(25 \times 10^3 \text{ cells per well})$ were placed in DMEM by using 24-well plates. After treating with different amount functional copolymers (C1, C1-B and, C1-B-PEO) (about 50-500 µg.mL⁻¹ in aqueous solutions) for 2-24 hours period, both attached and detached cells were collected, then washed with PBS and stained with Hoechst dye 3342 $(2 \ \mu g.mL^{-1})$, propodium iodide (PI) $(1 \ \mu g.mL^{-1})$ and DNAse free-RNAse (100 µg.mL⁻¹) for 15 min at room temperature. After that 10-50 µL of cell supension was smeared on slide and coverslip for examination by fluorescence microscopy [42,43]. The nuclei of normal cells were stained light blue but apoptotic cells were stained dark blue by the hoechst dye. The apoptotic cells were identified by their nuclear morphology as a nuclear fragmentation or chromatin condensation. Necrotic cells were staining red by PI. Necrotic cells lacking plasma membrane integrity and PI dye cross cell membrane, but PI dye don't cross non necrotic cell membrane. The number of apoptotic and necrotic cells in 10 randomly chosen microscopic fields were counted and the result expressed as a ratio of apoptotic and necrotic to normal cells.

2.6. M30 Immunostaining

The percentage of apoptotic cells was determined by M30 CytoDEATH antibody [44]. This is a monoclonal mouse immunoglobulin (Ig) G2b antibody (clone M30; Roche,

Mannheim, Germany) that binds to a caspase-cleaved, formalin-resistant epitope of cytokeratin 18 cytoskeletal protein. The immunoreactivity of the M30 antibody is confined to the cytoplasm of apoptotic cells. HeLa cells $(25x10^3 \text{ cells per well})$, treated with C1, C1-B and, C1-B-PEO copolymers (about 50-500 ug. mL⁻¹ in aqueous solutions) for about 2-24 h, were fixed in 10% neutral-buffered formalin for 15 min, treated with 0.3% hydrogen peroxide in methanol for 10 min to block the endogenous peroxidase activity, washed in the standard phosphate buffer solution, and then incubated with M30 antibody at room temperature for 1 h. In negative controls, preimmune mouse serum instead of primary antibody was used. Immunoreactions were revealed by the avidin-biotin complex technique using diaminobenzidine (DAB) as substrate. We counted the number of M30positive cytoplasmic staining cells in all fields found at x400 final magnification. For each image, three randomly selected microscopic fields were observed, and at least 100 cells/field were evaluated. M30 CytoDEATH antibody was not sensetive to L929 Fibroblast. On account of this reason, M30 CytoDEATH antibody did not applied to L929 Fibroblast cells.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of Organoboron Functional Copolymers

Boron-containing bioengineering functional copolymer (C1-B) and its α -hydoxy- ω -methoxy-poly(ethylene oxide) (PEO) long branched derivatives were synthesized by (1) amidolysis of succinic anhydride units of biocompatible poly(MA-*alt*-MVE) alternating copolymer (C1) with EAPB containing a primary amine group, and (2) esterification (grafting) of free anhydride units of partially amidolysed C1-B copolymer with PEO, containing an end hydoxyl group, respectively. General scheme of synthesis of the organoboron functional copolymer and its PEO branched derivative can be represented as follows (**Scheme 1**).

The synthesized boron-containing copolymers contain a combination of hydrophilic/ hydrophobic linkages, free carboxylic groups, positive charges and ionized organoboron linkage as antitumor sities, along with an ability to interact with canser biomacromolecules, especially with HeLa cells. The chemical and physical structure, composition and properties (temperature-responsiveness, glass-transition, melting and degradation temperatures, andantitumor activity and cytotoxicity) of synthesized copolymers were characterized by spectroscopy (FTIR, ¹H and ¹³C NMR), viscometry, DSC, X-ray diffraction and Fluorescence microscopy analyses.

The results of chemical structural analysis of the syn thesized organoboron copolymers FTIR (KBr pellet) and (¹H and ¹³C) NMR spectroscopy (in DMSO- d_6 solution)

were summarized in **Table 1** (FTIR analysis data for C1-B) and illustrated in **Figure 1** (NMR spectra of C1-B) and **Figure 2** (FTIR spectra of C1-B-PEO). The formation of amide, carboxyl and organoboron groups in the structure of C1-B copolymer as results of amidolysis reaction was confirmed by apearance of the correspoded characteristic absorption bands for each monomer unit and diphenylboronic fragment in the spectra. Absorption



Scheme 1. Schematic representation of the synthesis routes of organoboron functional copolymers (C1-B and C1-B-PEO) by the amidolysis of poly(MA-*alt*-MVE) (C1) with organoboron amine (AEPB) and esterification of poly (MA-*alt*-MVE)-*g* -AEPB) (C1-B) with PEO, respectively.

 Table 1. The results of FTIR analysis organoboron functional copolymer: Poly(MA-alt-MVE)-g-AEPBA) (C1-B).

Absorption bands (cm ⁻¹)	Band assignments
MA unit	
1980-1925 (w)	C=O (overtones)
1864 (m-s), 1781 (vs)	C=O stretching (anhydride)
1227 (s, broad), 1094 (s)	C–O and C–O–C bands
650 (w)	CH (in chain backbone)
MVE unit	
2942 (m-s)	CH ₃ C–H stretching
2854 (m)	CH ₂ C–H (chain backbone)
1475-1416 (m)	CH ₂ and CH ₃ deformation
1372 (m)	CH ₃ deformaton (in O–CH ₃)
975 (m)	CH3 rocking
926 (vs)	C–O deformation
735 (m-w), 720 (w)	CH ₂ and CH ₃ deformation
Maleamide unit	
1736 (m-s)	C=O strtching (in -COOH)
1575-1510 (m-w)	COO ⁻ stretching (H-bonding)
1650 (m),1720 (m-w)	NH–C=O amide I band
1315 (w)	amide III band
Organoboron linkage	
3240 (w), 3100,1600 (m)	CH= (in aromatic ring)
1545 (m-w)	B–O stretching
1443 (m), 1420 (w)	B–Ph aromatic ring
1180 (m)	CH in-put-bending
770 (w)	CH out-put-bending
702 (m)	O-B-Ph aromatic ring

bands at 1864 and 1781 cm⁻¹, relating to C=O groups of free anhydride units, indicated the partially amidolysis of these units as shown in **Scheme 1**.

From the comparative analysis of ¹H and ¹³C NMR spectra of virgin alternating copolymer (C1) and its organoboron derivative (C1-B) (Figure 2a, 2b), the following changes of the characteristic signals were observed: unlike the spectra of C1 copolymer having the peaks from chemical shifts of the CH and CH₂ backbone and CH₃ (in methoxy group) protons new signals from protons of amide NH, COOH and phenyl groups (in or ganoboron linkage) were appeared in the spectra of organoboron copolymer (C1-B). More detailed informations about micristructure of C1-B copolymer were pre-pared by analysis of ¹³C NMR spectra (Figure 2c). The following chemical shifts (δ , ppm) of carbon atoms were observed in the spectra: 174.4 (-C=O of the maleamide and anhydride units), 128-136 (-B-C₆H₅ mono-substitued benzene ring), 77.2 (-CH-NH in organoboron linkage), 58.01 (-CH-O), 49.08 (-CH-CH-chain backbone), 30.3 (–CH₃-O), and 30.15 (–CH₂).

Chemical structure of C1-B-PEO long branched copolymer was confirmed by the appearance in the FTIR spectra (Figure 3) the following characteristic absorption bands (cm⁻¹): 3400 (strong broad peak for OH in Hbonded carboxyl groups), 2933-2735 for C-H stretching in CH₂ and CH₃), 2667 and 2600 (C-H stretching in CH₂-O of PEO branched segments), 2280 and 2135 (Fermi doublet for C-N band), 1986 and 1966 (overtones of C=O), 1746 (C=O of ester groups), 1710 (C=O of carboxyl groups), 1630 (NH-C=O amide I band), 1592 (phenyl groups), 1558 (H-bonded COO⁻ stretching), 1545 (weak peak for B–O stretching), 1490 (C-H deformation for CH₂–O in PEO branches), 1480 and 1466 (CH₂ deformation), 1450 (B-Ph aromatic ring), 1405 (amide III band), 1372 and 1352 (CH₃ deformation in O-CH₃), 1115 (broad peak for C-O band in CH₂-O and CH₃-O of PEO and MVE units, respectively), 948 (strong peak for C-O deformation in PEO branchs), and etc.

The comparative analysis of the XRD patterns of alternating copolymer and its organoboron derivative show a significant difference between physical structures of these copolymers (Figure 3). C1 copolymer has an amorphous structure, while C1-B copolymer exhibits pseudo-crystallinity behavior (without re-crystallization process due to macromolecular physical interactions via H-bonding, hydrophobic-hydrophilic interactions, etc.) with degree of pseudo-crystallinity $\gamma_c = 26.2$ % (by XRD analysis), glass-transition $T_{\rm g}$ and pseudo-melt phase transition $T_{\rm m}$ at 84.2°C and 136.3 °C, respectively (by DSC analysis). It can be proposed that the producing the amphiphilic organoboron linkages in side chain of copolymer causes a formation of hydrophilic/hydrophobic balance, more polar amide and carboxyl groups, which are able to form strong H-bonded segments, and therefore, self-assembled suramacromolecular structure of C1-B

copolymer as in other organoboron polymer systems [37].

3.2. Cytotoxicity of the Copolymer and its B-Containing and PEO Branched Derivatives

In this study, the comparative analysis of HeLa cells (cancel cells) and L929 Fibroblast cells (normal cells) has been investigated. The cytotoxicities of C1 copolymer and corresponding C1-B, C1-B/PEO derivatives were inquired about the utility for antitumor drugs. Figures 4 and 5 give the number of viable cancer and normal cells in each group after incubation of the cells with copolymer and organoboron copolymers at their different concentrations for 24 h incubating time in cell culture media, respectively. Under the same conditions, the wells containing cells without copolymers were also studied as a control. The following important results can be drawn from this graph which is illustrated in these figures. The C1 copolymer does not exhibit any observable toxicity in the chosen range of copolymer concentration. The toxicity of polymers containing boron (C1-B and C1-B-PEO) was significant, most probably due to hydrogen bonding supramacromolecular structure of these copolymers containing a combination of hydrophilic/hydrophobic linkages, free carboxylic groups, which are formed after partial amidolysis of anhydride containing copolymer C1 and full hydrolysis of free anhydride units in the chosen physiological medium where positive charges and ionized organoboronoxy groups also exist as antitumor sites along with an ability to interact with cells.

It was observed that an increase of C1-B and C1-B-PEO concentrations in each well caused higher degree of dying cells as compared to virgin C1 copolymer tested under the same conditions. C1-B copolymer exhibits relatively higher in vitro cytotoxicity than C1-B-PEO branched copolymer which can be explained by the higher content of organoboron linkages in C1-B copolymer. It is important to note that the boron containing side chain linkages, rather than the individual copolymers, increase the cytotoxicity more profoundly; an important feature which has a significant role in leading us to the present study. C1 copolymer had less toxicity compared to cultured cells at various quantities and different incubation times. On the contrary, the toxicity of C1-B and C1-B-PEO organoboron copolymers towards the HeLa cells increased by increasing their quantity from 50 to 500 μ g.mL⁻¹, whereas, no significant change was observed with varying time. According to Figure 4, C1 did not show high toxicity at all although the copolymer amount was increased from 50 to 500 µg.mL⁻¹ whilst, a significant toxicity of C1-B andC1-B-PEO (100 µg.mL⁻¹ and above) started to be observed when cancer and normal cells (Figure 5) were incubated for about 4 h. As the amount of boron con taining polymers and their incubtion



Figure 1. ¹H NMR spectra of (A) C1 copolymer and (B) C1-B organoboron copolymer; (C) ¹³C NMR spectra of C1-B copolymer.

time increased, toxicity to cultured cells was increased. C1-B-PEO and especially C1-B showed higher toxicity at 500 μ g. mL⁻¹. Thus, it can be concluded that virgin C1 alternating copolymer does not exhibit any toxic effect on cultured HeLa cells, whereas, its organoboron and PEO branched derivatives are definitely toxic to cells. In particular, C1-B copolymer containing relatively high amount of organoboron linkages exhibits high toxicity toward cancer cells compared to normal cells at 500 μ g.mL⁻¹ for 24 h.

3.3. Staining Results

The important observations can be summarized as follows: we checked for apoptosis or necrosis with double staining (Hoescht 3342 and PI), M30 immunostaining for cancer cells. For the morphological observations, cancer and normal cells were stained by hematoxylen-eosin.

3.4. Hematoxylen-Eosin Staining Results

In this study, C-1 copolymers treated cancer and normal



Figure 2. FTIR spectra of PEO macrobranched organoboron copolymer (C1-B-PEO).



Figure 3. XRD patterns of (A) alternating copolymer (C1) and (B) its branched organoboron derivative.

cells have intact nucleus of about 50-200 µg.mL⁻¹ concentration during 2-14 h incubation. Cell morphology has not been changed at the same concentration for 2-14 h (**Figure 6b**). While C1-B and C1-B-PEO copolymers



Figure 4. In vitro cytotoxicity of C1, C1-B and C1-B-PEO copolymers with different amount at 24 h incubation. Number of viable HeLa cells in wells. Results are presented as means \pm SEM. *Significant difference from control (p < 0.05).



Figure 5. *In vitro* cytotoxicity of C1, C1-B and C1-B-PEO copolymers with different amount at 24 h incubation. Number of viable L929 Fibroblast cells in wells. Results are presented as means \pm SEM. *Significant difference from control (p < 0.05).

treated HeLa cells has no morphological changes at 50-200 μ g.mL⁻¹ concentration for about 2-4 h, they have vacuole formation in their cytoplasms with C1-B copolymer between 6-12 hours (**Figure 6c**). Vacuole formations determined rarely in normal cells (**Figure 6e, f**). In addition, cell membranes have lysed with C1-B copolymer around 12-24 h but, there was no change in their nuclei of cancer and normal cells. Moreover, some of the cells (30% and 15% for HeLa and fibroblast, respectively) have been detached from the well. Unaffected cells displayed similar morphological characteristics as with untreated (control) cells.

3.5. Double Staining and M30 Immunostaining Results

In this study, if the HeLa and L929 Fibroblast cells treated by C1, C1-B, C1-B-PEO copolymers at low conce ntration for a short time, the number of apoptotic and necrotic cells was not high (Tables 2 and 3). However, if the polymer concentration and incubation time were increased, the number of apoptotic and necrotic cells was increased as well. Especially, the number of apoptotic and necrotic cells was increased when they were treated by C1-B copolymer at 500 µg.mL⁻¹ concentration in cancer cell culture for 24 h (Table 2). The number of apoptotic and necrotic Fibroblast cells was not increased according to HeLa cells at the same concentration (Table 3). If cells were treated by the other copolymer under similar conditions, their apoptotic index was below 30 %. The results obtained at 500 μ g.mL⁻¹ concentration for 24 h are shown in Table 2. Meanwhile, apoptotic HeLa cells were immunostained by M30 antibody (Figure 7a, 7b). The double staining and M30 immunostaining results were similar to each other in HeLa cells. Apoptotic indexes of HeLa cells for M30 immunostaining were 12% for C1, 45 % for C1-B, 23 % for C1-B-PEO at 500 µg.mL⁻¹ and 24 h incubation. Apoptotic L929 Fibroblast cells were stained only double staining method (Table 3). In addition to these polymers, especially boron containing polymers had toxic effects towards cancer and normal cells. But toxic effect of boron containing polymers was lower to normal cells than cancer cells. After an incubation at 50-500 µg.mL⁻¹ for 24 h period, C1 resulted in less apoptosis, while incubation with C1-B and C1-B-PEO at the same concentration and incubation time led to high apoptosis of HeLa cells compared to L929 Fibroblast cells. Both C1-B and C1-B-PEO may well inhibit cell growth and viability in HeLa (Figure 7b, c, d) and L929 Fibroblast cells (Figure 7e, f). One the other hand, around 50-500 µg.mL⁻¹ of C1-B and C1-B-PEO copolymer contents for 24 h gave rise to an increase in necrosis stained with PI dye (Figure 7c, e and Tables 2 and 3). It is important to note that incubation for 24 h with 500 µg. mL⁻¹ C1-B produced apoptosis supporting its high toxicity and necrotic effect. Furthermore, incubation without polymers as control cells resulted in a few PI-positive cells. Whereas, cells exposed to C1-B and C1-B-PEObecame highly PI-positive, suggesting that they were in necrosis. HeLa cells incubated with a high dose of boron containing copolymers resulted in rupture of cell membrane at around 12-24 h incubation period. Cell cytoplasm was discharged out of HeLa cells. On the otherhand, great of number vacuole originated in most of HeLa cells cytoplasma. It may have given rise to metabolicchanges of cells, affected by boron containing copolymers. C1-B copolymer was more toxic than virgin counder the testing conditions determined by us.



Figure 6. Light microscope image of (A) non stained HeLa cell culture as a control, (B) C1-B-PEO copolymer/HeLa cells conjugate (stained with hematoxilen-eosin dye); dense spots were showed nucleus of cells, and distinct violet were indicated cytoplasma of cells as a control, (C) Light microscope image of vacuole of HeLa cells cytoplasma; dense spots were showed nucleus of cells in C1-B copolymers (500 µg.mL⁻¹ consantration) at 24 h incubation. Light microscope image of (D) non stained L929 Fibroblast cell culture as a control, (B) C1-B-PEO copolymer/L929 Fibroblast cells conjugate (stained with hematoxilen-eosin dve): dense spots were showed nucleus of cells. and distinct violet were indicated cytoplasma of cells as a control, (C) Light microscope image of vacuole of L929 Fibroblast cells cytoplasma; dense spots were showed nucleus of cells in C1-B copolymers (500 µg.mL⁻¹ consantration) at 24 h incubation. Images (A) and (D) taken under X200 magnification, others images taken under x400 magnification.

4. CONCLUSIONS

This work has attempted to develop novel bioengineering functional organoboron copolymers (C1-B and C1-B-PEO), namely, amphiphilic macromolecules of which contained hydrophilic/hydrophobic fragments, ethylene amidodiphenylborinate linkages, long branched PEO segments and free carboxylic groups with an ability to conjugate with cancer HeLa cells. These copolymers were synthesized by amidolysis and esterification of anhydride units of poly(MA-*alt*-MVE) (C1) as a bio-

Table 2. The comparative analysis of apoptotic and necrotic HeLa cell index for copolymer (C1), organo-boron (C1-B) and organoboron PEO branched (C1-B-PEO) copolymers at 24 h incubation.

Polymer amount	Apoptotic	Necrotic
(µg.mL ⁻¹)	cells (%)	cells (%)
C1		
control	3 ± 2	4 ± 2
50	3 ± 1	5 ± 1.5
100	6 ± 1.5	9 ± 3
250	8.5 ± 1	13.8 ± 1.5
500	15 ± 2	27 ± 3
C1-B		
control	5 ± 3	2 ± 1
50	13 ± 2	20 ± 3
100	18 ± 2.5	31 ± 4
250	32 ± 1	41 ± 2
500	43 ± 2	53 ± 3
C1-B-PEO		
control	3.5 ± 1.5	5 ± 1.5
50	9 ± 1	11 ± 1.5
100	12 ± 1	16 ± 2
250	15 ± 2	25 ± 3
500	28 ± 2.5	43.5 ± 5

Table 3. The comparative analysis of apoptotic L 929 Fibroblast cells index for copolymers (C1), organoboron (C1-B) and organoboron PEO branched (C1-B-PEO) copolymers at 24 h incubation.

Polymer amount	Apoptotic	Necrotic
(µg.mL ⁻¹)	cells (%)	cells (%)
C1		
control	2±1	3 ±1
50	3±1	3±1.5
100	4 ± 1	6±2
250	7±1	11±1.5
500	9±1.5	17±2
C1-B		
control	2±1	2±1
50	7±2	12±2
100	12±2.5	17±3
250	16±1	25±2
500	27±2	38±2
C1-B-PEO		
control	3±2	3±1
50	4±1	8±1.5
100	6±1	11±2
250	9±2	16±3
500	17±2.5	30±1

compatible and non toxic polymer matrix with organoboron amine and PEO, respectively. Chemical and physical structure of organoboron copolymers were confirmed by FTIR and ¹H (¹³C) NMR spectroscopy and X-ray powder diffraction methods. The comparative analysis of novel organoboron functional copolymers with antitumor acivity towards cancer and normal cells was achieved. It was found that unlike the virgin amorphous C1 copolymer, organoboron copolymer (C1-B) exhibited semi-crystalline phase transition behaviour due to the formation of self-assembled supramacromolecular structures through strong intra-and intermolecular hy-



Figure 7. Light microscopy images of (A) virgin (nonapoptotic) HeLa cells as a control group (stained with M30 immunostaining kit), and (B) organoboron copolymer C1-B copolymer/HeLa cells conjugate (stained with M30 immunostaining kit), where brown cytoplasma of cells image indicates the formation of apoptotic cells; Fluorescence microscopy image of (C) nucleus of HeLa cells (stained with PI), where formation of red spots demostrates nucleus of necrotic cells, and (D) nucleus of HeLa cells (stained with Hoescht 3342), where dense spots indicates nucleus of apoptotic cells. Fluorescence microscopy image of (E) nucleus of L929 Fibroblast cells (stained with PI), where formation of red spots demostrates nucleus of necrotic cells and green spots demostrates nucleus of living cells, and (F) nucleus of L929 Fibroblast cells (stained with Hoescht 3342), where dense spots indicates nucleus of apoptotic cells. İmages of Cand D were recorded with x400 magnification, others image were recorded with x200 magnification.

drogen bonding. The interactions of these copolymers with HeLa cells were investigated by using a combination of different methods such as cytotoxicity, statistical, hematoxylen/eosin staining, apoptotic and necrotic cell indexes, M30 immunostaining, double staining and M30 immunostaining, light and fluorescence microscopy analyses. In vitro cytotoxicities and antitumor activities of organoboron copolymers (C1-B and C1-B-PEO) against human cervix epithelioid carcinoma cell line (HeLa) was as well evaluated. It was observed that organoboron copolymers exhibited the most apoptotic and necrotic effects against HeLa cells whereas a minor effect relative to cancer cells was observed on L929 Fibroblast cells. Thus the obtained results allow us to propose that synthesized organoboron copolymers containing a combination of non toxic and biocompatibile polymer matrix and long branched PEO segments with functional groups as antitumor sities, can be utilized as therapeutic potential functional copolymer drugs, which are able to form an artificial bioconjugate with HeLa cells, in cancer chemotherapy.

5. ACKNOWLEDGEMENTS

The supports of the Turkish National Scientific and Technical Council (TÜBİTAK) through project TBAG-2486 and HU Scientific Research

Foundation (BAB) through the BAB-2601006 project are kindly acknowledged.

REFERENCES

- [1] Albertsson, P.A. (1986) *Partition of Cell Particles and Macromolecules*. New York, Wiley.
- [2] Herold, D.A., Keil, K., Bruns, D.E. (1989) Oxidation of polyethylene glycols by alcohol dehydrogenase.*Biochem Pharmacol*, 38(1), 73.
- [3] Sinha, V.R., Aggarwal, A., Trehan, A. (2004) Biodegradable PEGylated microspheres and nanospheres. *Amer J Drug Deliv*, 2(3), 157.
- [4] Köşeli, V., Rzaev, Z.M.O., Pişkin, E. (2003) Bioengineering functional copolymers. III. Synthesis of biocompatible poly(NIPA-co-MA)-g-PEO/PEI macrocomplexes and their thermostabilization effect on the activity of the enzyme penicillin G acylase. J Polym Sci Part A: Polym Chem, 41(11), 1580.
- [5] Mazi, H., Kibarer, G., Emregül, E., Rzaev, Z.M.O. (2006) Bioengineering functional copolymers. IX. Poly[(maleic anhydride-co-hexene-1)-g-poly(ethylene oxide)]. Macromol Biosci, 6(4), 311.
- [6] Mazi, H., Emregül, E., Rzaev, Z.M.O., Kibarer, G. (2006) Preparation and properties of invertase immobilized on a poly(maleic anhydride-hexen-1) membrane. *J Biomater Sci, Polym Ed*, 17, 821.
- [7] Rzaev, Z.M.O., Dinçer, S., Pişkin, E. (2007) Functional copolymers of *N*-isopropyl-acrylamide for bioengineering applications. *Prog Polym Sci*, **32**(**5**), 534.
- [8] Butler, G.B. (1992) Cyclopolymerization and cyclopolymerization. New York, Marcel Dekker.
- [9] Veron, L., Bignicount, M.C.D., Delair, T., Pichot, C., Mandrand, B. (1996) Syntheses of poly [*N*-(2,2-dimethoxyethyl)-*N*-methyl acrylamide] for the immobilization of oligo-nucleotides. *J Appl Polym Sci*, **60(2)**, 235.
- [10] Ladavière, C., Delair, T., Domard, A., Pichot, C., Man-drand, B. (1999) Covalent immobilization of biological molecules to maleic anhydride and methyl vinyl ether copolymers-A physico-chemical approach. J Appl Polym Sci, 71(6), 927.
- [11] Chaix, C., Minard-Basquin, C., Delair, T., Pichot, C., and Mandrand, B. (1998) Oligonucleotide synthesis maleic anhydride copolymers covalently bound to silica spherical support and characterization of the on obtained conjugates. *J Appl Polym Sci* **70**(12), 2487.
- [12] Voľkova, F., Gorshkova, M.Yu., Ivanov, P. E., Stotskaya, L. L. (2002) New scope for synthesis of divinyl ether and maleic anhydride copolymer with narrow molecular mass distribution. *Polym Adv Technol*, **13(10-12)**, 1067.
- [13] Izumrudov, V.A., Gorshkova, M.Yu., Volkova, F. (2005) Controlled phase separations in solution of soluble poly-electrolyte complex of DIVEMA (copolymer of divinyl ether and maleic anhydride. *Eur Polym J*, **41**(6), 1251.
- [14] Ladavière, C., Delair, T., Domard, A., Pichot, C., Mandrand, B. (1999) Covalent immobilization of bovine serum albumin onto (maleic anhydride-alt-methyl vinyl ether) copolymers. *J Appl Polym Sci*, 72(12), 1565.
- [15] Delair, T., Badey, B., Domard, A., Pichot, C., Mandrand, B. (1997) *Polym Adv Technol*, 8(5), 297.

- [16] Patel, H., Raval, D.A., Madamwar, D., Sinha, T.J.M. (1997) Polymeric prodrugs. Synthesis, release study and antim-icrobial properties of polymer-bound acriflavine. *Angew Makromol Chem*, 245(1), 1.
- [17] Patel, H., Raval, D.A., Madamwar, D., Patel, S.R. (1998) Polymeric prodrug: Synthesis, release study and antimicrobial property of poly(styrene-*co*-maleicanhydride)-bound acriflavine. *Angew Makromol Chem*, **263(1)**, 25.
- [18] Hirano, T., Todorski, T., Kato, S., Yamamoto, H., Caliceti, P. (1994) Synthesis of the conjugate of superoxide dismutase with the copolymer of divinyl ether and maleic anhydride retaining enzymatic activity. *J Control Release*, 28(1-3), 203.
- [19] Hirano, T., Todorski, T., Morita, R., Kato, S., Ito, Y., Kim, K., Shukla, G., Veronese, F., Maeda, H., Ohashi, S. (1997) Anti-inflammatory effect of the conjugate of superoxide dismutase with the copolymer of divinyl ether and maleic anhydride against rat re-expansion pulmonary edema. J Control Release, 48(2-3), 131.
- [20] Maeda, H.H. (1991) SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy. Adv Drug Delivery Rev, 6(2), 181.
- [21] Gam, G.-T., Jeong, J.-G., Lee, N.-J., Lee, W., Ha, C.-S., Cho, W.-J. (1995) Synthesis and biological activities of copolymers of *N*-glycinyl maleimide with methacrylic acid and vinyl acetate. *J Appl Polym Sci*, **57**(2), 219.
- [22] Claracq, J., Santos, S., Duhamel, J., Dumousseaux, C., Corpart, J.M. (2002) Rigid interior of styrene-maleic anhydride copolymer aggregates probes by fluorescence spectroscopy. *Langmuir*, **18**(10), 3829.
- [23] James, T.D., Sandanayake, S., Shinkay, S. (1996) Saccharide sensing with molecular receptors based on boronic acid. *Angew Chem Inter Ed Eng*, **35**(17), 1910.
- [24] Barth, R.F., Yang, W., Rotaru, J.H., Moeschberger, M.L., Boesel, C.P., Soloway, A.H., Joel, D.D., Nawrocky, M.M., Ono, K., Goodman, J.H. (2000) Boron neutron cupture therapy of brain tumors: enchanced survival and cure following blood-brain barrier disruption and intracarotid injection of sodium borocaptate and boronophenyl aniline. *Int J Radiat Oncol Biol Phys*, **47**(1), 209.
- [25] Siebert, W. (Ed.) (1887): Advances in Boron Chemistry. Cambridge, Royal Society Chemistry.
- [26] Mishima Y. (Ed.) (1996) *Cancer Neutron Capture Therapy*. New York, Plenum Press.
- [27] Kettner, C.A. and Shenvi, A.B. (1984) Inhibition of the serine proteases leukocyte elastase, pancreatic elastase, cathepsin G, and chymotrypsin by peptide boronic acids. J *Biol Chem*, 259(24), 15106.
- [28] Miyazaki, H., Kikuchi, A., Kitano, S., Koyama, Y., Okano, T., Sakurai, Y., Kataoka, K. (1993) Boronate-containing polymer as novel mitogen for lymphocytes. *Biochem Biophys Res Commun*, **195**(2), 829.
- [29] Aoki, T., Nagao, Y., Terada, E., Sanui, K., Ogata N., Yamada, N., Sakurai, Y., Kataoka, K., Okano, T. (1995) Endothelial cell differentiation into capillary structures by copolymer surfaces with phenylboronic acid group. J Biomater Sci Polym Ed, 7(7), 539.
- [30] Otsuka, H., Uchimura, E., Koshino, H., Okano, T., Kataoka, K. (2003) Anomalous binding profile of pheny-lboronic acid with *N*-acetylneuraminic acid (Neu5Ac) in aqueous solution with varying pH. J Am

Chem Soc, 125(12), 3493.

- [31] Uchimura, E., Otsuka, H., Okano, T., Sakurai, S., Kataoka, K. (2001) Totally synthetic polymer with lectin-like function: Induction of killer cells by the copolymer of 3-acryl-amidophenylboronic acid with *N*,*N*-dimethylacrylamide. *Biotech Bioeng*, **72(3)**, 307.
- [32] Otsuka,H., Ikeya, T., Okano, T., Kataoka, K. (2006) Activation of lymphocyte proliferation by boronatecontaining polymer immobilised on substrate: The effect of boron content on lymphocyte proliferation. *Eur Cells Mater*, **12**(1), 36.
- [33] Kataoka, K., Miyazaki, N., Okano, T., Sakurai, Y. (1994) Sensitive glucose-induced change of the lower critical solution temperature of poly [*N*,*N*-dimethylacrylamide*co*-3-(acrylamido) phenylboronic acid] in physiological saline. *Macromolecules*, 27(4), 1061.
- [34] Uguzdoğan, E., Denktaş, E.B., Tuncel, A. (2002) RNAsensitive *N*-isopropylacrylamide/vinylphenyl boronic acid random copolymer. *Macromol Biosci*, 2(5), 214.
- [35] Uguzdoğan, E., Kayi, H., Denktaş, E.B., Patir, S., Tuncel, A. (2003) Stimuli-responsive properties of aminophenylboronic acid carrying thermosensitive copolymers. *Polym Int*, **52(5)**, 649.
- [36] Shiomori, K., Ivanov, A.E., Galaev, I.Yu., Kawano, Y., Mattiasson, B. (2004) Thermo-responsive properties of sugar sensitive copolymer of *N*-isopropylacrylamide and 3-(acrylamido)phenylboronic acid *Macromol Chem Phys*, 205(1), 27.
- [37] Rzayev, Z.M.O., Beşkardeş, O. (2007) Boron-containing functional copolymers for bioengineering applications.

Collect Czech Chem Commun, 72(12), 1591.

- [38] Kahraman, G, Beşkardeş, O., Rzayev, Z.M.O., Pişkin, E. (2004) Bioengineering functional copolymers. VII. Synthesis and characterization of boron-containing self-assembled supramolecular architectures. *Polymer*, 45(17), 5813.
- [39] Çimen, E.K., Rzayev, Z.M.O., Pişkin, E. (2005) Bioengi-neering functional copolymers. V. Synthesis LCST, and thermal behavior of poly(*N*-isopropylacrylamide-*co-p*-vinyl-phenylboronic acid). *J Appl Polym Sci*, **95(3)**, 573.
- [40] Rzayev, Z.M.O., Erdoğan, D., Türk, M., Pişkin, E. (2008) Bioengineering functional copolymers. VIII. Stimuli-responsive boron-containing graft copolymers and their poly(ethylene imine) macrocomplexes and DNA conjugates. J Biol Chem, 36(2), 83.
- [41] Türk, M., Dincer, S., Yulug, I.G., Piskin, E. (2004) In vitro transfection of HeLa cells with temperature sensitive polycationic copolymers. *J Control Release*, 96(2), 325.
- [42] Choi, S.-J., Oh, J.-M., Choy, J.-H. (2009) Toxicological effects of inorganic nanoparticle nanoparticles on human lung cancer A549 cells. *J Inorg Biochem*, **103**(3), 463.
- [43] Ulukaya, E., Kurt, A., Wood, E.J. (2001) 4-(*N*-hydroxyphenyl)retinamide can selectively induce apoptosis in human epidermoid carcinoma cells but not in normal dermal fibroblasts.*Cancer Invest*, **19**(2), 145.
- [44] McPartland, J.L., Guzail, M.A., Kendall, C.H., Pringle, J.H. (2005) Apoptosis in chronic viral hepatitis parallels histological activity: an immunohistochemical investigation using antiactivated caspase-3 and M30 cytodeath antibody. *Int J Exp Pathol*, 86(1), 19.

The central nucleus of amygdala is involved in tolerance to the antinociceptive effect of NSAIDs

Merab G. Tsagareli¹, Nana Tsiklauri¹, Gulnazi Gurtskaia¹, Ivliane Nozadze¹, Elene Abzianidze²

¹Beritashvili Institute of Physiology, Tbilisi, Georgia; <u>tsagareli@biphysiol.ge</u> ²State Medical University of Tbilisi, Tbilisi, Georgia

Received 12 October 2009; revised 20 November 2009; accepted 27 November 2009.

ABSTRACT

Aim: Repeated microinjections of non-opioid analgesics into the midbrain periaqueductal gray matter and rostral ventro-medial medulla induce antinociception with development of tolerance. Antinociception following systemic administration of non-steroidal anti-inflammatory drugs (N SAIDs) also exhibit tolerance. Presently our aim was to investigate the development of tolerance to the antinociceptive effects of NSAIDs analgine, ketorolac, and xefocam microinjected into central nucleus of amygdala (Ce) in rats. Methods: Under anesthesia with thiopental a stainless steel guide cannula was stereotaxically implanted unilaterally or bilaterally into the Ce using stereotaxic atlas coordinates, and anchored to the cranium by dental cement. Five days after surgery, 3 µl of these NSAIDs were injected via the injection cannula while the rat was gently restrained. Twenty min post microiniection, i.e. 10-min before the peak of the drugs' effect is normally reached, animals were tested with tail flick (TF) and hot plate (HP) tests. On the 5th experimental day all animals received a Ce microiniection of morphine. Results: Daily microinjection of NSAIDs into the Ce uni- or bilaterally, produced antinociception with development of complete tolerance over a 5-day period. Following the treatment period, morphine microinjection into the Ce failed to elicit antinociception, indicating cross-tolerance to the antinociceptive effect of N SAIDs. In other words, the "non-opioid tolerant" rats showed cross-tolerance to morphine. Conclusions: Our data confirmed the suggestion that NSAIDs interact with endogenous opioid systems, which likely play a key role in the development of tolerance to the antinociceptive effects of NSA IDs.

Keywords: Descending Inhibition; Morphine Cross-Tolerance; Nociception

1. INTRODUCTION

Microinjection of non-opioid analgesics metamizol, and lysine-acetylsalicylate (LASA) into certain brain areas, including the midbrain periaqueductal gray matter (PAG) and rostral ventro-medial medulla (RVM), produces antinociception with development of some degree of tolerance [1-4]. We have also observed tolerance to the antinociceptive effects of analgine (metamizol), ketorolac, and xefocam administered systemically [5-7]. These studies are consistent with the possibility that endogenous opioidergic mechanisms associated with descending pain modulation may partly mediate the tolerance observed with non-steroidal anti-inflammatory drugs (NSAIDs) [8].

The amygdala receives massive input from the hippocampus and the neocortex and provides a major source of afferents to PAG [9]. Analgesia resulting from microinjection of opioid agonists into the basolateral amygdala is blocked by lidocaine inactivation of, or opioid antagonist injection into, the PAG [10-12]. Cortical afferents to the amygdala largely target its basolateral component. The basolateral amygdala then projects to the central nucleus of amygdala (Ce), which in turn projects densely to the PAG [13]. The Ce also receives nociceptive input, both directly from the spinal cord, and indirectly via a large projection from the dorsal horn to the parabrachial nucleus [14,15]. The Ce is an integral component of the endogenous pain-modulatory circuit and is critical for systemic morphine-induced suppression of spinal nociceptive reflexes [16].

The present study reports that microinjection of analgine, ketorolac, and xefocam into the Ce of rats elicits antinociception with the development of tolerance.

2. MATERIALS AND METHODS

The experiments were carried out using male Wistar rats, 200-250g in body weight, bred at the Beritashvili Institute of Physiology. The animals were kept under standard housing conditions (22 ± 2 °C, 65% humidity, light from

7:00 a.m. to 8:00 p.m.) and were fed a standard dry diet; water was freely available. Experiments were performed during the light phase of the circle between 10:00 and 14:00 clock. Guidelines of the International Association for the Study of Pain regarding animal experimentation and Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996) were followed throughout.

Under anesthesia with thiopental (55 mg/kg, i.p. "Kievmed" Ukraine) a 12-mm stainless steel guide cannula (Plastic One, Inc., USA) was stereotaxically implanted unilaterally (left side) or bilaterally into the Ce amygdala using coordinates from the atlas of Paxinos G. & Watson C. [17], and anchored to the cranium by dental cement. The guide cannula was plugged with a stainless steel stylet. Thereafter, the rats were handled in 3 daily 15 min periods to become habituated to the experimental environment and test protocol that involved removal of the stylet and insertion of injection cannula (10 mm length of PE tubing attached to a 50 µl Hamilton syringe; Hamilton, Inc., USA) without drug injection. Five days after surgery, three µl of drug was injected via the injection cannula while the rat was gently restrained. Drugs were: Analgine (metamizol sodium, 1.5mg/3µl, "Sanitas", Ltd, Lithuania), ketorolac (ketorolac tromethamine, 90µg/3µl, "Zee Drugs", India), xefocam (lornoxicam, 12µg/3µl, "Nycomed", GmbH, Austria), or saline (3µl) ("Galichpharm" Ltd. Ukraine). Twenty min post microinjection, i.e. 10min before the peak of the drugs' effect is normally reached, animals were tested with TF or HP. For the TF test, the distal part of the tail was stimulated with a light beam (IITC #33, IITC Life science, Inc., Woodland Hills, CA, USA) and the latency measured until the tail was reflexively flicked away from the beam. For the HP test, the rat was placed on a 52°C hot plate (IITC #39) and the latency to lick paw or jump was measured. The cut-off time was 20 s for both TF and HP latencies. Each rat was tested with both tail flick (TF) and hot plate (HP) latencies in the same session. The same procedure was followed to deliver repeated microinjections of each drug (analgine, ketorolac, xefocam) or vehicle (saline) over five consecutive days. On the 5th experimental day all animals received a Ce microinjection of morphine hydrochloride (3µg/2µl, "Laboratoires Stella", France) and TF and HP latencies were measured 20 min thereafter. At the conclusion of experiment on the fifth day, the microinjection site was marked with 2 µl of a saturated solution of Pontamine Sky Blue (Sigma Chemical Co., USA), and the animal was sacrificed by ester inhalation. After fixation by immersion in 10% formalin the brain was sectioned and the microinjection site was identified with the aid of Paxinos & Watson' stereotaxic atlas [17].

All data are presented as mean±S.E.M. Analysis of variance (ANOVA) with post-hoc Tukey-Kramer multiple comparisons were used for statistical evaluations. The

statistical software utilized was InStat 3.05 (GraphPad Software, Inc, USA). Statistical significance was ac-knowledged if P<0.05.

3. RESULTS

Only rats with microinjections into Ce were included for data analysis. Histological location of microinjection sites is shown in simplified drawing section from the Paxinos and Watson atlas [17] (Figure 1). These data consisted of 13 rats microinjected with analgine (6 uniand 7 bilaterally), 13 with ketorolac (6 and 7), 12 with xefocam (6 and 6), and 15 with control saline (8 and 7), respectively. Injection sites outside the boundaries of the Ce (the shaded region in Figure 1) were not included in data analysis. In special control experiments with intentionally microinjections of NSAIDs out of Ce we did not reveal significant changes in TF and HP latencies (data not shown).

On the first test day, unilateral microinjection of each NSAID into the Ce produced antinociception as revealed by significant increases in latency for TF [ANOVA: F(3,20)=21.251; P<0.001] and HP [ANOVA: F(3,20)= 15.872; P<0.001] compared to saline controls (P<0.001 for all drugs) (Figure 2). However, on successive days, microinjection of each NSAID had a progressively weaker antinociceptive effect such that on the fourth and/or fifth experimental days the TF and HP latencies were not significantly different compared to saline injections. This was similar to the development of tolerance to morphine administration to PAG in similar preparations [18,19], and we therefore refer to it as "non-opioid tolerance". Note, however, that tolerance to the antinociceptive effect of xefocam was slower compared to analgine and ketorolac. On day 5, both experimental and



Figure 1. Location of microinjection sites in the Ce. Only NSAIDs and saline injections within the shaded regions were included in data analysis. Coronal sections are taken from the atlas of Paxinos and Watson (1998). The Distances from interaural line ± 5.7 mm and from the bregma -3.3 mm respectively. All injections fell within ± 0.5 mm of this coronal plane.

control groups received a morphine microinjection at the same Ce sites, and only the saline group exhibited antinociception (P<0.001). The latencies of the non-opioid tolerant rats were not altered by the morphine microinjections, i.e. they showed cross-tolerance to morphine (**Figure 2**).

Bilateral microinjections into the Ce also increased latency of TF [ANOVA: F(3,20)=8.873; P=0.006] and HP [ANOVA: F(3,20)=11.933; P<0.001] compared to control rats on the first day for these NSAIDs, in TF (P<0.01) and in HP (P<0.001) respectively (**Figure 3**). Similar to the unilateral drug injections, there was a progressive decline in the antinociceptive effect elicited by bilateral injection of each drug over the 5-day period,



Figure 2. Response latencies in unilateral microinjections. Mean response latencies following unilateral microinjections of each NSAIDs are plotted over the 5-day period, followed by morphine, for TF (A) and HP (B) tests. Cutoff latency (20 sec) was reached in some animals for xefocam and morphine microinjections. Asterisks mark significant difference of latency in comparison to saline control as analyzed by post hoc tests (*p < 0.05, **p < 0.01, ***p < 0.001).



Figure 3. Response latencies in bilateral microinjections. Mean response latencies following bilateral microinjections of each NSAIDs are plotted over the 5-day period, followed by morphine, for TF (A) and HP (B) tests. Cutoff latency (20 sec) was reached in some animals for xefocam and morphine microinjections. Asterisks mark significant difference of latency in comparison to saline control as analyzed by post hoc tests (*p<0.05, **p<0.01, ***p<0.001).

such that TF and HP latencies were not significantly different from saline controls after the 4th-5th day (**Figure 3**). Again, xefocam exhibited a slower time course for development of tolerance. Bilateral microinjection of each NSAIDs also exhibited cross-tolerance to morphine as compared with saline controls (P<0.001) (**Figure 3**).

4. DISCUSSION

The present study revealed that microinjection of analgine, ketorolac, and xefocam into the Ce induced antinociception in awake rats. This confirmed our previous results with systemic (i.p.) administration of NSAIDs [5-7], and results of others using microinjection of the same NSAIDs into the PAG [2-4]. Importantly, repeated microinjections of NSAIDs into the Ce resulted in a progressive decrease in antinociceptive effectiveness (tolerance) similar to that observed with intra-PAG injections [2-4], and reminiscent of the effect of opiates.

A major involvement of opioidergic mechanisms in tolerance to the analgesic effect of NSAIDs was surprising, because traditionally the cellular and molecular actions of opioids were thought to differ from those of nonopioid analgesics. One interesting aspect of NSAIDs administration, namely tolerance, emphasizes their similarity to opioid analgesics. Indeed, microinjection of metamizol [3,4,20], or LASA [2,20] into PAG or into Ce, progressively led to a loss of their antinociceptive effects, i.e. produced tolerance. Furthermore, tolerance to metamizol or LASA was accompanied by cross-tolerance to morphine [2-4] as if opioid analgesics had been repeatedly administered. Interestingly, tolerance to the effect of PAG-microinjected metamizol can, like tolerance to morphine, be reversed by microinjection of proglumide, a cholecystokinin antagonist, at the same PAG site [3]. The latter finding constituted additional evidence that the PAG effects of non-opioid analgesics are similar to those of morphine. Moreover, the data suggested that Ce should be incorporated into current models of endogenous pain control circuitry [21].

It is well known that morphine injection after administration of NSAIDs or in combination, morphine plus NSAIDs usually potentiates their own analgesic effects [8]. We have recently tested each of NSAIDs for crosstolerance to morphine given over a 5-day period in two age groups of rats. There was a significant difference between adult and juvenile rat groups for the degree of morphine analgesia, which was most marked on the first and second experimental days. Furthermore, morphine-tolerant rats exhibited cross-tolerance to analgine, ketorolac, and xefocam for both TF and HP tests, respectively (data not shown).

In conclusion, our data confirmed previous studies indicating that the antinociceptive action of NSAIDs may be closely related to that of endogenous opioids, including the development of tolerance. In addition, the Ce along with PAG and RVM represents an important component of the endogenous antinociceptive system.

5. ACKNOWLEDGEMENTS

The authors would like to thank Professor E. Carstens for helpful comments and English revisions of the manuscript. This research was supported by the grant from Georgian National Science Foundation (GNSF/ST07/ 6-234).

REFERENCES

[1] Jones, S.L. (1996) Dipyrone into the nucleus raphe mag-

nus inhibits the rat nociceptive tail flick reflex. *Eur J Pharmacol*, **318**, 37-40.

- [2] Pernia-Andrade, A.J., Tortorici, V. and Venegas, H. (2002) Induction of opioid tolerance by lysine-acetylsalicylate in rats. *Pain*, **111**, 191-200.
- [3] Tortorici, V., Nogueira, L., Salas, R. and Venegas, H. (2003) Involvement of local cholecystokinin in the tolerance induced by morphine microinjections into the periaqueductal gray of rats. *Pain*, **102**, 9-16.
- [4] Tortorici, V. and Vanegas, H. (2000) Opioid tolerance induced by metamizol (dipyrone) microinjections into the periaqueductal gray of rats. *Eur J Neurosci*, **12**, 4074-4080.
- [5] Tsagareli, M.G., Tsiklauri, N., Lagidze, T., Gurtskaia, G. and Abzianidze, E. (2005) Tolerance induction by nonopioid analgesics in rats. *Proc Georgian Acad Sci Biol Series A*, **31**, 903-909.
- [6] Tsiklauri, N., Gurtskaia, G. and Tsagareli, M.G. (2006) Is endogenous opioid system involved in non-opioid analgesics tolerance? *Georgian Med News*, 8(137), 121-125.
- [7] Tsiklauri, N. and Tsagareli, M.G. (2006) Non-opioidinduced tolerance in rats. *Neurophysiol*, 38, 370-373.
- [8] Hernandez-Delgadillo, G.P. and Cruz, S.L. (2006) Endogenous opioids are involved in morphine and dipyrone analgesic potentiation in the tail flick test in rats. *Eur J Pharmacol*, **546**, 54-59.
- [9] Aggleton, J.P. (Ed) (1996) The amygdala: Neurobiological aspects of emotion, memory, mental dysfunction. *Willey-Liss*, New York
- [10] Bodnar, R.J. (2000) Supraspinal circuitry mediating opioid antinociception: Antagonist and synergy studies in multiple sites. *J Biomed Sci*, 7, 181-194.
- [11] Helmstetter, F.J., Tershner, S.A. and Poore, L.H. (1998) Antinociception following opioid stimulation of the basolateral amygdala is expressed through the periaqueductal grey and rostral ventromedial medulla. *Brain Res*, 779, 104-118.
- [12] Pavlovič, Z., Cooper, M. and Bodnar, R. (1996) Opioid antagonists in the periaqueductal gray inhibit morphine and beta-endorphin analgesia elicited from the amygdala of rats. *Brain Res*, **741**, 13-26.
- [13] Rivzi, T.A., Ennis, M. and Behbehani, M.M. (1991) Connections between the central nucleus of the amygdala and the midbrain periaqueductal gray: Topography and reciprocity. *J Comp Neurol*, **303**, 121-131.
- [14] Gauriau, C. and Bernard, J.F. (2002) Pain pathways and parabrachial circuits in the rat. *Exp Physiol*, 87, 251-258.
- [15] Gauriau, C. and Bernard, J.F. (2004) A comparative reappraisal of projections from the uperficial laminae of dorsal horn in the rat: forebrain. *J Comp Neurol*, 468, 24-56.
- [16] Manning, B.H. and Mayer, D.J. (1995) The central nucleus of the amygdala contributes to the production of morphine antinociception in the tail-flick test. *J Neurosci*, **15**, 8199-8213.
- [17] Paxinos, G. and Watson, C. (1998) The rat brain in stereotaxic coordinates, 4th edition. *Academic Press*, New York.
- [18] Morgan, M.M., Clayton, C.C. and Boyer-Quick, J.S. (2005) Differential susceptibility of the PAG and RVM to tolerance to the antinociceptive effect of morphine in the rat. *Pain*, **113**, 91-98.
- [19] Tortorici, V., Nogueira, L., Aponte, Y. and Vanegas, H.

Openly accessible at http://www.scirp.org/journal/HEALTH/

(2004) Involvement of cholecystokinin in the opioid tolerance induced by dipyrone (metamizol) microinjections into the periaqueductal gray matter of rats. *Pain*, **112**, 113-120.

[20] Vanegas, H. and Tortorici, V. (2002) Opioidergic effects

of non-opioid analgesics on the central nervous system. *Cell Mol Neurobiol*, **22**, 655-661.

[21] Price, D.D. (1999) Psychological mechanisms of pain and analgesia. *IASP Press*, Seattle.

Paediatric gallstones and laparoscopic cholecystectomy

D. G. Samuel, N. N. Naguib, A. Y. Izzidien

Prince Charles Hospital, Merthyr Tydfil, UK; daisams@doctors.org.uk

Received 19 October 2009; revised 30 November 2009; accepted 2 December 2009.

ABSTRACT

Introduction: Gall stones disease is a rare occurrence in paediatric patients and the diagnosis is often overlooked. Patients often present with non-specific symptoms of abdominal pain and the classic features of gallstones are sometimes absent [1]. The aim of our study is to increase the awareness of cholecystitis and acute pancreatitis being a possible occurrence in the paediatric age group and should therefore be in the differential diagnosis of acute abdominal pain in children. We undertook a retrospective analysis of all the paediatric patients recorded as having had a laparoscopic Cholecystectomy performed at Prince Charles Hospital. 8 paediatric patients underwent Laparoscopic cholecystecomy between 2000 and 2008 consisting of 5 female patients and 3 male patients. The average age of the cohort was 14.1 years [12-16]. Premorbid obesity was a feature in 4 patients and all patients reported high fat diet. Abdominal UItrasound used to assess all 8 patients who presented with acute abdomen showed gallstones to be present in all. 7 patients underwent an elective procedure 3-6 months after the initial diagnosis was made and 1 patient had laparoscopic Cholecystectomy within 72 hours of initial presentation. 1 patient was found to have an inherited haematological disorder and 2 of the patients were sisters with a family history of gallstone disease. 2 patients presented with acute pancreatitis. Gallstone related cholecystitis is a rare occurrence amongst paediatric patients and is often overlooked as a differential diagnosis. We report 8 patients over an 8 year period. Conclusion: It is important that clinicians include cholecystitis and biliary colic in the differential diagnosis of patients presenting with acute abdomen in childhood not explained by other diagnoses. Laparoscopic Cholecystectomy is the treatment of choice and has minimal complications.

Keywords: Gallstones; Cholecystitis; Paediatrics; Laparoscopic Cholecystecomy

1. INTRODUCTION

Gallstones in children are rare but can become a potentially serious condition [1]. It does not always present itself in the classical clinical picture of adult gallstones and is not considered as a typical differential diagnosis of abdominal pain [1,2]. Right upper quadrant pain, nausea and vomiting may not always be present and initial diagnosis may be overlooked or delayed. We present our experience as a paediatric surgical team in managing Paediatric gallstones at a District General Hospital in South Wales. In order to assess how common paediatric gallstones are at a typical District general Hospital we re- viewed our patient records was carried at Prince Charles Hospital to identify any paediatric patient (aged 16 years or under) who had undergone a Cholecystectomy. Theatre reports and full case history notes were reviewed and data was extracted accordingly. Correspondence were also re- viewed to note the outcome of any subsequent follow up and to identify any other medical problems which may have arisen after the procedure or had an impact on the patient developing gallstones. All 8 patients underwent a laparoscopic Cholecystectomy performed by a single Consultant Paediatric Surgeon (Mr Asal Y Izzidien) at Prince Charles Hospital, Merthyr Tydfil.

2. THE PATIENT COHORT

All patients presented with abdominal pain but only 3 (37.5%) patients localised this pain in the right upper quadrant. All patients eventually underwent Abdominal Ultrasound examination (USS) which showed the presence of gallstones. 3 patients underwent a Magnetic Retrograde Cholangio-Pancreotography and 1 patient underwent a HAIDA scan to assess gallbladder function. Every patient had a full history work up including a family history screen. Blood tests were assessed in each patient for the signs of haemolytic abnormalities. 2 patients (25%) presented with pancreatitis and 1 patient had a known hereditary haemolytic condition (hereditary spherocytosis). This patient presented with jaundice and had an obstructive biochemical pattern.

Appendicitis was the initial working diagnosis in 3 of our patients and a Urinary tract Infection was suspected and treated empirically in another 2 patients.

Following diagnostic confirmation of gallstones, all 8 patients underwent laparoscopic Cholecystectomy. 7 patients underwent the procedure electively 4-6 months after the initial diagnosis was made and one patient had the procedure within 72 hours of initial presentation. When we reviewed the histopathology of our sample all 8 patients had evidence of acute cholecystitis changes in their gallbladder with oedematous, thick walled gallbladders. Stones were analysed and in 7 patients the stones were reported as being mixed cholesterol pigment stones. 1 patient had pigmented stones in keeping with their hereditary haemolytic condition.

Our only short term complication was one patient who developed an infection in his umbilicus portal site scar. This was treated with Augmentin 250/62 mg tds. He recovered and was discharged without any further problems. One patient developed keloid at one of her scar sites although no further action was taken and the patient was discharged.

3. DISCUSSION

Paediatric gallstones and pancreatitis are rare occurrence and in most cases, caused by trauma or chronic illness [1-3]. Gallstone related disease can often present in a non-specific manner and the classical signs are often absent. Murphy's sign is unreliable and children may find it difficult to describe the typical pain of biliary colic and cholecystitis. Clinicians often misdiagnose the condition as a Urinary tract infection and Appendicitis is also commonly documented in differential diagnoses [1,2]. Usually there is a chronic history of generalised abdominal pain of some months or more. In several cases, this had not been followed up to identify an underlying cause. Clinicians should therefore hold an index of suspicion for a diagnosis of gallstones in any paediatric presentation of abdominal pains, raised White cell count and fever [3].

Patients should undergo screening and evaluation for hereditary haemolytic conditions [1] as an increased breakdown of haemoglobin can produce large amounts of pigmented gallstones. Other underlying causes such as obesity, hypercholesterolemia, chronic liver disease and Cystic fibrosis may also be the primary cause of paediatric gallstones and should be excluded. There may be also a need to investigate for genetic predisposition as 2 of the cases were sisters and had strong family history of gall stones.

In the paediatric group non-invasive investigations should be the preferred choice if possible. Abdominal Ultrasound provides an effective method of identifying gallstones [2,4]. MRCP may be used to exclude stones in the common bile dict [5].

Weight loss is almost always advised in adult patients and the same rule appears to hold true for the paediatric population. Most of our patients were obese and being overweight is recognised as a risk factor for developing gallstones. Modern diets are often high in fat and with an obesity epidemic being predicted by some corners of medical society, the medical profession needs to be aware that gallstones are likely to become more common in children in years to come. Other factors associated with gallstones include use of the Oral contraceptive pill [4] and pregnancy at an earlier age may also contribute to a rise on paediatric gallstones in years to come.

4. CONCLUSIONS

There is increase in the incidence of gallstones in the paediatric age group possibly due to the worldwide epidemics of obesity but genetic predisposition may be a factor that needs more study to explore. Our study shows that the condition remains an uncommon presentation but should be considered in children presenting with abdominal symptoms, especially when other diagnoses have been excluded. Laparoscopic Cholecystectomy app- ears to be and appropriate and safe procedure to carry out on paediatric patients and has a low incidence of morbidity. It should therefore be considered as the procedure of choice in paediatric patients [6,7] with gallstones. Due to early development of gall stones, hence the longer span of this pathology, paediatric patients may be more prone to developing complications including pancreatitis. It is paramount to prevent this condition as this carries a high risk of morbidity and mortality in the paediatric cohort [5.8.9].We advocate carrying out surgery at the earliest opportunity. However, decisions should be taken on an individual basis if the clinical status of the patient dictates that more immediate surgery would be appropriate. At present our hospital has not performed Endoscopic procedures on paediatric patients and cases are typically referred to tertiary centres. However, if the incidence of paediatric gallstones continues to rise in line with childhood obesity, the likelihood of having to performing Endoscopic retrograde Cholangio Pancreatography (ERCP) will increase. This will in itself will have implications for service provision, patient safety and long term implications for patients.

5. ACKNOWLEDGEMENTS

D. S. carried out the relevant literature review and wrote the paper. He also analysed the patient data and reviewed the patient notes. N. N. and A. I. reviewed the paper and critically analysed the data. A. I. performed all the operative procedures

REFERENCES

 Sutton, R. and Cheslyn-curtis, S. (2001) Acute gallstone pancreatitis in childhood. *Ann R Coll Surg Engl*, 83, 406-408.
- [2] Bruck, S.W., Sigmund, H.E., Rocchi, C. and Kim, P.C.W. The management of nonpigmented gallstones in children. *Journ Paediatr Surg*, **35**, 729-732.
- [3] Davenport, M. (2003) Laparoscopic surgery in children review. *Ann R Coll Surg Engl*, **85**, 324-330.
- [4] Haddock, G., Coupar, G., Youngson, G.G., MacKinlay, G.A. and Raine, P.A. (1994) Acute pancreatitis in children: A 15-year review. J Paediatr Surg, 29, 719-22
- [5] Lerner, A., Branski, D. and Lebenthal, E. (1996) Pancreatic diseases in children. *Paediatr Clin North Am*, 43, 125-56.
- [6] De Caluwe, D., Akl, U. and Corbally, M. Cholecystectomy

verus cholecystolithotomy for cholelithiasis in childhood: Long-term outcome. *Journ Paedr Surg.*, **36(10)**, 1518-1521.

- [7] St Peter, S.D., Keckler, S.J., Nair, A., Andrews, W.S., Sharp, R.J. *et al.* (2008) Laparascopic cholecystectomy in the paediatric population. *J Laparoendosc Adv Tech A.*, 18(1), 127-30.
- [8] Weizman, Z. and Durie, P.R. (1988) Acute pancreatitis in childhood. *J Paediatr*, **113**, 24-9
- [9] Lowe, M.E. and Greer, J.B. (2008) Pancreatitis in children and adolescents. *Curr Gastroenterol Rep.*, 10(2), 128-35.

Troponin based studies in search of a biomarker for cardiac arrest

Pasha Ghazal^{1,2}, Kaneez Fatima Shad¹, Nikhat Sidduiqui²

¹Dr Panjwani centre for molecular medicine and drug design, ICCBS, University of Karachi, Karachi, Pakistan ²Neurochemistry Research Unit, Department of Biochemistry, University of Karachi, Karachi, Pakistan; <u>ftmshad@yahoo.com</u>, <u>ftmshad@gmail.com</u>

Received 3 November, 2009; revised 13 December, 2009; accepted 14 December, 2009.

ABSTRACT

Cardiac arrest is shown to be a cause of a large number of deaths not only in Pakistan but around the globe. The prevalence of this disease demands identification of its etiology. The science of proteomics can be used to identify cardiac specific proteins. The subsequent over expression or under expression of these proteins can be utilized as targets not only for therapeutical interventions but also for identifving molecular signatures for Cardiac diseases. In context of a number of studies which have shown that the specificity of serum biomarkers like troponin (cTnI and cTnT) are questionable as they may also appear in serum in pathological conditions other than cardiac dysfunction, the search of a specific marker for cardiac arrest becomes imperative. In this study protein profiling of cardiac arrest patients was performed after its quantification through Bradford assay. SDS-PAGE and 2 DE techniques were used as to characterize proteins. The samples of the patients prior to characterizing of proteins were subjected to lipid and cardiac enzymes profiling. The results of these investigations have shown an increase in almost all of these parameters by many folds from that of normal values. In addition to this the samples were found out to be positive for troponin T which strongly confirms the incidence of the cardiac arrest. The results of SDS-PAGE exhibited the induction of three proteins of 100 kDa, 97 kDa and of 66 kDa with 100 kDa as the most highly expressed protein. In addition to that SDS-PAGE gels have shown the down regulation of 45 kDa protein, again indicating the changes as a result of cardiac arrest. 2DE gel patterns of cardiac arrest samples demonstrated higher number of protein spots as compare to control in the alkaline range, which might suggest their role in cardiac dysfunction. Therefore it can be concluded that this study may pave the grounds for identification of such proteins which can serve not only as potential therapeutical targets but also as candidate markers for accurate diagnosis of the disease.

Keywords: Cardiac arrest; Troponins; Proteomics, SDS-PAGE; 2DE; Therapeutical Targets.

1. INTRODUCTION

Cardiovascular diseases(CVD) have been identified as being the single most significant cause of morbidity [1] and global mortality, accounting for almost 17 million deaths annually i.e. 30% of global mortality, moreover survival rates from cardiac arrest is less than 1% [2]. This situation is prevalent not only in the developing countries but in the western industrialized part of the world as well. The statistical analysis is in evidence to this, in United States of the 2 400 000 US deaths in 1999, 720 000 (30%) were directly attributed to cardiac diseases. Of this number, the US Centers for Disease Control and Prevention estimated that 462 000, or 64% of the subtotal, were sudden cardiac deaths (SCDs) [3]. In the context of phenomenal advances in the physical and biological sciences, the limited number of viable therapeutic targets and effective cardiovascular therapies are a source of sheer surprise [4]. In addition to that, molecular causes underlying cardiac dysfunction in most heart diseases are unknown and expected to result from causal alterations in gene and protein expression [5]. Increasing body of evidence suggest that examining changes in the protein expressions arising due to intrinsic and extrinsic perturbations [4], offer insight into the understanding of cellular and molecular mechanisms that cannot be obtained from genomic analysis[6]. Proteins are considered to be the central executors of all life programs [7]. Up till now only 150 proteins have been identified from human heart tissue using 2D-gel electrophoresis and sequencing [8]. Therefore in context of this situation, the emerging global trends of CVD demands identification of upstream and downstream effectors forming the basis of the disease which could also serve as targets for therapeutical intervention. The myofilament proteins, including Troponin T are responsible for the contractile nature of the cardiomyocytes. These proteins are highly regulated by a number of specific post translational modifications (PTMs), some of which have been discovered through proteomic studies [9]. These altered PTMs lead to heart failure and ischemia. Proteomic analysis facilitates in comprehending the pathophysiology of the diseases in non-biased manner and also provides oppourtunity for the development of a suite of candidate biomarkers for the diagnosis, staging and tracking of disease .Still, the number of useful cardiovascular biomarkers are limited [5]. Although, Troponin as proteins have proved to be ideal biomarkers because of being low molecular weight and due to complete specificity of the cardiac isoforms of TnT and TnI. These protein markers arise from damaged cardiomyocytes which release their cellular components into blood after necrosis [1].

Although the value of Troponins cannot be negated, yet there is a room for identification of more specific biomarkers as, elevated serum troponins can be seen not only with acute cardiac injury but also with other non-cardiac disorders [10]. This risk factor creates possibility for pursuit of new biomarkers which decreases the risk of elevation of these in diseases secondary to myocardial infarction and may lead to wrong diagnosis of disease. The present study which employed the technology of proteomics is an effort in this regard, as with the advent of proteomics, it is possible to examine global alterations in the expression of diseased heart and hence has potential to provide insights into the molecular and cellular mechanisms of cardiac arrest. Ultimately, a better understanding of the role of these proteins should increase the likelihood of new effective therapies developed through rationale design [11].

2. MATERIALS AND METHODS

2.1. Study Group

Collection of serum samples was made from 50 apparently healthy Pakistani male from University of Karachi. All of the subjects belonged to the same age group (35-40), were non smokers and were not on any type of medication. An equal number of samples were collected from patients with cardiac arrest pathology who presented themselves at Karachi University clinic. Prior to sampling, written consent was obtained from both groups of subjects and studies were carried out in accordance with human ethical approval from PCMD, University of Karachi.

2.2. Sampling and Biochemical Analysis

The samples taken were stored at-70°c until used for protein analysis. Prior to protein profiling serum from cardiac arrest patients was also evaluated for lipid profile and cardiac enzymes assay followed by Troponin T quantification.

Assessment of different parameters of lipid profile was made, which included test for cholesterol Triglycerides, HDL and LDL and total lipids. HDL and LDL were measured using Cholestrol precipitant method.

Cholesterol levels were estimated using kit-method specifically, CHOD-POD enzymatic colourimetric method by spectrophotometer. The absorbance of the sample and the standards was read at 550 nm against the blank. TG levels were determined by using Bioscience kit specifically CPO-POD enzymatic colourimetric method. The absorbance of the samples and standards was read at 505nm against the blank.

HDL and LDL were measured using Cholestrol precipitant method.

Precipitation of HDL and LDL cholesterol was made by using precipitating reagent, according to the kit method and the absorbance of the samples and standards was read at 500 nm against the blank.

Cardiac enzyme profiling was carried out which included tests for SGOT, CPK, LDH, and CK-MB.

The SGOT levels in Cardiac arrest patients were estimated spectrophotometrically by kit method at 340nm wavelength. The CPK levels in cardiac arrest patients were estimated spectrophotometrically by kit method at 480 nm wavelength. The LDH levels in cardiac arrest patients were estimated spectrophotometrically by Breuer and Breuer kit method at 340,334 and 365 nm wavelengths.

The CK-MB levels in cardiac arrest patients were estimated spectrophotometrically by Breuer and Breuer kit method at 340 nm and 334 nm wavelengths.

2.3. Troponin T Quantification

Troponin T quantification was made through Roche Cardiac T reader.

2.4. Protein Quantification

After biochemical analysis serum samples were subjected to protein quantification by using Bradford assay.

2.5. Protein Characterization

Separation of serum proteins was carried out using Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) of cardiac arrest samples.

SDS-PAGE was performed in a Bio-Rad Mini-PROT-EAN III apparatus using Discontinuous gel system according to Laemmli, 1970 protocol. The experiment was conducted in the electrode tank buffer. Tris/Glycine (pH8.3) using 4% stacking gels and 10% separating gels. 100µg of protein was loaded on to the wells of the stacking gel. Glass plates were assembled with electrodes and electrode tank buffer was poured between the slabs. The comb was removed from stacking gel. 100µg protein samples were loaded into each well along with 5 µl of protein marker. Electrophoresis was carried out at constant voltage of 90 volts. After completion of electrophoresis, the glass plates were taken out and gel was removed. The gel was left overnight in staining solution. The staining solution was discarded and destaining solution was added until a clear background was obtained and bands were visible. The gel was then photographed and analyzed through Quantity One software.

Protein Profiling was carried out by Two Dimensional Gel Electrophoresis. The 2D electrophoresis was carried out by the modified procedure of O Farrell (1975). Denaturing Acrylamide tube gels were used to separate proteins (isoelectric focusing, IEF) and then run on SDS-Polyacrylamide gel (10%).0.688g of urea was dissolved in 0.5 ml of double distilled water at 40°C in a water bath. 0.16 ml monomer solution, 0.06 ml ampholytes and 0.025 ml NP-40 were added. The solution was then degassed.0.83ul of TEMED and 5.83 ul APS were added to solution and poured into the capillary tubes. Gels were allowed to polymerize for 1hr. Tubes and upper reservoir was filled with NaOH (2mM), while lower reservoir was filled was phosphoric acid. Gels were then pre-run at 200V for 10 minutes, then at 300V for 15 minutes followed by 400V for 15 minutes. After the pre-run, 100 µg of protein samples was then loaded on the gel surface. 9.0M urea (10µl) was used to overlay the samples. The tubes and upper reservoir were filled again with NaOH (2mM). Gels were run at 750V for 5hrs. After isoelectric focusing, the gels were protruded from the tubes and equilibrated with SDB for 15 minutes. Gels were then loaded on the prepared SDS polyacrylamide gel (10% separating, 4% stacking). 0.5% Agarose was layered on the IEF gel and left to solidify for 15min. Electrophoresis was performed as for SDS-PAGE. The gel was then stained with Coomassie brilliant blue overnight. This was followed by destaining of the gels until a clear background was obtained and bands were visible.

2.6. Silver Staining of Polyacrylamide Gels

Silver staining protocol was used as described by Swan *et al.*, (1995) for the visualization of proteins specifically low abundance protein. The gel was placed in fixative for 10 min and then rinsed with double distilled water for another 10 min. The double distilled water was replaced with fixer/sensitizer for 15 min. Gel was rinsed first with ethanol (40%) and then with double distilled water for 20 min each. For 1 min the gel was immersed in sensitizer, and then rinsed twice with double distilled water (1 min/wash). Gel was then placed in staining solution for

20 min and rinsed again in double distilled water for 1 min. The bands were developed in the developer until the desire level of staining was obtained after which the developer was discarded and the gel was immersed in stop solution for 5 min. The stop solution was replaced with storage solution and the photograph of the gel was taken. Throughout the procedure the gel was agitated on an orbital shaker.

2.7. Statistical Analysis

Statistical analysis using student's *t* test was carried out in order to establish the degree of significance for lipid profile and for assessment of cardiac enzymatic level for both the control and cardiac arrest samples. The p-value was found to be less than 0.001 with 95% confidence level which clearly depicts the statistical significance of the results.

3. RESULTS

3.1. Protein Yield

The protein yield of cardiac arrest patients and normal control were expressed as μg of protein per ml. As shown in **Figure 1** the yield of serum protein samples was ± 72 . The protein yield of the diseased samples was found out to be much less than that of the controls which is in direct relation to the cardiac arrest pathology.

3.2. Lipid Profile

The lipid profile test was performed on samples of cardiac arrest and on its respective control before protein profiling was carried out (see **Table 1**).

Comparison between normal serum and control of different plasma lipid parameters was performed in order to assess the degree of severity of the pathology as shown in **Figure 2**. It was found out that there is a 1.44796 fold increase in cholesterol levels followed by a 0.775 fold decrease in TG levels, 1.415 decrease in HDL levels, 1.266 fold increase in LDL levels and 1.322fold increase in total lipids when comparison was made between cardiac arrest sample and its respective control.



Figure 1. Quantification of proteins in cardiac arrest sample by ELISA using Bradford Assay.

 Table 1. Comparison and contrast between control and cardiac arrest sample of different plasma lipid parameters.

Parameters	Normal serum (mg/dl)	Cardiac arrest sample (mg/dl)
Cholesterol	200±14.61	288.9592 ± 14.59
Triglycerides(TG)	274 ± 14.14	212.3878 ± 16.86
HDL	59±14.152	41.69388±4.77
LDL	154 ± 14.16	195.0612 ± 16.22
Total. lipids	624±14.15	825±14.710

HDL: High density lipoproteins

LDL: Low density lipoproteins



(b)

Figure 2. (a) Standard error bars for different parameters of lipid profiling of controls. (n=50, p>0.001). (b) Standard error bars for different parameters of lipid profiling of cardiac arrest sample (n=50, p>0.001).

3.3. Cardiac Enzyme Test

Tests were performed on serum samples of cardiac arrest patients and their respective control for assessment of cardiac enzyme levels. When comparative studies were made it was found out that SGOT level demonstrated an increase by 2.988 fold, CPK by 1.246 fold LDH by 1.31935 fold and CK-MB by 2.34fold between serum of diseased patients and control (see **Table 2**).

3.4. Troponins T Test

The cardiac arrest samples used were found out to be troponin T positive. The tests for troponins are carried out in situations when there is a many fold increase in HDL, LDL and cholesterol levels of the patient. The troponin T values of all the healthy subjects were assumed to be within the reference range of (0-<0.05 ng /ml) while that



Figure 3. (a) Standard error bars for cardiac enzymatic levels of control. (n=50, p>0.001). (b) Standard error bars for cardiac enzymatic levels of cardiac arrest sample. (n=50, p>0.001).

Table 2. Comparisons and contrast of different cardiac enzymes in normal serum and cardiac arrest sample.

Cardiac enzymes	Normal serum (µ/l)	Cardiac arrest sample	
SGOT	24.5±1.414	73.2449±15.78	
СРК	173.35±7.77	216.1633±14.08	
LDH	324±14.142	427.4694 ± 22.87	
CK-MB	$22.4{\pm}1.414$	52.4898±13.73	

SGOT: Serum glutamic oxaloacetic transaminase

LDH: Lactate dehydrogenase

CK-MB: Creatinine Kinase

of patients were found out to be within $(0.8-1.04\pm0.1413)$ which is in absolute concordance with the established ranges for the determination of myocardial damage. (0.1-2.0 ng/ml).

3.5. Protein Components Analyzed Using SDS-PAGE (10%)

Serum protein $(100\mu g)$ of control and cardiac arrest patients (n=50 for each group) were subjected to SDS Page (10%). The proteins were visualized by Coomassie brilliant blue.

As shown in **Figure 4** the electrophoretic patterns of the components of control (normal serum) and of cardiac arrest sample demonstrated approximately 16 and 9 bands respectively in the silver stained gel. The molecular weight of the protein components ranged from 200-

CPK: Creatine phosphokinase

25kDa. The silver stained electrophoretic patterns were used for the molecular weight determination and semiquantitative analysis was made using Quantity one software. Protein components (P1, P7, P11, P14, and P16) with molecular weights (200kDa, 100kDa, 97kDa, 66kDa, 45kDa and 25 kDa) respectively were found to be both common in control and cardiac arrest sample as shown in Table 3. The difference in expression of protein components between control and cardiac arrest patient samples was obtained through R.Q values (see Table 4 The 135kDa (P4), 116kDa (P6), 87kDa (P9) protein components were present in the cardiac arrest sample but were absent in normal control. The results showed that the 45kDa (P14) protein present in control was under expressed or down regulated in cardiac arrest sample, where as the 100kDa (P7), 97kDa (P8) and 66kDa (P11) were over expressed in cardiac arrest samples (see Table 5).

There was a 5.6 fold up regulation of 100kDa protein, 4.5 fold up regulation of 97 kDa protein, while 2.13 fold up regulation of 66 kDa protein, and in contrast there was a 0.75 fold, down regulation of 45kDa protein in the serum of cardiac arrest patients. 100kDa molecular weight protein was the most highly expressed followed by 97kDa and 45kDa. Differential expression of above mentioned proteins in cardiac arrest patients illustrate their possible role in the pathogenesis of cardiac arrest. 100kDa, 97kDa and 45kDa proteins can be identified as potential markers of cardiac arrest. Up regulation of these proteins are involved in the pathogenesis of this disease. Low level of 45 kDa protein depicts that the high level of this protein should be maintained to avoid the pathogenesis.

3.6. Protein Components Resolved Using Isoelectric Focusing (IEF)

2 DE performed on cardiac arrest sample had shown large number of proteins in acidic range of pI range of 4.75-7.1 (see **Figure 6**), however those proteins which were exclusively expressed in cardiac arrest sample were alkaline in nature with pI range 7.6-10. The 2DE gel patterns of control showed large number of proteins towards acidic side with in the pI range of 4.3-6.4(see **Figure 5**).While there are only 8 protein spots present in control as well as in the diseased samples and were in the pI range 4.6 to 8.2. This is an important observation suggesting a possible role of alkaline proteins in the progression of cardiac arrest and can be confirmed in future after further characterization.

These findings suggest the potential role of these proteins in disease progression and pathology. The altered expression of several proteins observed in the present study strengthened the hypothesis of the involvement of these proteins in disease etiology. The proteins identified here could be used in future as diagnostic and therapeutic biomarker.



Figure 4. SDS-PAGE analysis of cardiac arrest sample, with normal serum and marker.



Figure 5. 2D gel electrophoresis serum proteins from controls resolved through IEF and visualized by silver staining. The spots show differentially expressed proteins. Protein marker used was with in the range of Mr 66-24kDa.



Figure 6. 2DE electrophoretic pattern of cardiac arrest proteins resolved through IEF and visualized by silver staining. The spots show differentially expressed proteins Protein marker use was with in the range of Mr 200-29 kDa.

 Table 3. Difference in expression of protein components between control and cardiac arrest patient samples as obtained through R.Q values using quantity one software.

Protein components	Molecular weight(kDa)	Relative quantity% (control)	Relative quantity% (cardiac arrest samples)
P1	200	3.8	3.7
P2	165	2.3	-
P3	155	3.3	-
P4	135	-	4.8
P5	120	2.6	-
P6	116	-	5.1
P7	100	2.4	13.6
P8	97	2.3	10.3
P9	87	-	4.4
P10	76	4.6	-
P11	66	4.5	9.6
P12	62	4.9	-
P13	52	2.7	-
P14	45	5.4	4.1
P15	35	3.0	-
P16	25	0.9	0.2

Table 4. Protein components differentially expressed as in cardiac arrest sample and in control. (n=50) as visualized by 10% SDS.

Protein	Molecular	Control Cardiac arrest samp	
components	weight(kDa)		
P1	200	+	+
P2	189	-	+
P3	165	+	-
P4	155	+	-
P5	120	+	-
P6	116	-	+
P7	100	+	+
P8	97	+	+
P9	87	-	+
P10	76	+	-
P11	66	+	+
P12	62	+	-
P13	52	+	-
P14	45	+	+
P15	35	+	-
P16	25	+	+

Table 5. Protein components of patient in control and cardiac arrest sample which have been up regulated or down regulated.

_				
	Protein components	Molecular weight(kDa)	Control	Cardiac arrest sample
	P7	100	normal	↑up regulate
	P8	97	normal	↑up regulate
	P11	66	normal	↑up regulate
	P14	45	normal	↓down regulate

4. DISCUSSION

Heart diseases resulting in heart failure are a leading cause of morbidity and mortality in developing countries [1]. In context of this situation, the importance of a specific biomarker which precisely marks the prognosis of the disease like cardiac arrest, increases by many folds. In terms of availability and ease of measurement, a protein that is very abundant in the target cell, has a means of reaching blood and ideally a specific form reflective only of the target cell in the tissue can serve out to be a good candidate marker. We focused on heart muscle contractile proteins as their levels in blood reflect the amount of damage to the heart due to the necrosis of cardiomyocytes [12]. The quest for a specific biomarker for ischemic injury and heart failure began in 1950 and in 1990's trponins, specifically cardiac TnT and TnI became ideal because of their low molecular weight, the apparent specificity of the cardiac isoforms of TnT and TnI for the myocardium and in addition to this, the ease with which they can be detected in serum by chemical laboratory analysis, 4-6 hours of an acute event and may remain elevated for 7-10 days after the event [10]. However, elevated serum troponins can be seen not only with acute cardiac injury but also with other non-cardiac disorders including pulmonary embolus, sepsis and renal failures, a number of studies [13-15], have confirmed that cTnI along with cTnT were found to be elevated in renal failure cases. This finding supported our search of a more specific protein as biomarker for cardiac arrest.

Nevertheless, the presence of troponins in the serum are detected only after the occurrence of cardiac injury.i.e when the ideal period for thereuptical intervention has passed by a large extent. Consequently, the present study was an effort in this regard. In this study, the serum sample of only those cardiac arrest patients were included, which were found out to be positive for troponins cTnT and cTnI so in the presence of these established biomarkers, some new proteins could be identified which could serve the purpose of new diagnostic or prognostic biomarkers. Apart, from that another move was made towards the discovery of such proteins which could serve as potential drug targets for the development of new thereuptical approaches for combating heart diseases. As a result of which in this study, protein components from cardiac arrest sample and their respective controls were differentially expressed on SDS-PAGE gel pattern and 2DE.Most of the diseases cause induction or suppression of some proteins associated with them. Differentially expressed three proteins P7(100kDa), P8(97kDa), and P11(66kDa) on SDS-PAGE were found out to be over expressed in the cardiac arrest sample , suggesting their role in progression of disease while it can be hypothesized that normal expression of the P14(45kDa) which was found out to be down regulated in this study can be conducive in rectification of the disease. From 2DE gel patterns of the cardiac arrest samples it can be concluded that that there is a role of alkaline proteins in the etiology of the disease. The overall study concludes that the above mentioned three proteins with 100kDa protein being the

Protein component	Molecular weight (kDa)	pI (approx.)	Control	Cardiac arrest sample
P1	168	7.6	-	+
P2	163	7.25	-	+
P3	156	8.2	-	+
P4	118	10	-	+
P5	112	8.7	-	+
P6	102	9.03	-	+
P7	99	7.68	+	+
P8	89.5	4.775	+	-
P9	85	4.9	-	+
P10	80	5.02	+	+
P11	76	7.1	+	+
P12	71	6.4	+	-
P13	67.4	5.715	+	+
P14	58	6.27	+	+
P15	54	6.61	+	+
P16	49	6.565	+	+
P17	43	4.6	+	+
P19	26	8.2	+	+
P20	22	4.3	+	-
P21	18	73	-	+

Table 6. Difference in expression of protein components between control and cardiac arrest patient samples as obtained through R.f values using graphical methods. The protein components were obtained through 2D gel electrophoresis.

most highly expressed and 45 kDa being suppressed can be identified as potential biomarkers and targets for therapeutical intervention. The nature of these proteins can be elucidated by employing technique of mass spectrometry and tools of bioinformatics.

5. CONCLUSIONS

This study had been successful in detection of three proteins P7(100kDa), P8(97kDa) and P11(66kDa) on SDS-PAGE which were found out to be over expressed in the cardiac arrest sample, with 100 kDa protein being the most highly expressed and 45 kDa being down regulated and can be identified as potential biomarkers and targets for therapeutical intervention. In addition to that 2 DE had been conducive in proposing that proteins of alkaline nature play a major role in cardiac arrest pathology.

REFERENCES

- White, M.Y., Edwards, A.V.G., Cordwell, J.S. and Van Eyk, J.E.M. (2008) Mitochondria: A mirror into cellular dysfunction in heart disease. *Proteomics Clin. Appl*, 2, 845-61.
- [2] Mehra, R. (2007) Global public health problem of sudden cardiac arrest. *Journal of electrophysiology*, **40**,118-21.
- [3] Arking, D.E., Chugh, S.S., Chakarvarti, A., Spooner, P.M. (2004). Genomics in sudden cardiac death. *Circ. Res*.94:712-23.
- [4] Arab, S., Gramolini, A.O., Ping, P., et al. (2006). Cardiovascular Proteomics: Tools to Develop Novel Biomarkers and Potential Applications. Journal of the American College of Cardiology, 48, doi:10.1016/j. jacc.2006.06.063
- [5] McGregor, E. and Dunn, M.J. (2006) Proteomics of the heart: unravelling disease. *Circ. Res*, 98, 309-21
- [6] Macri, J. and Rapundalo, S.T. (2001) Application of Proteomics to the Study of Cardiovascular Biology. *Trends in Cardiovascular Medicine* 11, Pages 66-75
- [7] Jäger, D., Jung Blut, P.R. and Müller-Werdan, U. (2002) Separation and identification of human heart proteins. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*,**771**, 131-153
- [8] Bahadori, M (2001) Proteomics in human disease: Awareness of new biomedical Opportunities. Arch Irn Med 3,144-149
- [9] Mayr, M. and Van Eyk, J.E. (2008) Cardiovascular proteomics. *Proteomics Clin. Appl*, 2, 785-786.
- [10] Jin, W., Brown, A. T. and Murphy, A. M. (2008) Cardiac myofilaments: from proteome to pathophysiology. *Proteomics Clin. Appl*, 2, 800-810.
- [11] Charles, R.L. and Eaton, P. (2008) Redox signalling in cardiovascular disease. *Proteomics Clin.Appl*, 2, 823-36.
- [12] Ladenson, J.H.A. (2007) Personal history of markers of myocyte injury myocardial infarction. *Clin Chim Acta*, **381**, 3-8.
- [13] Bhayana, V., Gougoulias, T, Cohoe, S. and Henderson, A.R. (1995) Discordance between results for serum troponin T and troponin I in renal disease. *Clin Chem*, **41**, 312–317.
- [14] Collinson, P.O., Hadcocks, L, Foo, Y, Rosalki, S.B, et al (1998). Cardiac troponins in patients with renal dysfunction. Ann Clin Biochem, 35, 380–386.
- [15] Apple, F.S, Murakami, M.M., Pearce, L.A. and Herzog, C.A. (2002) Predictive value of cardiac troponin I and T for subsequent death in end-stage renal disease. *Circulation*, **106**, 2941–2945.

Call for Papers



Editor-in-Chief

Prof. Kuo-Chen Chou

Editorial Board

Dr. Yiqiang Cai Dr. James CS Chim Dr. Yohichi Kumaki Dr. Petr Kuzmic Dr. CHIH MING LIN Prof. Baoliang Lu Prof. Baoliang Lu Prof. George Perry Prof. Kenji Sorimachi Dr. Yuande Yang Prof. Jun Zhang Prof. Jun Zhang Prof. Qun Zhao Dr. Wei Zhong Prof. Yang Zhong

Editorial Advisory Board

Dr. Wade Adams Prof. Dmitrios Braddock Dr. Athel Cornish-Bowden Dr. Louis J. Denis Prof. Reba Goodman Dr. Robert L. Heinrikson Prof. Denise Kirschner Dr. Claude Klee Prof. Harold A. Scheraga Prof. Kai Wucherpfennig Dr. W. Z. Zhong

HEALTH

A Journal Published by Scientific Research Publishing, USA www.scirp.org/journal/health

Gordon Life Science Institute, San Diego, California, USA

Yale University School of Medicine, USA University of Hong Kong, Hong Kong (China) Institute for Antiviral Research, Utah State University, USA BioKin Ltd., USA Johns Hopkins University, Singapore Shanghai Jiao Tong University, China University of Texas at San Antonio, USA Dokkyo Medical University, Japan Wuhan University, China University of Kentucky, USA Shanghai Jiao Tong University, China University of Georgia, USA University of Goorgia, USA University of South Carolina Upstate, USA Fudan University, China

Eurofins AvTech Laboratories, USA Yale University School of Medicine, USA Centre National de la Recherche Scientifique, France Clinical Research & Development, Pfizer, USA Columbia University, USA Proteos, Inc., Michigan, USA University of Michigan Medical School, USA National Institutes of Health (Cancer), Maryland, USA Cornell University, USA Harvard Medical School, USA Global Research & Development, Pfizer, USA

HEALTH is an international journal dedicated to the latest advancement of human health. The goal of this journal is to provide a platform for doctors and academicians all over the world to promote, share, and discuss various new issues and developments in health related problems. All manuscripts must be prepared in English, and are subject to a rigorous and fair peer-review process. Accepted papers will immediately appear online followed by printed hard copy. The journal publishes original papers including but not limited to the following fields:

- Blood, Heart and Circulation
- Brain and Nerves
- Cancers
- Chinese Medicine
- Diagnosis and Therapy
- Disorders and Disease
- Drug Therapy
- Epidemiology
- Exercise Physiology
- Food and Nutrition
- Genetics/Birth Defects
- Immune System
- Medical Physics
- Medicine
- Medical Equipment

- Mental Health and Behavior
- Nursing
- Nutrition and Dietetics
- Occupational Therapy
- Optometry
- Osteopathic Medicine
- Pharmacology
- Physical Therapy
- Psychoanalysis
- Psychotherapy
- Public Health
- Physical Education
- Surgery and Rehabilitation
- Toxicology and Environmental Health
- Virology

We are also interested in: 1) Short reports—2-5 page papers where an author can either present an idea with theoretical background but has not yet completed the research needed for a complete paper or preliminary data; 2) Book reviews— Comments and critiques.

Notes for Intending Authors

Submitted papers should not be previously published nor be currently under consideration for publication elsewhere. Paper submission will be handled electronically through the website. For more details, please access the website.

Website and E-Mail

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

health@scirp.org

TABLE OF CONTENTS

Volume 2, Number 1, January 2010

A low dose of nicotine is sufficient to produce nicotine withdrawal in mice
B. Morgane, S. Sandra, C. Jean-Pierre, G. Sylvie
THE role of intracellular sodium (Na ⁺) in the regulation of calcium (Ca ²⁺)-mediated signaling and toxicity
X. M. Yu, B. R. Groveman, X. Q. Fang, S. X. Lin
Translational research in acupunctureteleacupuncture bridges science and practice G. Litscher
Does conventional abdominal preoperative ultrasonography have a role in the detection of pancreatic insulinoma? A case report
L. Spadaro, O. Magliocco, G. Trovato, F. Purrello
Applicability of the P19Cl6 cells as a model of cardiomyocytes a transcriptome analysisI. Khodadadi, N. J. Plant, V. Mersinias, A. E. Thumser
Development of a high-throughput cell based 384-well influenza A quantification assay for interpandemic and highly pathogenic avian strains M. R. Gainey, A. M. Wasko, J. N. Garver, D. J. Guistino, E. M. Vela, J. E. Bigger
Gait spectral index (GSI): a new quantification method for assessing human gaitR. Héliot, C. Azevedo-Coste, L. Schwirtlich, B. Espiau
The effect of high fat food on erythrocyte phospholipids, fatty acids composition and glutathioneredox-system of rats with alimentary dyslipidemiaT. P. Novgorodtseva, Y. K. Karaman, N. V. Zhukova.45
Bioengineering functional copolymers. XII. nteraction of boron-containing and peo branched derivatives of poly(Ma- <i>alt</i> -Mve) with HeLa Cells M. Türk, Z. M. O. Rzayev, G. Kurucu
The central nucleus of amygdala is involved in tolerance to the antinociceptive effect of NsaidsM. G. Tsagareli, N. Tsiklauri, G. Gurtskaia, I. Nozadze, E. Abzianidze
Paediatric gallstones and laparoscopic cholecystectomyD. G. Samuel, N. N. Naguib, A. Y. Izzidien.67
Troponin based studies in search of a biomarker for cardiac arrest
P. Gnazal, K. F. Shad, Shadat, N. Sidduiqui

