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Short-Stay Surgery: Impact of Opening a New Unit in a Tertiary Urban Hospital

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Abstract

Objective: The number of procedures considered suitable for short-stay surgery has experienced a remarkable increase. The objective of the study was to determine whether a new short-stay surgical unit (SSSU) was an effective alternative to conventional Hospital Units (HU) for selected elective and urgent surgical conditions. **Methods:** A comparative analysis (Mann-Whitney test) was used to identify differences between patients admitted to HU (n = 2873) and those admitted to the SSSU (n = 544) during the following months (January 1, 2014 to August 31, 2014, and January 1, 2015 to August 31, 2015, respectively). **Results:** Statistically significant differences were found in terms of mean length of stay (HU: 4.8 days versus SSSU: 2.2 days; $P < 0.0001$), and a number of associated conditions (HU: 1.37 versus SSSU: 1.09; $P = 0.02$). There were no statistically significant differences regarding age and sex. **Conclusions:** We conclude that selected surgical patients with elective or acute conditions can be effectively treated in the SSSU.

Keywords

Surgery, Short-Stay, Hospital, Management

1. Introduction

In recent years, the number of procedures considered suitable for short-stay surgery has experienced a remarkable increase. Pre-operative preparation and protocol discharge have contributed to a safe and effective short-stay surgery for patients presenting elective conditions and some acute conditions requiring urgent surgery [1]. However, there are few data to establish the duration of hospitalization in individual patients to achieve maximal benefit and to identify those patients suitable for early discharge from the hospital.

The objective of the study was to determine whether a new short-stay surgical unit (SSSU) was an effective alternative to conventional Hospital Units (HU) for selected elective and urgent surgical conditions.

2. Methods

A comparative analysis (Mann-Whitney test) was used to identify differences between patients admitted to HU (n = 2873) and those admitted to the SSSU (n = 544) during the following months (January 1, 2014, to August 31, 2014, and January 1, 2015, to August 31, 2015, respectively). The study was performed at Sant Pau Hospital, a 500-bed teaching tertiary care referral centre in Barcelona, Spain. The emergency department attends about 130,000 emergency visits per year, including pediatrics and obstetrics. We retrospectively studied the characteristics of patients hospitalized during both periods of the study with the same diagnoses (n = 3417). We chose charts of patients from the hospital discharge database and selected according to the Ninth Revision of the International Classification of Diseases codes. We used the computerized database to obtain outcome data on all patients. Clinical and demographic factors were available through chart review. Patients were excluded from the study if they were intubated and ventilated on the day of admission.

3. Results

Statistically significant differences were found in terms of mean length of stay (HU: 4.8 days versus SSSU: 2.2 days; $P < 0.0001$), and number of associated conditions (HU: 1.37 versus SSSU: 1.09; $P = 0.02$). There were no statistically significant differences regarding age and sex.

4. Discussion

The strength of our study includes a large sample size. We demonstrated that the introduction of an SSSU at a tertiary university hospital was associated with a decrease in the patients' length of stay. It is likely that part of the increased length of stay for the HU group is a feature of the nature and function of inpatient services. It is important to note that the comorbidity, measured as a number of associated conditions, in HU was significantly higher, which cannot rule out a possible bias effect of less seriously ill patients admitted to the new SSSU.

The definition of short-stay surgery includes those patients admitted and discharged between 24 hours and 72 hours. The complexity of procedures performed with minimally invasive surgery leads to a wide range of patients to be implemented as short-term surgery [2] [3]. This management can improve the quality of patient care, particularly postoperative recovery, while reducing length of stay [4]. Shortened hospital stay is basic to modern patient care and also reduces hospital-acquired infections and venous thromboembolism [5].

A multidisciplinary approach is recommended for patient assessment including inclusion and exclusion criteria for short-stay surgery. Social, medical and surgical factors

should be appropriate for postoperative care. Documentation is important from preoperative preparation to discharge and follow-up. Patients should be provided with general as well as procedure-specific information. Integrated care pathways are useful for evaluation of outcomes [6].

Staff working in these units should be specifically trained in surgery care and must have a clinical lead with interest in the development of local guidelines and clinical governance.

However, several important limitations must be addressed. First, readmission rate was not recorded to compare the occurrence of relapse after discharge that could have suggested the need for intensified ambulatory care or home care in the weeks after a short admission in an SSSU. And, as we did not conduct a formal economic analysis, further work is needed to quantify the economic impact of the introduction of an SSSU in the management of surgical conditions.

We conclude that selected surgical patients with elective or acute conditions can be effectively treated in the SSSU. The identification of these patients proved to be an effective measure in hospital management and a helpful intervention that alleviated in-hospital bed crises.

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Airway Characteristics and Safe Management of Spontaneously Breathing Patients: Risks of Sedation and Analgesia and Changes in Wakefulness

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Abstract

The goal of safe airway management is to maintain a patent airway. Lack of knowledge of the anatomical morphology and changes that may occur in the upper airway during sedation and unconsciousness may lead to critical incidents and hazardous complications. This review focuses on the risks of sedation and analgesia and changes in wakefulness on airway patency in spontaneously breathing patients. Furthermore, key elements of airway management are presented and discussed.

Keywords

Airway, Analgesia, Anesthetics, Anesthesia, Breathing, Chin Lift, Diagnostics, Emergency, Jaw Thrust, Sedation, Maneuvers, Obesity, Obstructive Sleep Apnea, Posture, Sleep

1. Introduction

In emergency situations (pre- and in-hospital) and in sedated patients (e.g., patients undergoing diagnostic procedures), failure to maintain a patent airway can lead to severe hypoxia and death. A comprehensive understanding of the anatomical morphology and changes in the upper airway geometry is crucial. Prediction methods are useful for identifying a potentially difficult airway. However, the predictive value of these methods is limited. This overview presents the airway characteristics and safe management of spontaneously breathing sedated patients. "Sedation and analgesia" encompasses different states such as minimal sedation (anxiolysis), moderate sedation and analgesia (conscious sedation), and deep sedation/analgesia through general anesthesia [1]. This

overview determines the influence of moderate to deep sedation on upper airway patency. Definitions of levels of sedation are given in Table 1.

2. Wakefulness and Sleep: Differences in Airway Patency

The upper airway is a complex structure. Geometrical changes caused by changes in wakefulness are fundamental for understanding the limitations of airway patency. The tone of oropharyngeal muscles is maintained during spontaneous breathing. In conscious patients with a normal anatomical morphology, essential dynamic changes in the upper airway caliber occur during quiet respiration. During inspiration, negative intraluminal pressure is majorly balanced by the upper airway dilator muscles [2]. During expiration, the positive intraluminal pressure expands the upper airway [3]. These respiratory changes are more prominent in the lateral than in the anteroposterior dimension. Thus, in addition to the tongue and soft palate, lateral pharyngeal walls are one of the most important upper airway soft tissue structures [4]. During sleep, airway dimensional changes occur because of the changes in body position and soft tissue structures. The activity of upper airway dilator muscles decreases, resulting in a reduction in the upper airway size and an increase in upper airway resistance. The complex anatomical structure consists of muscles such as the tongue, palate muscles, pharyngeal constrictor muscles, genioglossal and geniohyoideus muscles, and the four infrahyoid muscles (sternohyoid, omohyoid, sternothyroid, and thyrohyoid). The male airway is substantially more collapsible than the female airway because of the pharyngeal airway length, an increased cross-sectional area of the soft palate, and an increased airway volume [4]. Magnetic resonance imaging (MRI) studies have shown that during sleep, the volume of the retropalatal airway decreased significantly by 19% ($P = 0.03$), whereas that of the retroglossal airway decreased slightly [5]. These findings suggest that the retropalatal region may be more likely to collapse.

3. Sedation and Upper Airway

Determinants of upper airway patency include airway caliber, transluminal pressure gradient, compliance of the airway wall, and upper airway muscle activation [6]. Sedatives impair upper airway stability, and upper airway obstruction is the only serious

Table 1. Differences in moderate and deep sedation and analgesia.

	Depth of Sedation	
Sedation and Analgesia	Moderate	Deep
Responsiveness	Purposeful response to verbal or tactile stimulation	Purposeful response after repeated or painful stimulation
Spontaneous breathing	Adequate	May be inadequate
Airway	No intervention required	Intervention may be required
Cardiovascular function	Usually maintained	Usually maintained

a. This table is adapted from [1].

adverse event that can occur during moderate or deep sedation [7]. Anatomical predisposition, unfavorable posture, and profound impairment of muscle activity increase the risk of airway obstruction. Airway narrowing leading to obstruction was investigated in children anesthetized with propofol [8]. Increasing depth of anesthesia lead to narrowing of the entire upper airway, which was most prominent at the level of the epiglottis. Mahmoud *et al.* compared propofol with dexmedetomidine and hypothesized that dexmedetomidine has lower effects on the upper airway tone than other sedatives or anesthetics and provides more favorable conditions, which are similar to those observed during natural sleep [9]. However, propofol is still the most commonly used intravenous anesthetic agent in many countries, since of its rapid onset and reversal of action. There is no reason to contraindicate propofol in patients allergic to eggs, soybean oil or peanuts [10].

Midazolam is the most commonly used benzodiazepine for sedation. Studies have shown that midazolam may lead to upper airway obstruction; however, its dose-dependent effect remains unclear [11].

Ketamine is the safest sedative for preventing upper airway collapse. In particular, during emergency situations, ketamine may be the drug of choice. It causes minimal respiratory drive suppression and minor impairment of respiratory muscle activity [12]. However, rapid administration of high doses of ketamine should be avoided because of the possibility of (transient) apnea [13].

Opioids depress both the ventilator and pharyngeal neuromotor drive [11]. Opioids impair upper airway patency through the activation of laryngeal adductor motoneurons and depression of laryngeal abductor and pharyngeal constrictor motoneurons.

The most common adverse reaction to opioids is nausea. In addition, except for fentanyl and its derivates, opioids can induce pseudoallergic reactions by triggering degranulation of mast cells and the direct release of histamine.

Because of high interindividual anatomic variability, different age-related characteristics, and various comorbidities, safe airway management must be tailored individually. Physiological derangements of the patient increase the risk of cardiovascular critical incidents and even cardiovascular collapse from airway management. Mosier *et al.* described the four physiologically difficult airways: hypoxemia, hypotension, severe metabolic acidosis, and right ventricular failure [14]. Thus, sedatives must be used cautiously in critically ill patients. Practice guidelines for sedation and analgesia by non-anesthesiologists have been outlined in a report by the American Society of Anesthesiologists Task Force [1]. Although capnography indicates respiratory depression earlier than pulse oximetry, its strict application during sedation is not established. However, as a tool providing breath-to-breath ventilation data, capnography has the potential to further reduce the incidence of serious adverse events due to inadvertent oversedation [15]. This non-invasive respiratory monitoring tool is applicable in different settings (e.g., emergency medical service, endoscopy units, radiology departments, and other treatment areas outside the operation theater).

4. Posture and Position of the Head in Sedated Patients

4.1. Position of the Body

Unfavorable postures such as the supine position, neck flexion, and abnormal mouth opening may lead to impaired airway patency. A simple adjustment of the body position may support patency and reduce the probability of upper airway obstruction [16]. Isono, Tanaka, and Nishino reported that in patients with obstructive sleep apnea (OSA) syndrome, the lateral position reduced the effect of gravity on the soft palate, tongue, and epiglottis, thus structurally improving the maintenance of the passive pharynx [16]. Changing the patient's position from supine to lateral prevents the pharyngeal soft tissue from falling backward against the posterior pharyngeal wall. Litman *et al.* showed that lateral positioning widens all non-cartilaginous parts of the upper airway in children [17]. In morbid obesity, a "ramped" position is achieved by using an elevated pillow or by arranging blankets underneath the patient's upper body and head until a horizontal alignment between the external auditory meatus and sternal notch is obtained [18].

4.2. Head and Neck Position

Both flexion and hyperextension of the head and neck increase upper airway resistance. Particularly in patients prone to upper airway collapse, optimal head and neck positioning is mandatory. In unconscious patients, overflexion of the head causes complete airway obstruction. Caution should be exercised in patients with pharyngeal tumors. Extension is associated with the risk of airway occlusion, particularly in children, where anatomy is already narrow.

In adults, sniffing position has been considered the optimal head and neck position, aligning the laryngeal, pharyngeal, and oral axes. According to Jonathan Benumof, the sniffing position necessitates approximately 30˚ - 35˚ of flexion of the neck axis on the chest axis [19]. In obese patients, anterior displacement of critical pharyngeal structures (such as epiglottis and base of the tongue) and positioning of the head may be limited. The sniffing position is prohibited in patients with atlantoaxial instability. In children with Down syndrome, the potential risk of cervical spine instability must be considered [20].

In infants, the prominent occiput reduces the degree of cervical extension during shoulder elevation; resulting in an equally favorable position compared with the sniffing position [21]. Vialet, Nau, Chaumoître, and Martin demonstrated that slight extension of the head in infants and young children considerably improved the alignment of the axes of the airway [22]. Another MRI study showed that application of a soft neck collar in children aged 2 - 4 years may expand the retropalatal and retroglossal airway dimension during sedation in the supine position [23]. The underlying mechanism may be the slight extension of the head with anterior protrusion of the mandible achieved by the neck collar. Mustafa, Emara, and Nouh suggested that displacing the mandible pulls the tongue forward and subsequently increases the caliber of the retroglossal airway [23]. In addition, the retropalatal airway improves as the soft palate is displaced for-

ward because it is coupled to the tongue through the fauces [24].

The optimal head position for maintaining airway patency depends on age. Head extension and neutral head position angles differ in preschool and school children. In preschool children, a neutral head position or head extension with an angle of −1˚ or 13˚, and in school children, a head extension of 16˚ may be used to achieve optimal ventilation in an unprotected airway [25]. MRI studies have shown that the upper airway configuration in snorers and apneic patients is different from that in normal people [3] (**Figure 1**).

Head rotation is practiced routinely (e.g., during endoscopy). In infants and newborns, this maneuver increases the collapsibility of the passive pharynx [26]. During drug-induced sleep endoscopy, patients with OSA syndrome showed similar sites, severity, and patterns of upper airway collapse with rotation of the head in the supine position and lateral head and trunk position [27]. However, the severity of the anteroposterior collapse at the level of the soft palate during head rotation is lower in the supine position than in the lateral and trunk position.

5. Posture and Position of the Head in Sedated Patients

Depending on the depth of sedation/analgesia and pre-existing airway abnormalities, airway collapsibility has to be prevented through body/head positioning and appropriate airway maneuvers.

5.1. Chin Lift: A Single-Handed Maneuver

Chin lift is the simplest method of ensuring an open airway in an unresponsive patient. The chin of the patient is lifted at the anterior border of the mental protuberance without protruding the mandible; in chin lift, the lips are in close contact. A patent nasal airway is crucial [28]. MRI findings have shown that combined mouth closure and chin lift widens the anteroposterior and transverse diameters of the entire pharyngeal airway. The degree of muscular tone in geniohyoid and genioglossus muscles plays a vital role. During general anesthesia with muscle paralysis, chin lift improves the flaccid upper airway state by increasing the glottic opening. However, in obligate mouth breathers such as children with large adenotonsillar hypertrophy (ATH) or patients with extreme obesity, chin lift should be avoided [29] [30].

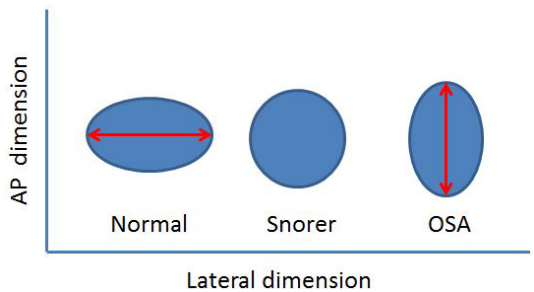

Figure 1. Differences in upper airway dimensions. This figure is adapted from [3]. AP = anteroposterior; OSA = obstructive sleep apnea.

5.2. Jaw Thrust: A Two-Handed Maneuver

The jaws are displaced at the mandibular angles with both hands upward and anterior to open the mouth. In 90% of unconscious children (1 - 9 years old), jaw thrust restored the patent airway with no clinical signs of obstruction [31]. Chin lift had only a 50% success rate in these patients, probably because of the high incidence of large ATH in this age group. However, jaw thrust can deteriorate upper airway patency in patients with large cervical masses in their lateral pharyngeal walls, which are probably caused by medial displacement of the tumorous tissue [32].

In unconscious infants, excessive jaw thrust with head tilt should be avoided because of the potential critical narrowing of the soft and pliable trachea. The pediatric basic life support guidelines of the American Heart Association still recommend the head tilt–chin lift maneuver for both injured and non-injured patients [33]. These recommendations have not been discussed further in 2015 [33]. For children, discussions should be held to determine whether jaw thrust should be used carefully beginning with a slight pressure to open a potentially obstructed airway and to assess the patient's consciousness [34].

In patients with an unstable upper cervical spine, the jaw thrust maneuver results in less motion at an unstable C1-C2 injury compared with the head tilt–chin lift maneuver [35].

5.3. Continuous Positive Airway Pressure

Predicting the response of the patients to sedatives and analgesics may be difficult. In patients where the level of sedation becomes deeper than expected, respiratory instability has to be managed using ventilatory support. Continuous positive airway pressure (CPAP) applied to the airways throughout both inspiration and expiration is effective at maintaining airway patency in most patients. Jun *et al.* demonstrated that nasal CPAP produced more effective tidal volumes than full face mask CPAP and ventilation in unconscious patients [36].

In children with ATH and depending on open mouth breathing, chin lift does not improve or aggravate airway patency [28]. Moreover, application of jaw thrust with CPAP has been the most effective maneuver to overcome airway obstruction in these patients [37].

6. Obesity and OSA

Positioning of morbidly obese patients may be challenging. The reverse Trendelenburg position increases pulmonary compliance and functional residual capacity, thereby improving oxygenation compared with the supine position [38]. Obese patients have fatty tissue externally and increased adipose tissue in the upper airway soft tissue structures, mainly in the tongue, soft palate, lateral pharyngeal walls, and parapharyngeal fat pads [3]. Thus, the upper airways show increased collapsibility. Pharyngeal critical closing pressure is associated with the hyoid position [39]. Computed tomography analysis showed that tongue dimensions, pharyngeal length, and mandibular plane to hyoid

muscles are associated with obesity variables (body mass index and neck and abdominal circumferences) [39]. In apneic patients, the thickness of the lateral pharyngeal walls and the more collapsible velopharynx are the predominant anatomic factors causing airway narrowing [3] [40].

Pien *et al.* re-examined the use of determining the critical closing pressure value from direct observation of occluded breaths (=no flow) [41]. They found that during overnight polysomnography, observed critical closing pressure values provide a consistent metric for describing hypotonic airway collapsibility in both subjects with and without OSA. OSA is a sleep-related breathing disorder characterized by repetitive episodes of airflow cessation. OSA patients are at increased risk of upper airway collapse and respiratory complications under the influence of sedatives and opioids [42]. CPAP application could improve airway patency in these patients [43]. Appropriate safety precautions must be taken to minimize the possibility of aspiration of gastric content [44].

7. Conclusion

Airway management in spontaneously breathing patients under moderate to deep sedation/analgesia is challenging. The goal is to provide these patients with the benefits of sedation/analgesia while minimizing the associated risks. A comprehensive understanding of the anatomical morphology and changes in the upper airway geometry is crucial. Because of high interindividual anatomic variability, different age-related characteristics, and various comorbidities, safe airway management with appropriate positioning and application of airway maneuvers has to be tailored individually. Emergency equipment should be available when sedatives or analgesic drugs are administered in order to rescue patients whose sedation level becomes deeper than initially intended.

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Meta-Analysis of Invasive versus Non-Invasive Techniques to Predict Fluid Responsiveness by Passive Leg Raising in the Critically Ill

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Abstract

Objective: To analyze the accuracy and specificity of recent studies to compare the ability of predicting fluid responsiveness with Passive Leg Raising (PLR) by using invasive or non-invasive techniques during passive leg raising. Data Sources: MEDLINE, EMBASE and the Cochrane Database of Systematic Reviews were systematically searched. Study Selection: Clinical trials that reported the sensitivity, specificity and area under the receiver operating characteristic curve (AUC) between the responder and non-responder induced by passive leg raising and Volume Expansion (VE) in critical ill patients were selected. 246 studies were screened, 14 studies were included for data extraction, which met our inclusion criteria. Data Extraction: Data were abstracted on study characteristics, patient population, type and amount of VE, time of VE, definition of responders, position, techniques used for measuring hemodynamic change, number and percentage of responders, the correlation coefficient, sensitivity, specificity, best threshold and area under the ROC curve (AUC). Meta-analytic techniques were used to summarize the data. Data Synthesis: A total of 524 critical ill patients from 14 studies were analyzed. Data are reported as point estimate (95% confidence intervals). The pooled sensitivity and specificity of invasive techniques were 80% (73% - 85%) and 89% (84% - 93%) respectively with the area under the sROC of 0.94. While, the pooled sensitivity and specificity of non-invasive techniques were 88% (84% - 92%) and 91% (86% - 94%) respectively with the area under the sROC of 0.95. The pooled DOR of invasive techniques was 32.2 (13.6 - 76.8), which was much lower than that of non-invasive techniques with the value of 64.3 (33.9 - 121.7). Conclusions: The hemodynamic indexes changes induced by PLR could reliably predict fluid responsiveness. Non-invasive hemodynamic techniques with their accuracy and safety can benefit the daily work in ICUs. Because the num-

ber of patients included in the present trials was small, further studies should be undertaken to confirm these findings.

Keywords

Invasive, Non-Invasive, Fluid Responsiveness, Meta-Analysis

1. Introduction

Fluid therapy is an essential part in Intensive Care Unit (ICU) to survive patients with hypovolemia. In fact, that's not easy. Studies have shown that about 50% of critically ill patients do not exhibit the desired effect [1]. How to assess intravascular volume accurately has been a critical problem.

Passive Leg Raising (PLR) is a reversible maneuver that mimics rapid Volume Expansion (VE) by shifting venous blood from the lower limbs toward the intrathoracic compartment [2]. Thus, PLR increases the cardiac preload. PLR has been validated to predict fluid responsiveness, but it requires the determination of CO or its surrogates with a fast-response device, because the hemodynamic changes may be transient [3] [4].

There are a lot of "fast-response devices" and all of them can be divided into 2 categories: invasive and non-invasive. Invasive hemodynamic techniques such as transpulmonarythermodilution (PiCCO), Vigileo, arterial BP transducer, pulmonary artery catheter are widely used in intensive units. Over the past few years, new techniques assessed for rapid and non-invasive prediction of fluid responsiveness have been introduced in clinical practice. Transthoracic echocardiography (TTE), transesophageal echocardiography (TEE), transthoracic Doppler ultrasonography (USCOM), Bioreactance technology-based system (NICOM), Continuous Non-invasive Arterial Pressure (CNAP) have been developed to predict fluid responsiveness.

Evidence shows that various studies have confirmed the ability of predicting fluid responsiveness by these techniques, but the predictive value of the hemodynamic response after PLR as a dynamic index of fluid responsiveness between invasive and non-invasive techniques has not been compared yet. The aim of this systematic review is to answer the question: can non-invasive techniques be better than invasive ones to be used as a tool for predicting volume responsiveness in critically ill during PLR maneuver and VE?

Data reporting conformed to the Standards for Reporting of Diagnostic Accuracy (STARD) [5].

2. Materials and Methods

2.1. Search Strategy

Two authors independently performed a search in MEDLINE (using PubMed as the search engine, from 1947), EMBASE (from 1974) and the Cochrane Database of Systematic Reviews for prospective studies in January 2014 with the following key words:

"Passive leg raising" AND (fluid therapy OR fluid responsiveness OR fluid expansion OR fluid load* OR volume therapy OR volume responsiveness OR volume expansion).

2.2. Study Selection

Only full-text articles in indexed journals were included. Reviews, chapter, case reports, reference network and studies published in abstract form were excluded. No language restriction was imposed. We included only studies with patients admitted in intensive care unit (ICU). Children and pregnant women would be excluded. Articles were collected by one reviewer and crosschecked by another reviewer and references of included papers were examined to identify other studies of interest.

2.3. Inclusion Criteria

We included full-text studies with the following criteria: 1. PLR was performed and followed with VE; 2. the number of patients and boluses had been counted; 3. the reference standard of predicting fluid responsiveness had been described; 4. the number of responsive patients and non-responsive patients had been counted; 5. sensitivity, specificity and the threshold of the index in identifying those patients who subsequently responded to VE (responders) had been calculated.

2.4. Data Extraction and Quality Assessment

Data were extracted using a structured data collection sheet including the following items: authors, year of publication, study setting, population, age of patients, number of patients included, ventilation mode, cardiac rhythm (sinus vs. arrhythmias), type and amount of VE, time of VE, definition of responders, position, assessments used for measuring hemodynamic change, number of VE administered, number and percentage of responders, sensitivity, specificity, best threshold and area under the ROC curve (AUC). We use QUADAS-2 (quality assessment of diagnostic accuracy-2) [6] to assess the quality of included studies on diagnostic accuracy in systematic reviews. The checklist was structured with 4 parts: patient selection, index test, reference standard and flow and timing.

2.5. Statistical Analysis

We used RevMan 5.2 (Cochrane Collaboration, Oxford, UK) to make the QUADAS-2 scale to assess quality of studies on diagnostic accuracy to be included in systematic reviews. To calculate pooled values of sensitivity, specificity, diagnostic odds ratio (DOR) and area under summary receiver operating characteristic (sROC) curve we used MetaDiSC 1.4 (Unit of Clinical Biostatisticsteam of the Ramon y Cajal Hospital, Madrid, Spain). P-values of less than 0.05 were considered statistically significant. Publication bias was performed by STATA statistical software 12.0 (StataCorp, College Station, TX).

We used the Cochran Q statistic [7] to evaluate heterogeneity between studies. When the value of p less than or equal to 0.10 and I^2 more than 50%, it could be regarded as

heterogenerity significantly and a random effect model was used to perform meta-analysis. For sensitivity and specificity, the Spearman correlation coefficient between those two parameters was calculated to evaluate a threshold effect determining heterogeneity [8].

For each study, sensitivity, specificity, positive likelihood ratio (+LR), negative likelihood ratio (−LR), and DOR were calculated after constructing a 2 × 2 contingency table. Pooled estimates with 95% confidence intervals (CIs) were calculated using a random-effects model. A summary receiver operating characteristic (sROC) curve was drawn according to the regression model proposed by Moses *et al.* [9] and it was performed to assess the interaction between sensitivity and specificity. The area under the sROC curve (AUC) was obtained to assess the diagnostic performance of hemodynamic techniques. Potential presence of publication bias was tested using the Egger [10] and Begg test [11].

3. Results

3.1. Process of Study Selection

The initial search yielded 246 articles after the first query in the three databases. Among them, 86 were excluded for not directly concerning item of interest. In the 160 full-articles, 103 were excluded because they were reviews, chapters or abstracts. 16 were excluded because they didn't perform PLR and another 14 were excluded because they didn't use VE. 13 were excluded because they didn't satisfy our inclusion criteria. Therefore, 14 studies [12]-[25] were included for final analysis.

3.2. Characteristics of Included Studies

The clinical characteristics of the 14 included studies were summarized in **Table 1** and main results were reported in **Table 2** and **Table 3**. The results of QUADAS-2 were showed in **Figure 1**. All the included 14 studies were prospective studies with enrollment of patients with sign of inadequate tissue perfusion. We found good compliance with appropriate population selection, index test adequately described, appropriate reference standard, and adequate flow and timing. Population selection bias was minimized, as the inclusion criteria of the patients were close. However, no study described the blinding of the assessors to the outcome measurement of the results. 2 out of the 14 included studies didn't report the lasting time of PLR.

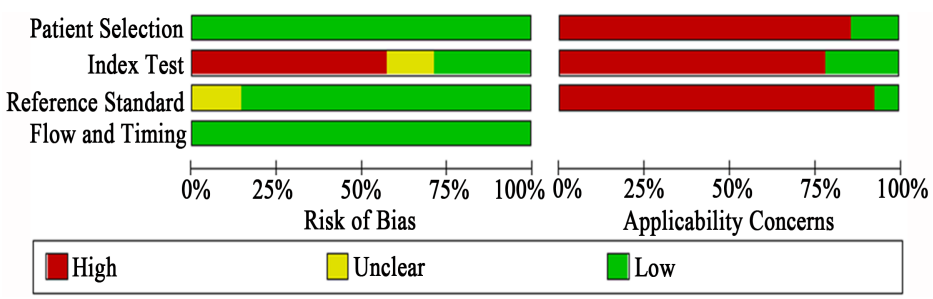

Figure 1. Results of QUADAS-2 (software RevMan 5.2).

Table 1. Main characteristics of the included studies.

Authors	Year	No.	Ventilation	Rhythm	VE	Position	Responder	Index	Techniques
Lafanechère [12]	2006	22	MV	sinus	500cc saline	supine position	ΔABF≥15%	cABF-TEE cPP	TEE arterial BP transducer
Monnet [13]	2006	71	MV	sinus/arr	500cc saline	semi-recumbent	ΔABF≥15%	cPP cABF-TEE	arterial BP transducer TEE
Lamia [14]	2007	24	MV/SB	sinus/AF	500cc saline	semi-recumbent	ΔSVI≥15%	cVTIAo-TTE cCO-TTE	TTE TTE
Maizel [15]	2007	34	SB	sinus	500cc saline	supine position	ΔCO-TTE≥12%	cCO-TTE cSV-TTE	TTE TTE
Thiel [16]	2009	89	MV/SB	sinus/arr	500cc saline, Ringer's lactate, HES	semi-recumbent	ΔSV≥15%	cSV-TTE	TTE(USCOM)
Monnet [17]	2009	34	MV	sinus/arr	500cc saline	semi-recumbent	ΔCI≥15%	cCI cPP	PiCCO arterial BP transducer
Biais [18]	2009	30	MV/SB	sinus	500cc saline	semi-recumbent	ΔSV-TTE≥15%	cSV cSV-TTE	Vigileo TTE
Préau [19]	2010	34	SB	sinus	500cc HES	semi-recumbent	ΔSV≥15%	cSV-TTE cPP	TTE arterial BP transducer
Guinot [20]	2011	17	MV	sinus/arr	500cc saline	semi-recumbent	ΔSV-TTE>15%	cSV-TTE cCO-TTE	TTE TTE
Liu [21]	2011	20	MV	sinus/arr	250cc saline	semi-recumbent	ΔSV≥10%	cSV	PiCCO
Wang [22]	2011	33	MV/SB	sinus/arr	500cc saline	semi-recumbent	ΔSV-TTE≥15%	cSV-TTE cSV-USCOM	TTE USCOM
Monnet [23]	2012	39	MV	sinus	500cc saline	semi-recumbent	ΔCI≥15%	cPPV cPPV-CNAP	PiCCO CNAP
García [24]	2012	37	MV	sinus/arr	500cc HES	semi-recumbent	ΔCO≥15%	cCO-TEE cPP	TEE arterial BP transducer
Monnet [25]	2013	40	MV	sinus/arr	500cc saline	semi-recumbent	ΔCI≥15%	cCI	PiCCO

MV: mechanical ventilation, arr: arrhythmia, AF: atrial fibrillation, VE: volume expansion, min minutes, BP: blood pressure, Δ: variation; c: PLR-induced changes, TTE: transthoracic echocardiography, TEE: transesophageal echocardiography, CI: cardiac index, CO: cardiac output, SV: stroke volume, PP: pulse pressure, PPV: pulse pressure variation, ABF: aortic blood flow, VTIAo: aortic velocity-time integral, USCOM: transthoracic Doppler ultrasonography, CNAP: continuous non-invasive arterial pressure.

Table 2. Pooled results for predictive capacity of invasive hemodynamic techniques.

Authors	Index	Boluses	TP	FP	FN	TN	AUC	Best Threshold	Sens.	Spec.	DOR	+LR	−LR
Lafanechère [12]	cPP	22	7	1	3	11	0.78	12	70	92	25.7	8.4	0.3
Monnet [13]	cPP	71	22	5	15	29	0.96	12	60	85	8.5	4	0.5
Monnet [17]	cCI	34	21	0	2	11	0.94	10	91	100	197.8	21.5	0.1
Biais [18]	cSV	30	20	2	0	8	0.96	13	100	80	139.4	4.3	0
Préau [19]	cPP	34	11	3	3	17	0.86	9	79	85	20.8	5.2	0.3
Liu [21]	cSV	46	12	2	3	29	0.85	12.5	80	93.5	58	12.4	0.2
Monnet [23]	cPPV	39	15	2	2	20	0.89	10	88	91	75	9.7	0.1
García [24]	cPP	37	14	3	7	13	0.73	11	67	81	8.7	3.6	0.4
Monnet [25]	cCI	40	20	1	1	18	0.98	15	95	95	360	18.1	0.1
Overall (95% CIs)		353							80 (73 - 85)	89 (84 - 93)	32.2 (13.5 - 76.8)	5.8 (3.8 - 8.8)	0.2 (0.1 - 0.4)

TP: true-positive, FP: false-positive, FN: false-negative, TN: true-negative, AUC: area under the receiver operating characteristics curve, 95% CIs: 95% confidence intervals, Sens: sensitivity, Spec: specificity, DOR: diagnostic odds ratio, +LR: positive likelihood ratio, -LR: negative likelihood ratio, CI: cardiac index, SV: stroke volume, PP: pulse pressure, PPV: pulse pressure variation.

Table 3. Pooled results for predictive capacity of non-invasive hemodynamic techniques.

Authors	Index	boluses	TP	FP	FN	TN	AUC	Best Threshold	Sens.	Spec.	DOR	+LR	−LR
Lafanechère [12]	cABF-TEE	22	9	2	1	10	0.95	8	90	83	45	5.4	0.1
Monnet [13]	cABF-TEE	71	36	2	1	32	0.75	10	97	94	576	16.5	0
Lamia [14]	cVTIAo-TTE	24	10	0	3	11	0.96	12.5	77	100	69	18	0.3
Maizel [15]	cSV-TTE	34	15	3	2	14	0.9	8	88	83	35	5	0.1
Thiel [16]	cSV-TTE	102	38	4	9	51	0.89	15	81	93	53.8	11.1	0.2
Biais [18]	cSV-TTE	30	17	1	3	9	0.92	16	85	90	51	8.5	0.2
Préau [19]	cSV-TTE	34	12	2	2	18	0.94	10	86	90	54	8.6	0.2
Guinot [20]	cCO-TTE	25	11	2	2	10	0.87	5	85	83	27.5	5.1	0.2
Wang [22]	cSV-TTE	36	24	2	0	10	0.95	15	100	83.3	205.8	5.1	0
Monnet [23]	cPPV-CNAP	39	14	2	3	20	0.89	11	82	91	46.7	9.1	0.2
García [24]	cCO-TEE	37	20	1	1	15	0.97	12	95	94	300	15.2	0.1
Overall (95%CIs)		454							88 (84 - 92)	91 (86 - 94)	64.3 (33.9 - 121.7)	7.8 (5.3 - 11.6)	0.17 (0.12 - 0.24)

TP: true-positive, FP: false-positive, FN: false-negative, TN: true-negative, AUC: area under the receiver operating characteristics curve, 95% CIs: 95% confidence intervals, Sens: sensitivity, Spec: specificity, DOR: diagnostic odds ratio, SV: stroke volume, ABF: aortic blood flow, VTIAo: aortic velocity-time integral, TTE: transthoracic echocardiography, TEE: transesophageal echocardiography, CNAP: continuous non-invasive arterial pressure.

A total of 524 patients were enrolled (range 17 - 89 for single paper) and a total of 574 VE were administered. The mean responder rate was 52.8%.

All studies were conducted in intensive care units (ICU) on patients with hypovolemia, whose attending physician decided to perform a fluid challenge. 2 study [15] [19] enrolled patients had spontaneous breathing without mechanical ventilator in sinus rhythm. The others [12] [13] [14] [16] [17] [18] [20]-[25] enrolled patients with mechanical ventilation and/or arrhythmias. The reference standard for definition of responders after fluid bolus as CO or its surrogates ranged between 10 and 15%. 11 out of 14 studies [12] [13] [14] [15] [17] [18] [20] [21] [22] [23] [25] used saline for volume expansion (VE). 2 studies [19] [24] used hetastarch for VE. Only 1 study used either saline, ringer's lactate or hetastarch for VE. PLR was starting from a supine position in 2 studies [12] [15], and from semi-recumbent position in 12 studies [13] [14] [16]-[25]. 9 studies [12] [13] [17] [18] [19] [21] [23] [24] [25] used invasive hemodynamic techniques like PiCCO, Vigileo and arterial BP transducer and 11 studies [12] [13] [14] [15] [16] [18] [19] [20] [22] [23] [24] used non-invasive techniques, such as TEE, TTE, NICOM, USCOM and CNAP.

3.3. Diagnostic Accuracy of Invasive Techniques

We first divided the 14 studies into 2 groups: invasive group [12] [13] [17] [18] [19] [21] [23] [24] [25] and non-invasive group [12] [13] [14] [15] [16] [18] [19] [20] [22] [23] [24]. Then we meta-analyzed all papers into each group. Results were reported in Table 2 and Table 3. When a study used both invasive and non-invasive techniques

[12] [13] [18] [19] [23] [24], the indices of both techniques could be included. When a study reported analysis for two indices of the same category [14] [15] [17] [20] [21] [22] reported by the same technique only one was included in the meta-analysis in order to avoid duplication of sample size.

There were 9 papers (327 patients, 353 boluses) in the invasive group. The results I^2 = 39.6% (<50%) and p = 0.1037 (>0.05) showed that heterogeneity was not significant among the trials. Forest plots of the pooled sensitivity and specificity were shown in Figure 2. The sensitivity ranged from 60% - 100% (pooled sensitivity 80%, 95% CI: 73% - 85%), while specificity ranged from 85% - 100% (pooled specificity 89%, 95% CI: 84% - 93%). DOR was 32.2 (95% CI: 13.5 - 76.8). Pooled values for positive likelihood ratio (+LR) and negative likelihood ratio (−LR) were 5.8 (95% CI: 3.8 - 8.8) and 0.2 (95% CI: 0.1 - 0.4). The threshold for predicting fluid responsiveness varied between 9% and 15%.

After excluded the threshold effect with spearman correlation coefficient = 0.233 and p = 0.546 (>0.05), we used Moses-Shapiro-Littenberg method to draw the symmetrical summary ROC curve (SROC) with AUC of 0.94.

3.4. Diagnostic Accuracy of Non-Invasive Techniques

There were 11 papers (430 patients, 454 boluses) in the non-invasive group. The results

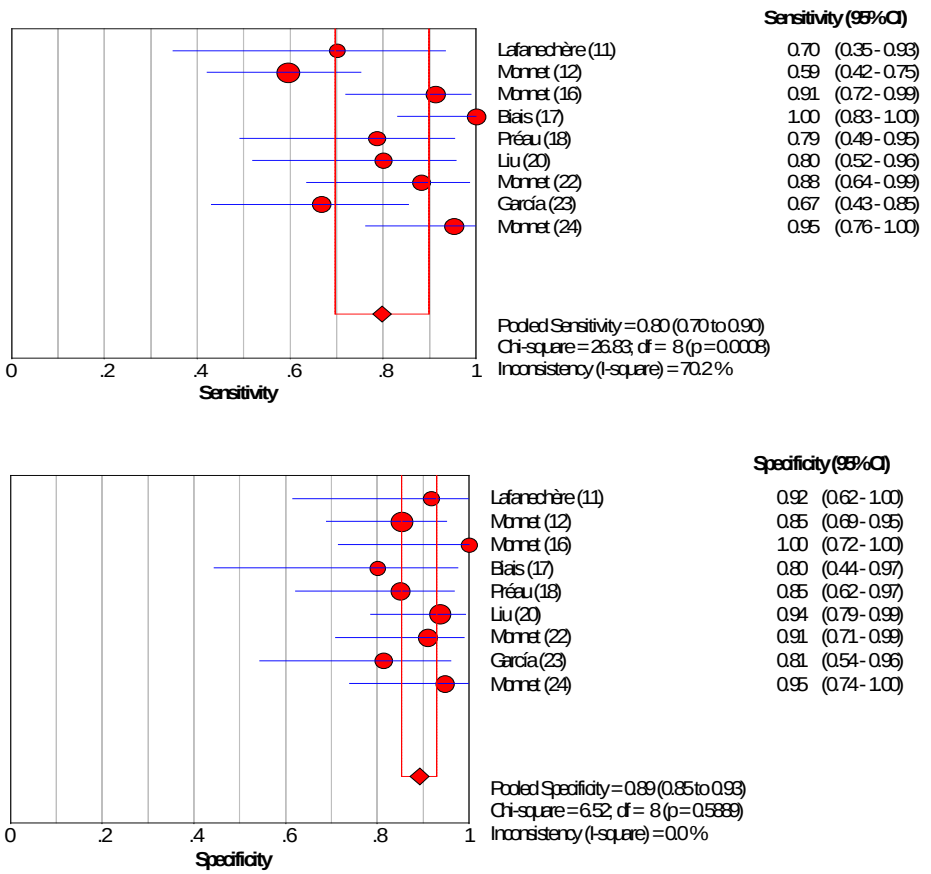

Figure 2. Forest plots of pooled sensitivity between invasive and non-invasive techniques.

I^2 = 0.0% (<50%) and p = 0.809 (>0.05) showed that heterogeneity was not significant. Forest plots of the pooled sensitivity and specificity were shown in **Figure 3**. The sensitivity ranged from 77% - 100% (pooled sensitivity 88%, 95% CI: 84% - 92%), while specificity ranged from 83% - 100% (pooled specificity 91%, 95% CI: 86% - 94%). DOR was 64.3 (95% CI: 33.9 - 121.7). Pooled values for positive likelihood ratio (+LR) and negative likelihood ratio (−LR) were 7.8 (95% CI: 5.3 - 11.6) and 0.2 (95% CI: 0.1 - 0.2). The threshold for predicting fluid responsiveness varied between 5 and 15%.

After excluded the threshold effect with spearman correlation coefficient = 0.361 and p = 0.276 (>0.05), we drew the symmetrical summary ROC curve (SROC) (**Figure 4**), with AUC of 0.95.

3.5. Publication Bias

The result of Egger test and Begg test showed that the potential publication bias was significant (P > 0.05), which indicated a potential for publication bias.

4. Discussion

The main finding of our systematic review are as follows: (1) The result of pooled sensi-

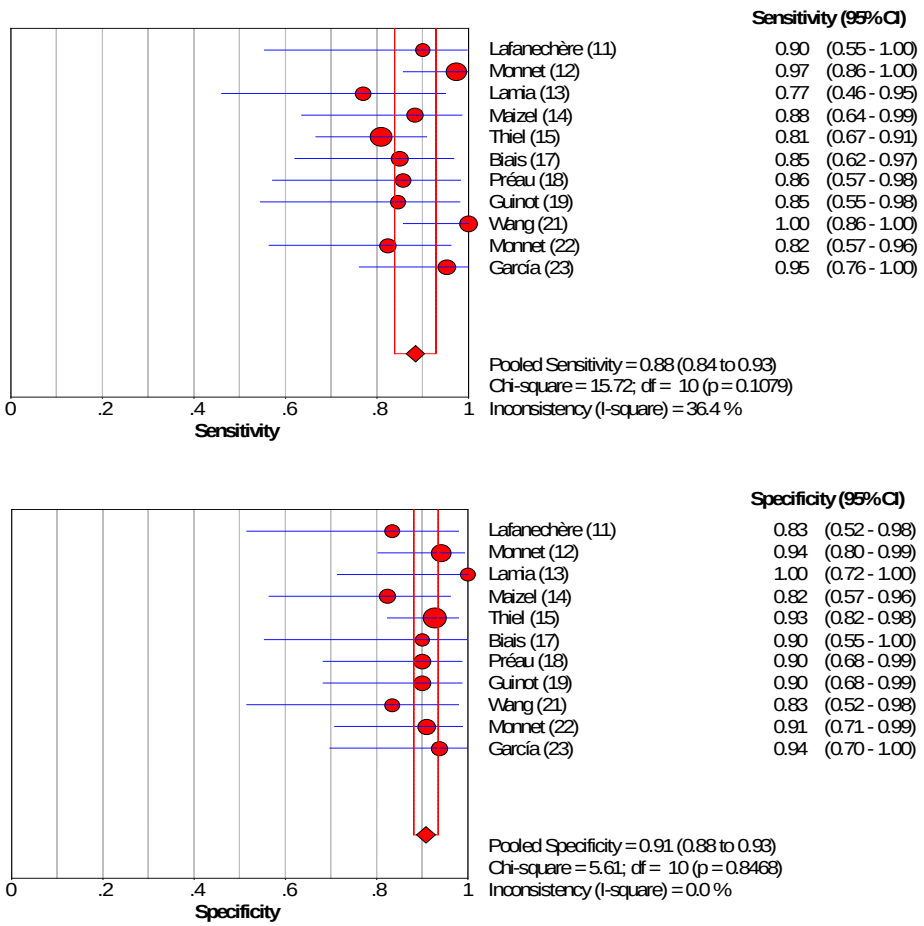

Figure 3. Forest plots of pooled specificity between invasive and non-invasive techniques.

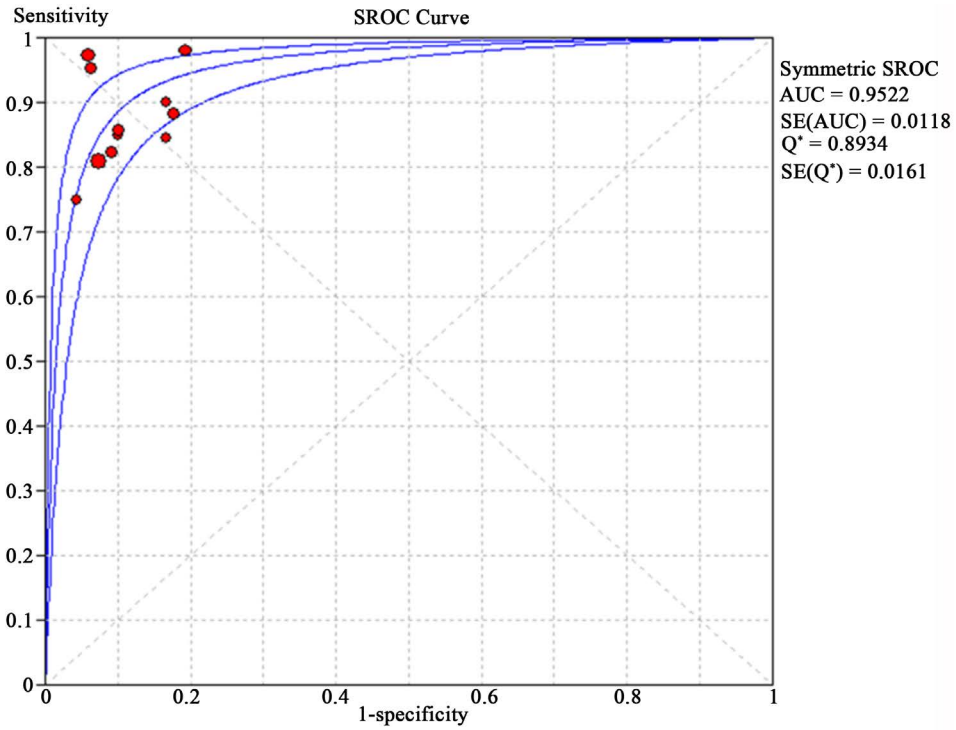

Figure 4. Summary receiver operating characteristics curve for the ability of non-invasive techniques discriminate between responders and non-responders.

tivity and specificity between invasive and non-invasive techniques are 80% (73% - 85%) vs. 88% (84% - 92%) and 89% (84% - 93%) vs. 91% (86% - 94%), which cannot conclude inferior or superior; (2) The results of pooled DOR between invasive and non-invasive is 32.2 (13.6 - 76.8) vs. 64.3 (33.9 - 121.7), which indicate using non-invasive techniques have better discriminatory test performance with higher DOR values [8].

Knowing that dynamic indexes such as CO, CI, SV, ABF, SVV, PPV make use of provoked cardiac reaction assessed with fluid bolus and postural change can predict fluid responsiveness. A recent analysis by Vallee F shows that increase in thermodilution CO following a fluid bolus can predict fluid responsiveness [26]. The invasive techniques such as PiCCO, Vigileo, and arterial BP transducer are widely used in ICU to assess the patients' volume statue. Also, a systematic review by Mandeville *et al.* [27] assessed the value of TTE in predicting fluid responsiveness in critically ill. In our review, both invasive and non-invasive hemodynamic techniques can accurately predict fluid responsiveness. DOR is the ratio of the odds of positive test results between the diseased and nondiseased groups. Non-invasive techniques have higher values of DOR can better discriminate test performance. Importantly, non-invasive techniques are much safer, more convenient than invasive ones. But the non-invasive techniques, especially for TTE and TEE require an experienced echocardiography practitioner, who can take echocardiography pictures to answer clinical questions arising in critical illness. Jensen showed that with only limited training, a diagnostic transthoracic window

was achieved 97 percent of the time when used in the evaluation of shock [28].

Strengths and Limitations

The strengths of our meta-analysis lie in the methods adhering to recent guidelines for diagnostic reviews [6] [29] [30], as well as in the advanced statistical methods used [31], which analyze all reported thresholds, sensitivity, specificity and their correlated results simultaneously. Also no other review has compared the ability to predict fluid responsiveness between these 2 groups of hemodynamic techniques. The results of our review could guide the using of the techniques to assess patients' volume statue in our clinical practice.

Limitations still exist in our meta-analysis. First, the pooling of diagnostic accuracy data inevitably contributed to sources of bias [7], which could be revealed in the significant amount of statistical heterogeneity across studies. Second, the number of patients included in the present trials was small (14 studies, 524 patients). A better review needs larger sample of studies. Third, the criteria of the included studies are based on clinical manifestation and the confounding factors such as cardiac function, respiratory function, severity of disease have not been analyzed.

5. Conclusion

The hemodynamic indexes induced by PLR can well discriminate between fluid responders and non-responders regardless of arrhythmia and ventilation mode. Non-invasive hemodynamic techniques with their accuracy and safety can benefit the daily work in ICUs.

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Inevitability of an Enhanced Monitoring Strategy to Reduce Water Borne Illness Combining Indicators of Sanitary Protection and Measuring Water Quality

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Abstract

Background: Diarrhoea is the second leading cause of childhood mortality in children under five years old. Water is a major contributing risk factor for this disease that is a common illness and a global killer. Material and Methods: Water samples from different areas of Karachi were collected and were processed by MPN technique to evaluate the presence of microbiological substances. Results: Out of the processed samples, 64% were positive for the presence of mix enteric pathogen from different areas of Karachi; from Bhens colony, 74% were positive for the presence of fecal coli forms, among which 4% were positive for EHEC isolated from storage tank and water tank. Conclusion: The abovementioned results reflect the presence of organisms of public health importance in different sources of drinking water. According to WHO guidelines, there should be zero tolerance of these organisms in water.

Keywords

Acute Watery Diarrhea, Parasites, Pathogens

1. Introduction

Worldwide 1.1 billion people lack access to improved water supplies and 2.4 billion do not have access to sanitation services while hundreds of millions rely on unimproved water supplies that are not safe because of microbial or chemical contamination [1].

Diarrheal diseases are infections caused by a group of bacterial, viral and parasitic organisms and the second leading cause of death in children under five years old, responsible for killing around 760,000 children every year [2] [3]. Children who are malnourished or immune compromised are at high risk of life-threatening diarrhea. The mode of transmission of such pathogens is food and water sources contaminated with human and animal feces. Most common source of entry is sewage, septic tanks and latrines [4] [5].

Approximately 25% Karachi's total population of 16 million people lives below the poverty line. Overcrowding, poverty and low socio economic status, frequent episodes of illness, poor sanitation and hygienic practice, use of contaminated drinking water and food, limited access of populations to their basic needs and high number of reported diarrheal cases from these areas were the major reasons to conduct this study in those areas. Unfortunately, environmental factors, diseases, inadequate sanitation and lack of safe drinking water even make the living condition worse in above mentioned study areas. Due to all of the above mentioned factors, the increase number of reported cases from these areas was the major reason to conduct this study there to relate the relationship of drinking water with waterborne diseases [6] [7] [8].

Public and environmental health protection requires safe drinking water, which means that it must be free of pathogens. Among the pathogens disseminated in water sources, enteric pathogens are the ones that are most frequently encountered [6]. As a consequence, sources of fecal pollution in waters devoted to human activity must be strictly controlled. Enteric pathogens, such as *Escherichia coli* O157:H7, are generally present at very low concentrations in environmental water within a diversified micro flora [2]. Food is another major cause of diarrhea when it is prepared by water stored in unhygienic conditions. Water can contaminate food during irrigation. Fish and seafood from polluted water may also contribute to the disease.

In developing countries, children under five years old experience three episodes of diarrhea on average every year. Each episode deprives the child of the nutrition necessary for growth. Infection is more common when there is a shortage of adequate sanitation and hygiene and safe water for drinking, cooking and cleaning. Interventions to prevent diarrhea, including safe Diarrhea, can be treated with a solution of clean water provision [7].

In a local study conducted presence of trace metals (nickel, copper, chromium, lead, cobalt, manganese and iron) was confirmed in drinking water samples when the concentration of metals was compared with the World Health Organization (WHO) drinking water quality guideline values, reflecting the adverse health effects to the inhabitants of Karachi due to the oral ingestion and dermal absorption of water containing these metals [9]. Another study says that microbial and chemical pollutants are the main factors responsible exclusively or in combination for various public health problems due to violation of standards set by WHO of drinking water quality in Pakistan [10].

The present study is designed to have a demographic survey of the portability of drinking water in a metropolitan city, Karachi. This study was aimed to investigate the

common sources of fecal contamination in drinking water. This study has helped us to design a few recommendations to avoid this waterborne deadly disease.

2. Methodology

2.1. Inclusion Criteria

Random selection of local water source used for human and animal consumption in the above mentioned localities, samples were un-boiled un-chlorinated

2.2. Exclusion Criteria

Any bottled water available in market as well chlorinated and boiled water samples were excluded.

2.3. Informed consent

Meeting was scheduled with every individual participant whose water source was selected and informed consent was read aloud.

Ethical consideration included informed consent form provided participants with an overview of the study objectives. The right of the participants to refuse to answer any questions, as well to end the interview at any time was briefed, before inclusion in the study. Every subject had been informed about the procedure of collecting water samples in the meeting.

2.4. Administration of Questionnaire

Eligible candidates were first briefed about the objectives of the study followed by face to face interview.

2.5. Data Collection Instrument

Questionnaire included socio economic status and personal information questions as well set of questions to predict the contributing factors towards the objectives of the study. The following principal variables were included. Use of un-boiled/un-boiled drinking water, socio economic living conditions, water source, and cleaning procedure and duration of storage tank at the time of interview.

2.6. Debriefing and Referrals

After the completion of interview, a debriefing session was held with participants to allow the interviews to respond to any question that the participants may have had. Information was also provided to participants during this session on the mode of transmission as well prevention and contributing factors of diarrheal infection due to use of contaminated water. All participates were referred to the lab for collection of reports after evaluation of water samples.

2.7. Water Collection and Laboratory Processing

Following areas were selected for the study after being constantly reported for high

number of diarrheal cases by the residing population during another surveillance to evaluate water as contributing factor for the disease. About 50 water samples from different sources and places of Karachi and 50 samples from Bhens coloney used for humans and animals consumption were selected with in the period of five months started from May 2015 till Sep 2015. Water samples from areas of Karachi including Landhi, orangi, Layari, LaloKheet, Sohrabgoath, slum Areas of Gulshan e-Iqbal, Sheer shah, Nazam-e-Abad and Malir were collected. Sterile plastic bottle in duplicates were filled with sample, placed it in a box with ice packs and brought it to IIDRL [Immunology nd infectious diseases reference laboratory] at the university of Karachi. Within 24 hours of collection, water samples were processed by MPN technique to evaluate the load of microorganism.

Water samples from municipal tap water, storage tanks, water carry tanks, water cooler and also include public filter plants of the locality were included. These are all sources that are being used by local population for drinking purpose. Water sample that is chlorinated, boiled or branded bottle water available in the locality were excluded and not processed in this study.

2.8. Water Culture by Multiple Tube/MPN Technique

Coliform examination consists of the inoculation of measured volumes of water into tubes of MacConkey Broth (Purple) which are incubated at 35˚C for 48 hours. Volumes for inoculation are one 50 ml, five 10 ml and five 1ml quantities of water-50 ml and 10 ml amounts being added to their own volume of double-strength Mac Conkey broth, while the 1 ml amounts are each added to 5ml of single-strength. Water samples from fifty different areas of Karachi city were collected in duplicate and transported in sterile water containers to the lab within 12 hours of collection. In MacConkey Broth Acid formation is indicated by yellow colorations of the broth, and gas formation is indicated by an amount of gas at least sufficient to fill the concavity at the top of the Durham tube. From the number of tubes showing the presence of acid and gas, the most probable number of [presumed] coliform bacteria present in 100ml of the original water may be estimated by reference to probability tables.

For the differential coliform test, each MacConkey tube showing acid and gas is then subcultured into a fresh tube of MacConkey Broth and incubated at 44˚C. Formation of gas within 48 hours is practically specific for *Escherichia coli* and indicative of fecal pollution of the original water sample. Organisms were processed for the identification by conventional methods using biochemical reactions. Gram staining was done from these lactose fermenting and non fermenting colonies and the isolates were found to be gram negative straight rods [1]. The strains were further identified on the basis of motility, Indole, MR , VP , Citrate, Urease, Oxidase Melanin and gas production from glucose were labeled according to specific reactions [2] [8]. *E. coli* samples were further processed for the detection of EHEC presence. PCR was done with following primers while extraction and amplification was done by kit according to manufacturers' guidelines [DNA extraction kit, 761001D, PCR master mix, 4390939, Invitrogen].

The optimized method utilized, a 0.025 μM concentration of the *stx*2 probe, 1.25 U

of AmpliTaq Gold (Applied Biosystems), and 0.5 µl of sample template in a total volume of 25 µl. The amplification program included an initial polymerase activation step, 10 min at 94˚C, and 40 cycles of 20 s at 94˚C and 25 s at 63˚C, performed on a Smart Cycler thermal cycler. Fluorescence values were recorded in each round during the 25-s, 63˚C annealing-extension step [9].

Strain	Target Gene	Oligonucleotide Sequence	Amplicon Size (bp)
EHEC	*stx*2	GGGTACTGTGTGCCTGTTACTGG	510
		GCTCTGGATGCATCTCTGGT	

3. Results

Out of fifty processed samples 32 (64%) were positive for the presence of different microorganisms. Fecal coli forms 8/19 (16%) were isolated from different sources. There were 3 (6%) samples, which contain *Klebsiella spp*. and 6 (12%) *Pseudomonas spp.* There were 5 (10%) samples, which contain the presence of combination of different organisms in the same sample (Table 1). This shows poor sanitation and high level of contamination in water used for the local community as shown by bar graphs in Figure 1.

Similarly water samples processed from Bhens colony showed even worse situation where 37 (74%) samples were positive for the presence of microorganisms. There were 16 (32%) samples indicative of fecal coliforms while 4% were *Klebsiella spp*. Also, 4 (8%) samples were positive for the presence of *Pseudomonas spp.* It is very important to note as indicated in Figure 2, that 2 (4%) samples were positive for the presence of *Salmonella spp*. [non typhoid]. Similarly 8 (16%) samples showed mix combination of different organisms (Table 2).

The positive samples were further processed for the presence of EHEC isolates and 2 samples were positive for EHEC. They were isolated from storage tank and water carry tank. This high level of contamination clearly showed how diarrheal pathogens act as major contributing factor in spread of diarrhea through water sources.

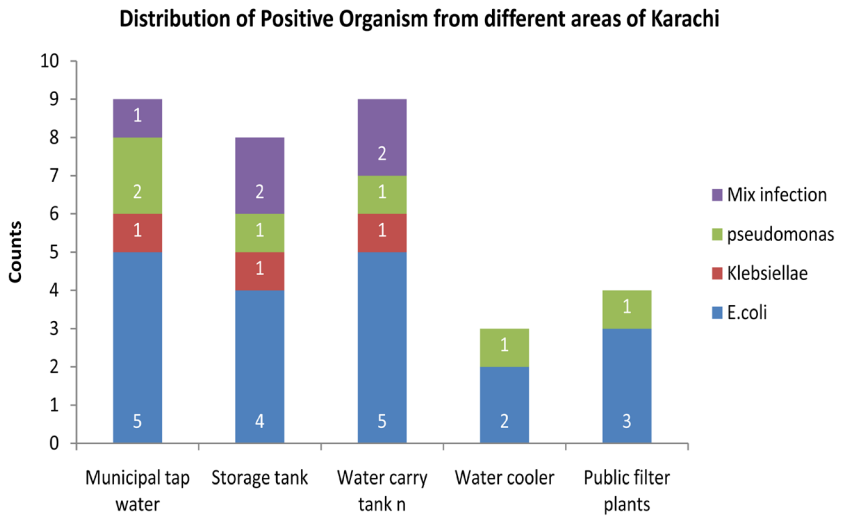

Figure 1. Distribution of positive organism from different areas of Karachi.

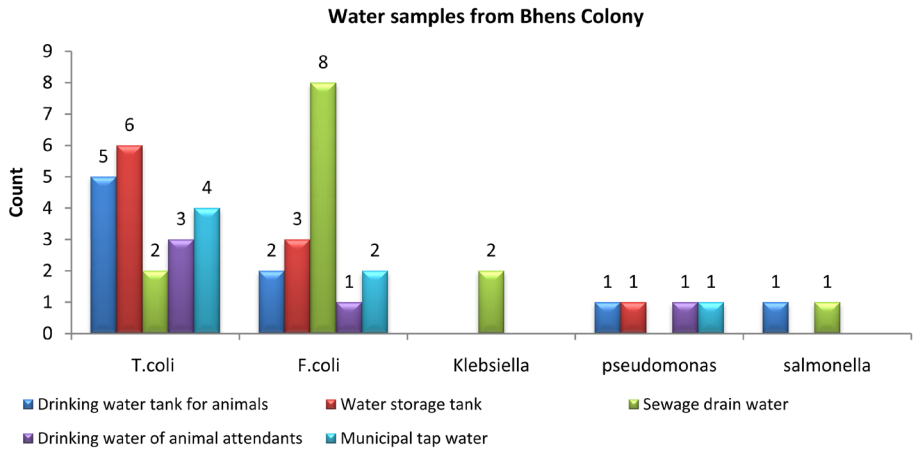

Figure 2. Water samples from Bhens Colony.

Table 1. Demographic study for the presence of enteric pathogens of water samples from different localities of Karachi.

Source of water	Municipal tap water	Storage tank	Water carry tank	Water cooler	Public filter plants	Total samples
	N = 10	N = 10	N = 10	N = 10	N = 10	N = 50
Positive samples	9	8	8	3	4	32
E.coli	5 T. coli for m 3 F. coli for m 2	4 T. coli for m 1 F. coli for m 3	5 T. coli for m 3 F. coli for m 2	2 T. coli for m 2 F. coli for m 0	3 T. coli for m 2 F. coli for m 1	19 T. coli for m 11 F. coli for m 08
Klebsiellae	1	1	1	0	0	3
pseudomonas	2	1	1	1	1	6
Mix infection	1	2	2	0	0	5
Combination of Organisms	*E. coli* + *Seudomonas* + *Salmonella*	*E. coli* + *Pseudomonas* and *E. coli* + *Klebsiella*	*E. coli* + *Klebsiellae* spp + *Pseudomonas* and *E. coli* + *Salmonella*	-	-	6

Table 2. Water samples from Bhens colony.

Source of water	Drinking water tank for animals	Water storage tank	Sewage drain water	Drinking water of animal attendants	Municipal tap water
Total 50	10	10	10	10	10
E.coli	7 T. coli 5 F. coli 2	9 T. coli 6 F. coli 3	10 T. coli 2 F. coli 8	5 T. coli 3 F. coli 1	6 T. coli 4 F. coli 2
Klebsiella	-	-	2	-	-
Pseudomonas	1	1	-	1	1
Salmonella	1	-	1	-	-
Mix	*E.coli* + *Pseudomonas*	*Salmo* + *E. coli*	4 diff combination	-	*E. coli* + *Pseudo*

4. Discussion

Analysis of water samples showed that only 18 out of 50 samples were satisfactory for drinking purpose. In our results, the worse was the situation in water samples collected from Bhens colony where only 13/50 samples were satisfactory, which reflected the presence of organisms of public health importance including *E. coli, Pseudomonas spp*., *Klebsiella spp*., *Salmonella spp*. The presence of high levels of Total Coliforms Count (TCC), Total Fecal Coliform count (TFC) is a matter of great concern because, according to WHO guideline there should be zero tolerance of these organisms in drinking water. Municipal pipe lines running parallel to drinking water pipe lines, water stored in the household contaminated containers or water transported in contaminated tankers from far of places is more likely to be the reasons of contamination. Inadequate chlorination or dilution of it due to presence of high level of organic matter is associated along with poor hygiene and inadequate sanitation. Specific risks are identified through sanitary inspection of the water source and its surrounding. It was found that at certain points there were open sewage lines or sewage lines running parallel to drinking water lines. The presence of EHEC in Bhens colony water samples is indicative of contamination of animal feces in local water sources used for human consumption and most probably can cause diarrheal disease among residing population. This study has some limitations, like shortage of funds to limited available resources, and specified time period. Lack of funds and limited available resources, this study was conducted to a short period of time during summer season, in order to get clear and perfect view of the worse situation, while the study duration should be expended to at least one year to depict seasonal variation as well as co-relation with spread of diseases. More samples from different sources and areas should be included to avoid limitations of study. In spite of all above mentioned factors it was found that there was lack of awareness about these water borne infections and importance of boiled water for human health. Government should promote national policies and investments that support case management of diarrhea and its complications. Government also needs to make sure the access to safe drinking-water and sanitation in the country. Research should be to develop and test all possible water sources in order to elevate hospitalization burden due to diarrhea where water is involved as contributing factor in the spread of diarrhea. It is needed to build capacity in implementing preventive interventions, including sanitation, source water improvements, and household water treatment and safe storage.

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Psychiatric Co-Morbidity and Quality of Life in Egyptian Type 2 Diabetic Patients

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Abstract

Background: Diabetes is a risk factor for depression, but little is known about anxiety and other psychiatric disorders and quality of life. The aim of this study was to assess the prevalence of depression, anxiety in diabetic patients in our locality and to assess the quality of life in type 2 DM. Subjects & Methods: This study was a cross-sectional study and was carried out in outpatient clinics of specialized medical hospital, Mansoura university for a period of one year. From 217 diabetes mellitus subjects, only 202 patients were matched with 247 healthy people as a control group. All subjects were examined by using socioeconomic data, clinical data, and anthropometric examinations to assess body mass index and waist circumference. All patients were interviewed by using the Mini-International Neuropsychiatric Interview (MINI) version 5, MINI, Hospital Anxiety and Depression scale (HAD) and health-related quality of life (HRQOL) scales. Laboratory investigation in the form of fasting and two-hour postprandial blood sugar (FBS & 2hpp) and HbA1C levels were done. Results: 18.3% were found to be major depressive disorder; and 2.5% panic disorder, 1% other phobia. Generalized anxiety disorder and obsessive-compulsive disorder were found in one patient, no patients were found to be diagnosed as Bipolar disorder, schizophrenia, or substance abuse. Although there was no statistically significant difference between subjects and control groups regarding height, there was statistically significant difference between weights, BMI, with more scores among DM group. Moreover our study showed that HbA1c, fasting blood sugar, two hours post prandial blood sugar were more among DM patients and control groups. Anxiety, depression, and poorer quality of life were found to be more prevalent among DM patients than control groups. Conclusion: DM is associated with depression anxiety disorder with poorer quality of life.

Keywords

Diabetes, Stress, Anxiety, Depression, Psychiatric Co-Morbidities, Diabetic Complications, Glycemic Control

1. Introduction

An individual's health behavior is influenced by his or her social, economic, cultural, and physical environment. Medical experts have reported on the psychological components of almost all diseases, particularly chronic illnesses such as diabetes mellitus [1].

Diabetes increases the risk of depression. In a meta-analysis, the odds of having depression were two-fold in patients with diabetes compared with those without [2]. In addition, anxiety and eating disorders have also been reported to be common in patients with diabetes [3]. The prevalence of anxiety disorders among patients with diabetes is considerably higher compared to the general population [4]. Anxiety symptoms have been found to be significant risk factors for development of diabetes [5]. Negative correlations have been observed between prevalence of anxiety disorders and levels of HbA1c [6].

Quality of life is difficult to define. It is further complicated by related terms being used interchangeably, such as well-being, health status, and satisfaction. The burdens associated with diabetes, such as anxiety, regimented lifestyle and long-term complications, have prompted researchers and clinicians to examine the impact of the disease on the health-related quality of life (HRQOL) of people with diabetes [7]. Several studies have demonstrated that diabetes has a negative influence on the overall HRQOL and its domains of physical, psychological and social relationships and environment [8] [9] [10].

The DAWN Study (Diabetes Attitudes, Wishes, and Needs) was the world's largest international psychosocial study in persons with diabetes. It included 5000 people with diabetes and 3000 diabetes healthcare professionals across 13 countries. The results of the DAWN Study showed that as many as 41% of the patients had poor psychological well-being [9].

The aim of this study was to assess the prevalence of depression, anxiety in diabetic patients in our locality and to assess the quality of life in type 2 DM.

2. Subjects & Methods

2.1. Study Locality and Duration

This study was carried out in outpatient clinics of specialized medical hospital, Mansoura University for a period of one year between 1st March 2013 till 28th February 2014.

2.2. Study Design

The study is a cross-sectional comparative study for one-year duration.

2.3. Target Population

All patients came to outpatient clinics of specialized medical hospital, Mansoura, Egypt for treatment from type 2 diabetes mellitus (217 subjects). Eight refuse to participate in this study and seven subjects were excluded due to fulfillment of one or more exclusion criteria. Therefore, the study was conducted on 202 patients matched with 247 healthy

people as a control group. Control subjects were chosen from workers of specialized medical hospital, Mansoura University, Mansoura city. They were medically healthy (No evidence for any disease was found clinically by medicine specialist or by routine general investigations e.g. laboratory test for complete blood picture, liver and kidney function test). Moreover, all control subjects were free from any psychiatric disorders or substance abuse.

An inclusion criterion includes sex, Age range 25 - 70 years old and type 2 diabetes mellitus. **Exclusion criteria includes:** Type 1 diabetes mellitus; Gestational diabetes; Secondary diabetes due to another disease; The use of medications that affect food intake (Appetite suppressants and other anti-obesity drugs); The incapacity to self completes the questionnaires of depression; and Past history of depression or depression treatment or any other psychiatric illness.

The study was approved by the Mansoura Faculty of medicine, ethics committee, and then it has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. A written informed consent was obtained from all participants before inclusion in the study.

2.4. Study Tools

All Subjects were examined using especially designed sheet to collect socioeconomic data; clinical data (Comprehensive general examination); and anthropometric examinations to assess body mass index which was calculated as weight divided by height squared (kg/m^2) and waist circumference was measured with a flexible tape placed on a horizontal plane at the level of the iliac crest as seen from the anterior view. All patients were interviewed using the Mini-International Neuropsychiatric Interview (MINI) version 5. MINI is a short structured diagnostic interview. The scale had been previously translated and validated into Arabic [11]. All patients were diagnosed using DSM-5 criteria [12]. Furthermore, the severity of anxiety and depression were measured using hospital anxiety and depression scale (HAD) [13]. The Arabic version of the HAD scale was validated by [14]. To examine the impact of the disease on the health-related quality of life (HRQOL) we used the World Health Organization (WHO) quality of life questionnaire, short version (WHOQOL-BREF) [15]. The Arabic version of WHO-HRQOL was translated and validated by [7]. The WHOQOL-BREF is a 26-item self-report instrument, scored on a 5-point scale ranging from one (strongly agree) to five (strongly disagree), with the highest scores representing better HRQOL. There are four sub-scales within the instrument which measure the four domains of HRQOL: physical (e.g. body pain), psychological (e.g. self-esteem), social relationships (e.g. social support), and environment (e.g. physical safety). Laboratory investigation in the form of fasting and two-hour postprandial blood sugar (FBS& 2hpp) and HbA1C levels were done.

3. Statistical Methods

Data were analyzed using SPSS (Statistical Package for Social Sciences) version 20. Qu-

alitative variables were presented as number and percent. Chi-square was used for comparison between groups. Quantitative variables were tested for normality distribution by Kolomogorov-Smirnov test. Normally distributed variables were presented as mean ± SD and unpaired t test was used for group comparison. Non-parametric variables were presented as median (minimum–maximum). Student t-test was used to compare between two groups. Significant predictors for depression, anxiety, quality of life were entered into a logistic regression analysis using forward Wald methods. Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated. P value less than 0.05 was considered statistically significant.

4. Results

37 patients (18.3%) were found to fulfill DSM-IV-TR criteria for Major depressive disorder; and 5 patients (2.5%) fulfill Panic attach criteria, other phobia were found in two patients (1%), generalized anxiety disorder and Obsessive compulsive disorder were found in one patients(0.5%), No patients was found to be diagnosed as Bipolar disorder, schizophrenia, or substance abuse. In control group, no subject fulfills any DSM-IV-TR criteria for any disorder.

Table 1 demonstrated that the only statistically significant difference between control and subjects group were more anxiety and depression with poor quality of life in patients with diabetes than control groups. Anxiety were found to had significant difference between both group diabetic subjects were 86 (42.6%), 84 (41.6%) vs. control 3 (1.2%) 0 (0%). Depression in diabetic group showed significant difference 35 (17.3%), 74 (36.6%) compared to control subjects 5 (2%) 2 (0.8%). Quality of life in patients with diabetic group showed statistically significant difference 98 (48.5%) with bad QOL than control groups 0(0%). Although there were no statistically significant difference between subjects and control groups regarding height, there was statistically significant difference between BMI, with more scores among DM group 29.2260 Vs control 3.86901. Table 2 showed that HbA1c, fasting blood sugar, two hours post prandial blood sugar were more among DM patients and control groups. Anxiety 10.4307, Depression 9.3762, and poorer quality of life were 61.1386 found to be more prevalent among DM patients than control groups Anxiety 4.11860, Depression 4.84250, quality of life 29.50151. Among different predictors for anxiety, depression, quality of life, HBA1c was found to be the only predictor for the three examined variables. In Addition Age was found to be predictor for bad quality of life in DM patients (Table 3). Longer duration of DM and bad control of HbA1c were found to be associated with more anxiety disorders, more depression, and poorer quality of life (Table 4).

5. Discussion

Relation of anxiety disorders and diabetes has not been explored as systematically and extensively as that of depression and diabetes. Anxiety in the context of diabetes has been studied mostly in association with depression [16].

Present study shows that five patients (2.5%) fulfill panic attack criteria, other pho-

Table 1. Demonstration of socio-demographic and clinical data of both studied group.

		Diagnosis		Total	X2	P
		Control	DM			
Sex	Male	103 (41.7%)	85 (42.1%)	188 (41.9%)	0.007	0.935
	Female	144 (58.3%)	117 (57.9%)	261 (58.1%)		
Residence	Urban	91 (36.8%)	74 (36.6%)	165 (36.7%)	0.002	0.964
	Rural	156 (63.2%)	128 (63.4%)	284 (63.3%)		
Work	No work	113 (45.7%)	165 (81.7%)	278 (61.9%)	60.85	0.000
	Working	134 (54.3%)	37 (18.3%)	171 (38.1%)		
Anxiety	Normal	224 (98.8%)	32 (15.8%)	276 (61.5%)	322.98	0.000
	Borderline abnormal	3 (1.2%)	86 (42.6%)	89 (19.8%)		
	Abnormal	0 (0%)	84 (41.6%)	84 (18.7%)		
Depression	Normal	240 (97.2%)	93 (46%)	333 (74.2%)	152.63	0.000
	Borderline abnormal	5 (2%)	35 (17.3%)	40 (8.9%)		
	Abnormal	2 (0.8%)	74 (36.6%)	76 (16.9%)		
Degree of HRQOL	Poor or bad HRQOL	0 (0%)	98 (48.5%)	98 (21.8%)	247.55	0.000
	Moderate HRQOL	4 (1.6%)	47 (23.3%)	51 (11.4%)		
	High HRQOL	243 (98.4%)	57 (28.2%)	300 (66.8%)		
Total		247	202	449		
		100.0%	100.0%	100.0%		

Table 2. Demonstration of data and scores for anthropometric examinations, psychiatric scales, and laboratory test.

	DM (202)		Control (247)		t	P
	Mean	Std. Deviation	Mean	Std. Deviation		
Body mass index	29.2260	3.86901	32.6400	3.14405	−10.316	0.000
Weight	74.7673	8.77527	83.6316	6.27858	12.45	0.000
Height	160.1733	4.82476	160.2834	4.71986	−0.244	0.808
HbA1C	7.2649	0.98390	9.3279	0.40594	−29.98	0.000
Fasting blood sugar	241.3515	30.16984	89.9879	11.95230	72.24	0.000
2 hours Post-Prandial blood sugar	309.0000	32.86063	165.6235	15.29892	60.98	0.000
Anxiety Score	10.4307	4.11860	4.6073	1.58615	20.45	0.000
Depression Score	9.3762	4.84250	4.4899	1.98749	14.44	0.000
Quality of life (Physical)	61.1386	29.50151	88.0567	12.51295	−12.99	0.000
Quality of life (psychological)	44.3069	25.40253	85.2227	13.30730	−21.9	0.000
Quality of life (social)	49.0099	27.29988	70.3441	23.39531	−8.92	0.000
Quality of life (environmental)	44.3069	27.06202	84.0081	13.98053	−20.02	0.000
Total	49.6906	21.87169	81.9079	7.67809	−21.59	0.000

Table 3. Demonstration of logistic regression analysis for depression, anxiety, and quality of life.

Dependent Variable	Depression			Anxiety			Quality of life		
Model	SQ* Beta	T	P.	SQ* Beta	T	P	SQ* Beta	T	P
(Constant)		0.421	0.675		2.333	0.021		−1.339	0.182
Age	0.090	1.425	0.156	0.002	0.055	0.956	−0.109	−2.949	0.004
Weight	0.546	0.601	0.549	0.408	0.975	0.331	−0.445	−0.831	0.407
Height	−0.271	−0.584	0.560	−0.188	−0.878	0.381	0.240	0.878	0.381
Body mass index	−0.638	−0.619	0.537	−0.461	−0.973	0.332	0.552	0.910	0.364
HbA1C	−0.189	−3.016	0.003	−0.924	−32.093	0.000	0.837	22.739	0.000
Fasting blood sugar	−0.028	−0.453	0.651	−0.028	−1.011	0.313	−0.034	−0.934	0.351
2 hours Post Prandial blood sugar	0.479	7.908	0.000	−0.027	−0.986	0.326	0.066	1.850	0.066

SSQ*: Standardizes; *: standardized coefficients.

Table 4. Study effect of DM duration and HbA1c on the presence or absence on anxiety and depression and quality of life.

		Diagnosis				Total		X^2	P	R.R*	95% Confidence Interval	
		presence		Absent		N	%				Lower	Upper
Anxiety Diagnosis												
DM Duration	10 years or more	91	56.9	32	76.2	123	60.9	5.212	0.032	0.847	0.741	0.969
	below 10 years	69	43.1	10	23.8	79	39.1					
HbA1c	Below 7	82	51.2	10	23.8	92	45.5	10.1	0.002	1.257	1.093	1.445
	7 or more	78	48.8	32	76.2	110	54.5					
Depression Diagnosis												
DM Duration	10 years or more	25	37.9	98	72.1	123	60.9	21.8	<0.001	0.392	0.26	0.59
	below 10 years	41	62.1	38	27.9	79	39.1					
HbA1c	Below 7	43	65.2	49	36.0	92	45.5	15.2	<0.001	2.235	1.463	3.415
	7 or more	23	34.8	87	64.0	110	54.5					
Quality of life												
		poor		Good								
DM Duration	10 years or more	22	22.4	101	97.1	123	60.9	118.1	<0.001	0.186	0.127	0.272
	below 10 years	76	77.6	3	2.9	79	39.1					
HbA1c	Below 7	89	90.8	3	2.9	92	45.5	157.3	<0.001	11.824	6.315	22.137
	7 or more	9	9.2	101	97.1	110	54.5					

bia are found in two patients (1%), generalized anxiety disorder and obsessive compulsive disorder are found in one patient (0.5%), no patients are found to be diagnosed as bipolar disorder, schizophrenia, or substance abuse. In control group, no subject fulfills any DSM-IV-TR criteria for any disorder. Anxiety symptoms have been found to be significant risk factors for development of diabetes [5]. Negative correlations have been observed between prevalence of anxiety disorders and levels of HbA1c [6].

Clinical features such as sweating, anxiety, tremor, tachycardia, and confusion are shared by both hypoglycemic episodes and anxiety disorders. This could present a diagnostic challenge especially among individuals having phobia of hypoglycemic episodes. Chronically anxious individuals may be more likely either to fail to perceive the initial warning signs of hypoglycemia or to confuse these with anxiety [16]. Moreover, medications used in management of anxiety disorders such as SSRIs, benzodiazepines, and beta adrenergic blockers could potentially interfere with glycemic control and normal physiological warning signs of an impending hypoglycemic episode [16].

Present study shows that, thirty-seven patients (18.3%) were found to fulfill DSM-IV-TR criteria for major depressive disorder depression and diabetes shared a bidirectional causal association. Depression has been postulated to play a causal role in emergence of diabetes. A meta-analysis has reported that depressed individuals have a 60% increased risk of developing diabetes [17]. A specific association has been found between risk of developing diabetes and non-severe depression, persistent depression, and untreated depression [18]. Similarly, diabetes has been recognized as a "depressogenic" condition [19]. Biochemical changes (including neuro-endocrinal changes such as hyper-cortisolemia, leptin activity in limbic system, altered glucose transportation, pro-inflammatory cytokines) associated with diabetes or its treatment, psychological factors (such as stress associated with living with diabetes, poor treatment adherence), and behavioral factors (sedentary lifestyles, smoking, overeating) have been implicated in this causal association [20]. There is a modest association between use of most antidepressants and incidence of diabetes with long-term use of antidepressants at moderate or higher doses increasing risk of diabetes by almost two fold [2]. Similarly factors such as poor diet, habitual inactivity, excessive nicotine use, psychotropic medications used for treatment of bipolar disorder have been implicated in association between BPAD and diabetes.

Present study found that poor quality of life was found to be more prevalent among diabetic patients with longer duration and with bad control of blood sugar. A number of studies have been done to assess health-related quality of life in patients with diabetes [21] [22]. In general, these studies have been able to demonstrate a reduced quality of life in patients with diabetes [9]. The quality of life of diabetic patients is significantly reduced in the presence of both microvascular and macrovascular complications [3] [9] [23]. Poor quality of life in these patients is attributable to psychological effects of reduced general well-being, lack of acceptance and support from family members, feelings of restriction when complying with treatment, and self-monitoring strategies among others [3] [9] [23]. Vileikyte reported a poor quality of life in patients

with foot involvement [22]. An assessment of patients with diabetic neuropathy using the Nottingham Health Profile showed that symptomatic diabetic neuropathy was associated with impaired quality of life in five out of six domains: emotional reaction, energy, pain, physical mobility, and sleep [24].

From our study, we can conclude that anxiety and depression were associated with hyperglycemia and poor metabolic control, which may increase the risk of complications from T2DM. Recognition of all psychiatric co-morbidities among individuals with diabetes is suboptimal, therefore global approaches to establish coordinated, multifaceted interventions to improve early recognition and early initiation of treatment for all psychiatric commodities are required to reduce the burden among individuals with diabetes; this may achieve greater efficiency and success in the treatment of T2DM.

Therefore, our recommendation is that it would be advantageous to have other longitudinal studies to better understand the nature of those associations between diabetes and different psychiatric illness. Diabetes health professionals require basic training in identification and management of associated psychiatric illness in patients with diabetes. In our locality, there is a need for adequate communication/interview skills, motivational techniques and counseling skills for health professionals treating individuals with diabetes. Effective management of patients with diabetes and psychiatric co morbidities requires collaborative efforts between a number of health care disciplines, including primary care, endocrinology, psychiatry, psychology, nursing, pharmacy, and allied health professions.

6. Limitations of the Study

First limitation in our study is the small number of patients. The second limitation is that we have done our study in one center that was Internal Medicine Hospital (diabetes clinic and diabetes inpatient department), Mansoura University instead of being multicenter. These limitations are due to high cost needed to include large numbers of patients in different centers.

Compliance with Ethical Standards

1) There is no fund to our study.
2) Author 1) Alaa Wafa has no conflict of interest. Author 2) Mohamed Adel El-Hadidy has no conflict of interest.
3) The study was approved by the Mansoura Faculty of Medicine, ethics committee, and then it has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.
4) A written informed consent was obtained from all participants before inclusion in the study.

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Serological Evidence of Human Coinfection by Brazilian Spotted Fever and Bartonellosis

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Abstract

Brazilian spotted fever and bartonellosis are zoonotic, emerging and under diagnosed diseases. Pets may be co-infected by multiple pathogens and become transmissions sources to humans. The study reports the first case of active co-infection by Brazilian spotted fever and bartonellosis based on serological evidence. The authors aim to demonstrate the importance of performing systematic syndromic investigations on nonspecific febrile syndromes, guided by the epidemiological history and considering the possibility of co-infection by zoonosis sharing the same ecological niche.

Keywords

Rickettsia rickettsii, Bartonella henselae, Brazilian Spotted Fever, Bartonellosis, Co-Infection

1. Introduction

Brazilian Spotted Fever (BSF) and bartonellosis are zoonotic diseases respectively considered reemerging and emerging. They are transmitted to humans by accident and are generally under diagnosed.

BSF, described in Brazil since the 1920s, is caused by *Rickettsia rickettsii,* and ticks are its vectors and reservoirs, especially those from *Amblyomma sculptum* species (from the *Amblyomma cajennense* complex), although other infected species may participate in its transmission [1]. It has wide geographical distribution with limited outbreaks in the Brazilian southeastern and southern regions, and in some locations in the midwest, northeast and north regions [2]. Its distribution is seasonal, between May and

October. On average, 55 cases are annually recorded in Brazil [3] and lethality ranges from 20% to 30% [2] [3]. The initial clinical presentation is nonspecific and may progress to sepsis and death mainly due to late diagnosis and consequent delay in the introduction of the specific treatment [1] [2] [3] [4].

Bartonellosis is caused by species from the *Bartonella* genus. The *B. henselae* species is the main pathogen for humans and it is often transmitted by scratching, biting, licking, or simple contact with cat's fleas and other ectoparasites. In addition to cats, several other mammals may be reservoir and vector. The species has worldwide distribution and seasonality period between January and July [5] [6]. Its incidence is of 3.7/100.000 in habitants. Most cases are benign and self-limiting, but the disease may progress to prolonged bacteremia, get worse and lead to cutaneous, hepatic or splenic vasoproliferative effects, especially in immunocompromised patients [5].

Veterinary studies demonstrate that animals, especially dogs, may be co-infected by multiple *Proteobacterium* pathogens, and become transmission sources to humans who manipulate them. Co-infection by *R. rickettsii* and by some Bartonella species, except for *B. henselae*, had been reported [7] [8]. However, *B. henselae* had been identified in domestic dog fleas [9] [10]. Additionally, there are records of human co-infection by *R. rickettssii* and *Ehrlichia chaffeensis* [11]. Kordich performed a serological survey and found antibodies to *B. henselae* and *R. rickettsii* on healthy dog breeder [7]. Despite the finding could not be correlated with the presence of simultaneous infectious syndrome, the co-infection possibility could be taken under consideration. Nevertheless, literature has no report on human active co-infection by BSF and bartonellosis.

The current study aims to report the first human case with serological evidence of active co-infection by *R. rickettsii* and *B. henselae*, recorded in Rio de Janeiro, Brazil. It is an alert to health professionals about the possibility of co-infection, the impact of this event on the research algorithm, the following up of complications and the empirical choice for specific therapies.

2. Case Report

MAJD, female, 47 years old, lived in São João de Meriti (metropolitan region of Rio de Janeiro/Brazil). She was previously healthy with nocomorbidities. In April 2014, she started presenting fever, chills, occipital headache, myalgia, hyporexia and hypogeusia. The patient sought the primary care unit several times between the 2nd and 13th day of the disease, time when the diagnostic hypothesis of dengue was considered and its supportive treatment was introduced. Between the 6th and 10th day of the disease, she presented aqueous diarrhea and vomiting. Since fever persisted, the patient was referred to the National Institute of Infectious Diseases in the 14th day of the disease. She reported the habit of collecting dogs and cats often parasitized by ectoparasites, which were abandoned. She also reported that one dog and one cat were being treated for "tick disease" and "infectious disease without etiology", respectively. She took care of her pets without using personal protective equipment. At the 14th day she was febrile (38˚C) and hemodynamically stable, without cutaneous rash or lymphadenopathy. La-

boratory tests performed in the14th day showed increased C-reactive protein with no others hematological, renal and hepatic changes. Previous laboratory tests showed leukocytosis without deviation in the 2nd day of the disease and thrombocytopenia between the 3rd and 13th day of the disease. The diagnostic hypothesis of zoonosis: BSF, leptospirosis or bartonellosis was consideration and specific tests were performed. In addition, dengue, viral hepatitis and other nonspecific bacterial infections investigated were complemented. Empirical treatment with doxycycline was immediately introduced at the dose of 200 mg/day. The patient evolved with defervescence and clinical improvement within 48 hours after started the antibiotic, and was discharged within 60 days (Figure 1). All test results—except for the BSF and bartonellosis serological tests—were negative, including the molecular analysis (PCR) for both *Proteobacteria*. Indirect immunofluorescence presented IgG antibody title of 1/512 for *R. rickettsii* and 1/128 for *B. henselae* on the 15th day of the disease, and on the 50th day the IgG antibody title was 1/1024 for *R. rickettsii* and 1/256 for *B. henselae* (Table 1).

3. Discussion

The patient presented initially a nospecific febrile syndrome. There was a delay in diagnosis and treatment due to the relative unawareness about the occurrence of the zoonosis among humans. Additionally the overlap of hyperendemic diseases, such as Dengue, has also been a frequent confounder factor. Epidemiological history suggests that the infection has been acquired through the unprotected handling of her own pets, some of them known to be sick. The delay in the first sampling and effective treatment can in part explain our inability to demonstrate the perfect seroconversion and the

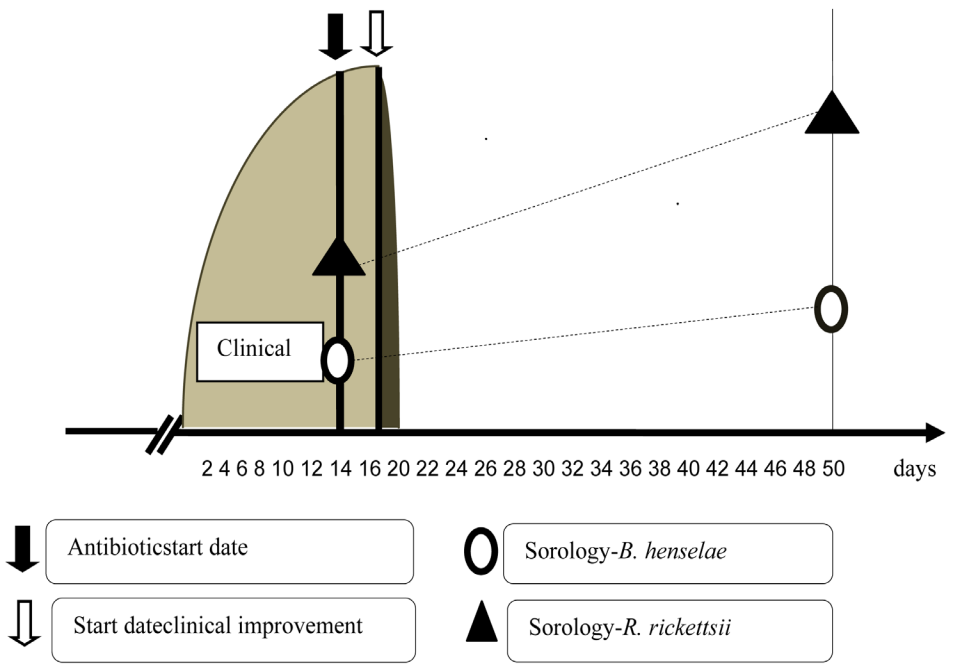

Figure 1. Clinical and laboratory time evolution.

Table 1. Representation of the relevant laboratory tests.

	Disease time in days	Complete Blood Count				CRP (mg/dL)	Rr (IgG)	Bh (IgG)
		Hb (g/dL)	Ht (%)	Leuk (mm^3)	Plat (mm^3)			
HUPC	2nd	13.8	42.0	**15,400**	180,000	-	-	-
	3rd	12.6	37.8	8,200	**82,000**	-	-	-
	4th	11.7	34.7	7,200	**53,000**	-	-	-
	6th	11.9	35.6	7,000	**57000**	-	-	-
	7th	12.2	36.1	7,900	101,000	-	-	-
	13th	12.2	35.1	8,100	**65,000**	-	-	-
	14th	11.6	36.3	9,140	209,000	**8.11**	-	-
INI	15th	11.8	36.4	6,500	113,000	0.45	**1:512**	**1:128**
	50th	-	-	-	-	-	**1:1020**	**1:256**

Nomenclature and benchmarks: HUPC = Health Unit Primary Care; INI = National Institute of Infectious Diseases; Hb = Hemoglobin (11 - 16 g/dL). Ht = Hematocrit (34 - 45 g/dL); Leuk = leukocytes (4000 - 10,000 mm^3); Plat = Platelets (150,000 - 450,000 mm^3). CRP = C-reative protein (0 - 0.3 mg/dL); Rr = *Rickettsii rickettsii.* Bh = *Bartonellahenselae;* IgG = *Immunoglobulin G.*

negative specific Polymerase Chain Reaction (PCR). The possibility of *R. rickettsii* and *B. henselae* molecular detection is higher in the early stages of the disease, with greater sensitivity in severe and fatal cases. Furthermore, the excellent therapeutic response, as well as the epidemiological history, reinforces the BSF and bartonellosis diagnostic. Usually serology is an essential and largely available method to laboratory confirmation for both zoonosis, however, it depends on the opportunity of the investigation [1] [4] [12].

The authors of the current study intend, by means of this report, to emphasize the importance of performing a syndromic investigation strongly guided by the epidemiological history, especially in an undifferentiated febrile syndrome. In addition, the possibility of co-infection by *Proteobacteria* zoonosis which shares the same ecological niche must be considered, and this possibility needs to be included in diagnostic algorithm for the choice of the most appropriate antimicrobial medication in order to decrease morbidity and mortality caused by the zoonosis.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Consent

Written consent was obtained from the patient under a research project approved by the Ethic Committees of IPEC/FIOCRUZ: "Detecção de formas não usuais de dengue a partir da vigilância de síndromes febris agudas", CAAE 0026.0.009.000-07.

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BIGH3: A Negative Regulator of Human Osteosarcoma Large Multicellular Spheroids

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Abstract

Numerous studies have demonstrated a relationship between the extracellular matrix protein BIGH3 and variations in the malignant properties of different cancer cell types, including osteosarcoma cells. BIGH3 protein can suppress and promote tumor growth, even on the same cancer cell type, indicating that contextual cues regulate BIGH3-mediated divergent outcomes. We employed a multicellular tumor spheroid model to study the effects of BIGH3 with respect to physical and molecular features of three-dimensional tumor growth. The results demonstrated that exogenous recombinant BIGH3 blocked the development of multicellular large tumor spheroids so that only small spheroids formed. The effect was dependent on the BIGH3 concentration in the growth medium and the time of incubation of BIGH3 with the osteosarcoma cells in the spheroid model. TGF-β1 signaling induced multicellular tumor spheroids to synthesize a greater quantity of BIGH3 relative to non-treated spheroids. The TGF-β1-mediated increase in BIGH3 protein antagonized the development of multicellular large spheroids. Anti-BIGH3 antibody, and an inhibitor of TGF-β1 signaling, blocked the antagonistic effect induced through TGF-β1 stimulation and BIGH3 protein expression, resulting in the formation of multicellular large spheroids. Immunohistochemistry detected BIGH3 at cell bodies within the spheroid stroma, suggesting osteosarcoma cell-surface proteins bind BIGH3. Flow cytometry demonstrates that osteosarcoma cells interact with soluble BIGH3, and solid-phase cell adhesion assays show that osteosarcoma adhesion to BIGH3 substratum is mediated by integrin $\alpha 4\beta 1$. However, anti-$\alpha 4$ antibody did not attenuate the BIGH3-mediated antagonism toward formation of multicellular large spheroids. We conclude that TGFβ1 and BIGH3 suppress the development of large osteosarcoma tumors.

Keywords

Cancer, 3-Dimensional, TGFβ1, Extracellular Matrix, TGFBI, Beta-ig

1. Introduction

As an *in vitro* model of tumor progression, propagation of Multicellular Tumor Spheroids (MTS) provides a convenient means to study the effects of the extracellular matrix (ECM) on tumor formation. Various tumor cell types have been propagated as MTS comprising cells and their ECM, and exhibiting behavior similar to that of tumors *in vivo* [1]. It is well established that cells on two-dimensional substrates of various polyresin plastics provide valuable information regarding ECM synthesis, cell signaling, propagation and viability, tissue regeneration, and cellular and molecular mechanisms underlying disease. Relative to cells propagated as a 2-dimensional monolayer, MTS are superior representations of the physiology of solid tumorigenesis. Extending *in vitro* cell culture methodology, three-dimensional intermediate model systems provide the convenience of culturing cells in the context of a complex environment of one or more cell types and their provisional ECM [2] [3] [4]. Relative to cells propagated as a 2-dimensional monolayer, MTS are superior representations of the physiology of solid tumorigenesis. Tumor spheroids comprise cells that are at the spheroid surface, within intermediate stroma, and in a centralcore, which can become a necrotic environment [5]. Molecules of the ECM can play significant roles in tumor progression, mechanically stabilizing and affecting tumor morphology and tumor cell viability and metastasis [1] [6] [7]. The human gene called *TGFBI* encodes for the ECM protein BIGH3, which was discovered investigating the effects of the cytokine TGFβ1 on adenocarcinoma tumor progression [8]. The BIGH3 gene was cloned and sequenced in 1992 [8], and subsequently BIGH3 was proposed to function as a tumor suppressor protein [9]. T*GFBI*$^{-/-}$ mice exhibited predisposition to develop various tumors, underscoring BIGH3's tumor suppressing property. Isolated T*GFBI*$^{-/-}$ mouse embryonic fibroblasts showed chromosomal abnormalities, increased cyclin D1 synthesis, and cell proliferation [10]. BIGH3 suppressed proliferation of osteosarcoma [11], lung [12] [13] [14] [15], ovarian [16] [17] [18], breast [19], prostate cancer cells [12], and exhibited anti-angi- ogenic and anti-tumorigenic activities [20]. In contrast, other studies show that BIGH3 protein stimulates cancer cell aggressiveness, including osteosarcoma metastasis [21], colon cancer [22], oral squamous carcinoma [23] [24], and astrocytoma progression [25]. Integrins, apoptosis [11], chromosomal abnormalities [26], and cell motility and chemoresistance to lung and ovarian cancer cells [27] have been implicated in molecular mechanisms that underlie BIGH3's divergent effects on tumors progression. Central to BIGH3 biology is a strong response of the BIGH3 gene to TGF-β1 stimulation [9], and the regulatory action in play between TGF-β1 and integrins [28] [29]. Thus, TGF-β1 staining is a contextual cue for transcription, translation and secretion of BIGH3. Despite the accumulating evidence of differential roles that BIGH3 mediates in different cancer and cancer cell types, there has been a paucity of information on the effect of BIGH3 protein on cancer cell behavior in the context of a three-dimensional tumor environment. We previously reported BIGH3 mediates apoptosis in MG-63 osteosarcoma cell monolayers [11]. To this end we sought to examine the effect of BIGH3 on developing osteosarcoma tumor spheroids by using a simple yet well-recognized MTS model

system as a convenient method to examine tumor response to ECM protein. Here, we utilized osteosarcoma MG-63 cells, MG-63 BIGH3, human recombinant BIGH3 protein, and TGF-β1 to test for BIGH3' influence on osteosarcoma MTS.

2. Methods

MG-63 cells were purchased from the American Type Culture Collection (ATCC # CCL-1427; Rockville, MD). Chinese Hamster Ovary (CHO) cells expressing human recombinant BIGH3 have been described [30]. Dulbecco's Modified Eagle Medium (DMEM), Glasgow Minimum Essential Medium (GMEM), non-essential amino acids, antibiotics, salmon sperm DNA and sodium pyruvate were purchased from Invitrogen (San Diego, CA). FBS was from Irvine Scientific (Santa Ana, CA). Paraformaldehyde, Cell Dissociation Solution, EDTA, l-asparagine monohydrate, nucleosides, methionine sulfoximine, agarose, heparin-agarose, Proteinase K, 3,3'-diaminobenzidine tetrahydrochloride (DAB), cycloheximide, Hoechst 33258 (bisBenzamide) and rabbit IgG were purchased from Sigma- Aldrich (St. Louis, MO). Flow cell YM membrane and Centricon microfiltration capsules were purchased from Amicon, Inc. (Beverly, MA). Mono-Q and hydroxyapatite bio-scale columns were purchased from Bio-Rad Laboratories (Hercules, CA). Superfrost Plus microscope slides were purchased from VWR Scientific Products (Sugarland, TX). Purchased from Chemicon International (Temecula, CA) were anti-bovine fibronectin antibodies and monoclonal antibodies against human integrin subunit β1 (clone 6S6) α4 (clone P1H4). Goat anti-rabbit immunoglobulin-G (IgG) conjugated to fluorescein-5-isothiocyanate (FITC), goat anti-mouse IgG conjugated to FITC and Cell Proliferation Reagent WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]- 1,3-benzene disulfonate) were purchased from Roche Molecular (Indianapolis, IN). Antibodies used for the immunohistochemical detection of BIGH3 in sectioned MTS were a gift from K. Bennett (Bristol Myers Squibb; Seattle, WA) or were polyclonal antibodies generated in New Zealand White rabbits against a BIGH3 bacterial fusion protein corresponding to amino acids 70 - 683 of the reported protein sequence [8] [9] [31] [32] [33] [34].

2.1. Purification of BIGH3

The serum-free conditioned medium taken from CHO cells expressing human BIGH3 was applied over heparin, hydroxyapatite and anion exchange resin bed volumes of 10 ml, 2 ml, and 2 ml, respectively. Chromatography buffers were Buffer A; 50 mM Tris, 50 mM NaCl, pH 5.5. Buffer B; Buffer A containing 1 M NaCl. Buffer C; 10 mM $NaPO_4$ buffer, pH 6.8. Buffer D; 0.4 M $NaPO_4$ buffer, pH 6.8. Buffer E; 10 mM Tris, 10 mM NaCl, pH 5.5. Buffer F was comprised of Buffer E containing 1 M NaCl. Purity and concentration of BIGH3 was assessed using SDS PAGE and bicinchoninic acid protein quantification (Pierce) respectively. Further details are described in the results section.

2.2. Cell Culture

Growth media contained 50 μg/ml each of penicillin and streptomycin sulfate. Cells

were maintained in a 37˚C incubator saturated with humidified 5% CO_2 and 95% ambient air. CHO cells expressing human BIGH3 were propagated in GMEM containing 10% heat-treated, dialyzed FBS and 25 μM of the glutamine synthetase inhibitor methionine sulfoximine (MSX). For purification purposes, the growth medium was removed, the monolayer was rinsed three times with Hank's Balanced Salt Solution (in mM; 1 $CaCl_2$, 5.4 KCl, 0.4 KH_2PO_4, 0.5 $MgCl_2 \cdot 6H_2O$, 0.4 $MgSO_4 \cdot 7H_2O$, 137 NaCl, 4.2 $NaHCO_3$, 0.4 Na_2HPO_4) and then the cells were maintained in serum-free GMEM for 25 - 48 hours. Osteosarcoma cells and MTS were propagated in DMEM containing 0.1 mM non-essential amino acids, 2 mM L-glutamine and 10% FBS ($DMEM^+$). Cells were tested for mycoplasma by immunofluorescence and found negative.

2.3. MTS Culture

As a convenient means to study the effects of BIGH3 on MTS, we utilized an *in vitro* model that fosters formation of avascular MTS [35] [36] [37]. Microtiter wells coated with a solution of 0.75% agarose in DMEM were seeded with 10^5 osteosarcoma cells in $DMEM^+$. Unless otherwise noted in the text, purified recombinant BIGH3 was added to the medium to yield a final concentration of 10 μg/ml at the time of seeding.

2.4. MTS Cross-Sectional Area Measurements

Digital images of MTS were recorded utilizing a 4x objective on a Nikon Diaphot 200 inverted microscope interfaced with a Dage DC330 CCD camera (MCI, Inc., Michigan City, IN). The surface area is defined as the area in μm^2 of contiguous cell contact of MTS in the culture well. Recorded images were analyzed and surface areas quantified offline using Image-Pro Plus software from Media Cybernetics (Silver Spring, MD).

2.5. MTS Fixation, Embedding and Immunohistochemical Staining

Day 3 osteosarcoma MTS were fixed in 4% paraformaldehyde and embedded in paraffin. A Microm HM325 microtome (Walldorf, Germany) cut 10-μm thick sections, which were then mounted onto charged microscope slides. Following deparaffinization and rehydration, sections were incubated in a solution of 0.005% trypsin-EDTA as described [38] and blocked with 10% normal goat serum. BIGH3 was detected by incubating sections at 4˚C overnight with anti-BIGH3 antibody followed by a second antibody conjugated to horseradish peroxidase. DAB served as the chromogen substrate to localize antibodies.

2.6. Flow Cytometry

Osteosarcoma cells were incubated for three hours with 10 μg/ml BIGH3 and then washed and mixed with anti-BIGH3 antibody, 1 mg/ml anti-integrin $\beta 1$ antibody, or negative control antibody. Cells were then washed and incubated for 30 minutes with 1 mg/ml fluorescein-conjugated goat anti-rabbit or goat anti-mouse antibody. Nonspecific fluorescence was determined by a 30-minute incubation of osteosarcoma cells with 1 mg/ml fluorescein-conjugated second antibody only. Following washes, the cells were

fixed with 4% paraformaldehyde in PBS (PBS in mM; 0.7 $CaCl_2$, 1.5 KH_2PO_4, 0.5 $MgCl_2 \cdot 6H_2O$, 137 NaCl, 8.1 $Na_2HPO_4 \cdot 7H_2O$) and kept at 4˚C until analysis. In three separate experiments flow cytometry data were collected using a Becton-Dickinson FACScan 200 fluorescent cell sorter.

2.7. Cell Adhesion

Substratum for osteosarcoma cells were prepared by treating microtiter wells with 10 μg/ml human recombinant BIGH3. 8 μg/ml human fibronectin and 1% BSA in PBS. To reduce the possibility that endogenous protein synthesis may affect the outcome of adhesion experiments, DMEM containing 10 μg/ml cycloheximide was used to pre- incubate cells 1 hour before experiments. An identical concentration of cycloheximide was included in all subsequent adhesion assay solutions. Cells used in adhesion assays were released from monolayer culture using 1 mM EDTA in divalent cation-free PBS, washed, and suspended at a density of 5×10^5 cells/ml $DMEM^+$. For integrin blocking experiments, cells were pre-incubated for one hour with function-perturbing anti-integrin antibodies (1:200 in DMEM) or control antibody. Following incubation with anti-integrin antibodies, 5×10^4 cells were added to each substratum and incubated for 90 minutes at 37˚C. Unattached cells were rinsed from wells and the cells remaining attached were quantified by addition of WST-1 in $DMEM^+$ followed by a two-hour incubation at 37˚C and recording absorbance at 450 nm.

2.8. Statistical Analysis

Analysis of the cell adhesion results was assessed by analysis of variance (ANOVA) and MTS formation data by the Mann-Whitney U nonparametric test. Statistical significance was accepted when $P < 0.05$.

3. Results

Recombinant BIGH3 Purification. Serum-free medium conditioned by CHO cells expressing human BIGH3 was dialyzed against distilled water and lyophilized. The lyophilized material was rehydrated in Buffer A (**Figure 1(a)** and **Figure 1(b)**, lane 1) and applied over heparin-agarose affinity column. The column was washed with three column-volumes of Buffer A, and BIGH3 was eluted between 150 - 250 mM NaCl utilizing a linear gradient of 50 - 500 mM NaCl (applied material, lane 1 (**Figure 1(a)**), heparin eluted, lane 2 **Figure 1(b)**, lane 2 and **Figure 1(c)**). Protein immunoblots identified BIGH3-con- taining fractions that were pooled and applied over a hydroxyapatite column. Following washing with three column volumes of Buffer C, a 10-to-400 mM linear $NaPO_4$ gradient eluted BIGH3 at 300 mM $NaPO_4$ (**Figure 1(a)** and **Figure 1(b)**, lane 3 and **Figure 1(d)**). Fractions that were immunoreactive for BIGH3 antiserum were pooled, applied over an anion exchange column that was then washed using three column volumes of Buffer E. A linear gradient ranging from 0.05 to 1 M NaCl was applied and BIGH3 eluted between 450 - 500 mM NaCl (**Figure 1(a)** and **Figure 1(b)**, lane 4 and **Figure 1(e)**). Fractions containing BIGH3 were combined and then concentrated by application

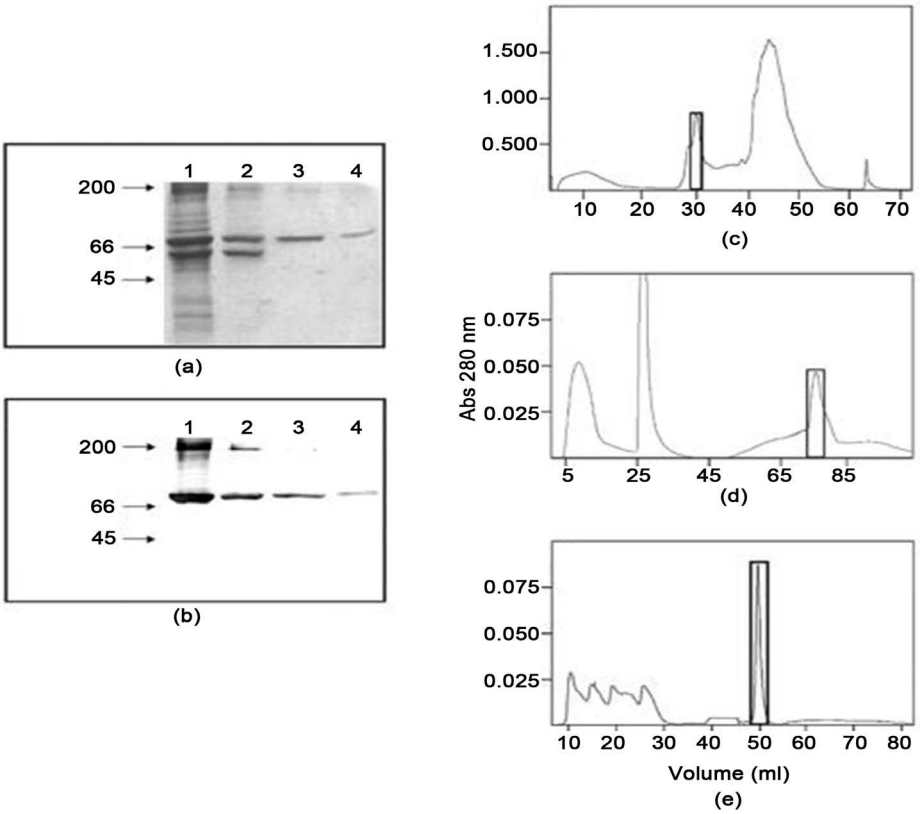

Figure 1. Recombinant BIGH3 is purified by heparin affinity, hydroxyapatite and anion exchange column chromatography. A polyacrylamide gel (a) and immunoblot (b) illustrate results of the purification paradigm for BIGH3. Starting material from growth medium conditioned by CHO cells expressing human BIGH3 (lane 1, (a) and (b)) was applied sequentially over heparin (c), hydroxyapatite (d) and anion exchange (e) resins. BIGH3 in the fractions indicated by the boxed peaks is shown ((a) and (b), lanes 2, 3 and 4, boxed peaks in (c), (d) and (e), respectively). Protein molecular mass standards are indicated as kDa.

over a 30,000 cutoff Centricon YM membrane. The average yield from a liter of conditioned medium was 75 - 100 μg of purified BIGH3 in PBS. The protein was stored at −20˚C until used for experiments.

BIGH3 Antagonizes the Formation of Osteosarcoma Spheroids. At the time of seeding recombinant BIGH3 was included in the growth medium to achieve a final concentration of 10 μg/ml. Unless noted otherwise in the individual experiments, additional BIGH3 was not added following the initiation of the MTS cultures. During days 1 and 2, no distinct differences were detected when cells in medium containing BIGH3 were compared to osteosarcoma cells cultured under otherwise identical conditions minus exogenous BIGH3. Small MTS comprised of 3 - 30 cells were typical in the control medium, and in the medium containing BIGH3 (**Figure 2(a)** and **Figure 2(b)**, respectively). Within 3 days BIGH3-treated MTS exhibited a marked impediment on the formation of MTS (**Figure 2(c)**) when compared to osteosarcoma MTS in control medium without BIGH3 (**Figure 2(d)**). Cells in the control medium began forming a large sheet-like aggregate that included the majority of cells in the well; few, if any single cells

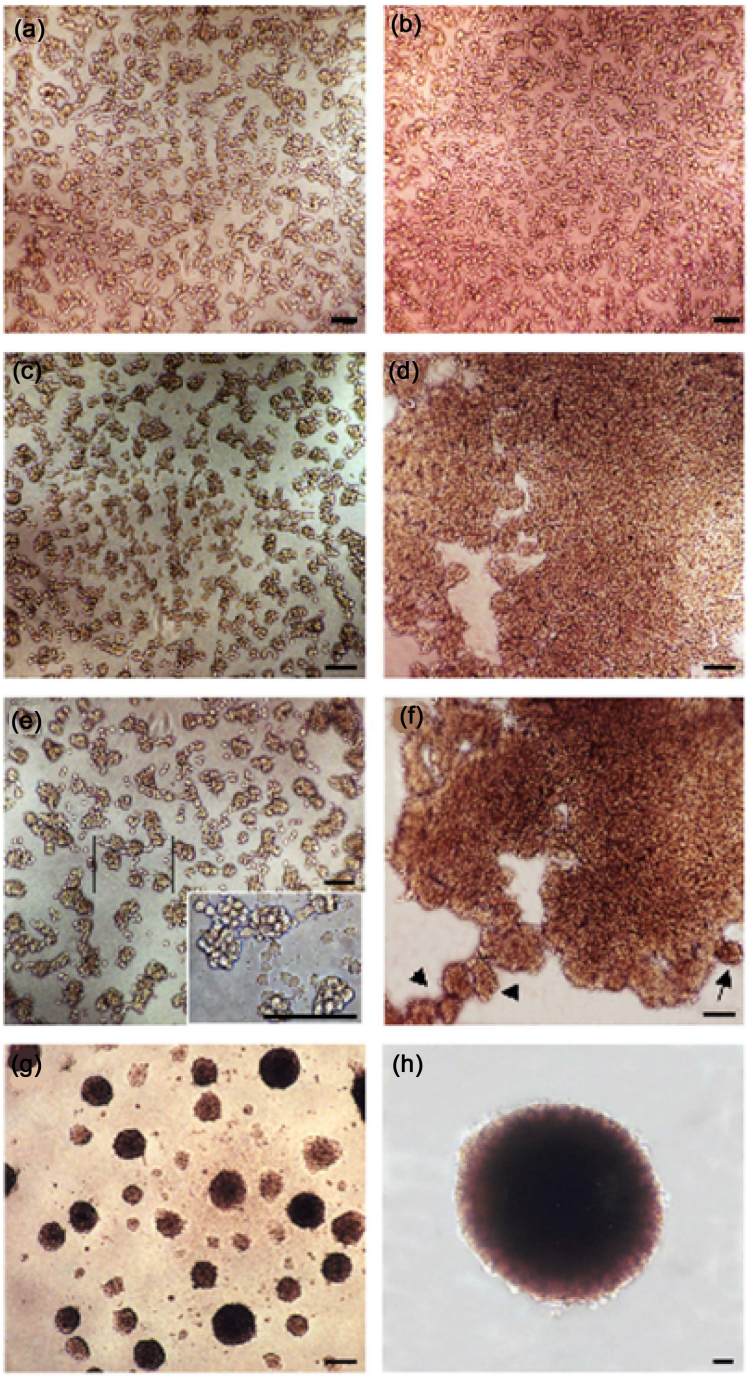

Figure 2. BIGH3 blocks progression of cells into an organized large MTS. Osteosarcoma cells were cultured in DMEM$^+$ with 10 μg/ml BIGH3 for 1, 3, 5 and 7 days ((a), (c), (e) and (g) respectively) or in identical medium without exogenous BIGH3 ((b), (d), (f) and (h), respectively). Beginning on day 3 a distinct difference is apparent when the MTS phenotype in BIGH3- treated medium (c) is compared to MTS cells in control medium (d). On day 5 small MTS only were observed in medium containing BIGH3 when compared to MTS grown in the absence of exogenous BIGH3 ((e) and (f)). The inset in (e) is an increased magnification of the small MTS formed by osteosarcoma cells in the presence of BIGH3. Differential MTS dimensions become increasingly obvious and distinct by day 7 ((g) and (h)). Scale bars represent 50 μm.

and small MTS were observed in the entirety of the well (Figure 2(d)). By day 5, small MTS only were observed in medium containing BIGH3 (Figure 2(e)) when compared to non-treated cells that formed a large MTS from juxtaposed smaller aggregates of cells (Figure 2(f), arrow) and from more distant, but bound cell aggregates (Figure 2(f), arrowheads).At day 7 the MTS size in BIGH3-containing medium was markedly distinguished from controls. Small MTS had formed in medium containing exogenous BIGH3 (Figure 2(g)) whereas cells in the control medium had culminated as one, or sometimes two or three large MTS (Figure 2(h)).

Surface Tracings Show MTS Development. When the two-dimensional surface areas of BIGH3-treated and non-treated MTS were compared at 24 hours culture (Figure 2, day 1), the traced MTS surface areas were similar (Figure 3). By day 3 a marked distinction in MTS size was evident. Cells in control medium exhibited a sheet-like aggregate forming a contiguous cell mass, thus a large surface area was quantified when traced. However, there was significantly less contiguous area of cells in the BIGH3-containing medium when compared to non-treated MTS (Figure 3, day 3). By day 5 the BIGH3-treated MTS surface areas exhibited little change whereas in non-treated conditions the MTS surface area decreased as the contiguous cell sheet further compacted and organized into a MTS (Figure 3, day 5). The outcome seen at day 5 progressed further; by day 7 small MTS were combined to form a large MTS, and in the control medium the large MTS exhibited compaction. Analysis of the differences between the surface areas of BIGH3-treated and control non-treated MTS achieved statistical significance at days 3 and 5 but not at day 7, indicating that the BIGH3-mediated antagonistic effect diminished over time.

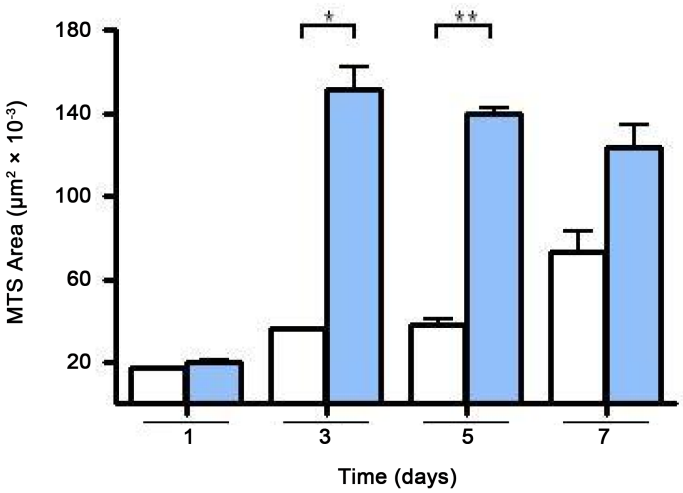

Figure 3. BIGH3 decreases the surface area of osteosarcoma cell MTS. The average two- dimensional surface area of MTS was quantified at days 1, 3, 5, and 7. At day 3 the contiguous MTS surface areas formed in the presence of exogenous BIGH3 (white columns) was significantly less than the surface area of non-treated MTS (blue columns). A statistically significant difference from the control group was achieved at days 3 and 5 ($P \leq 0.02$ and 0.007, respectively) as determined by the Mann-Whitney U test. Differences did not achieve significance at days 1 or 7, n = 18.

TGFβ1 Promotes Development of Small MTS. With little, if any TGFβ1 stimulus MG-63 cells synthesize BIGH3, however, the quantity is insufficient to antagonize formation of large MTS. To determine whether the failure of non-treated cells to form large MTS was related to the quantity of BIGH3 protein in the MTS environment, we used the cytokine TGFβ1 that up regulates expression of the gene *TGFBI*, which encodes BIGH3 [11]. Since exogenous BIGH3 itself was sufficient to block development of large MTS phenotype, we reasoned that excluding exogenous BIGH3 from the MTS culture, and adding exogenous TGFβ1 only, would result in an increase in BIGH3 protein synthesis and block development of large MTS. Real-time PCR and Western blot analyses show that TGFβ1-treated spheroids synthesize a greater quantity of BIGH3 transcripts and protein relative to non-treated spheroids. SB-431542, a small chemical inhibitor of TGFβ1 receptor signaling blocked the increase in TGF-β1-induced BIGH3 expression (**Figure 4(a)** and **Figure 4(b)**). The inhibitor SB-431542 lowered BIGH3 protein in the MTS itself and in the MTS medium conditioned (**Figure 4(c)** and **Figure 4(d)**). Although non-treated MTS synthesized BIGH3 large MTS formed (**Figure 5(a)** and **Figure 5(b)**). The TGFβ1-treated MTS synthesized a greater quantity of BIGH3 that was sufficient to block development of large MTS so that small MTS only developed (**Figure 5(a)** and **Figure 5(c)**). This result too implicates TGFβ1 and its activated receptor, and BIGH3 synthesis and secretion in the antagonistic pathway.

Anti-BIGH3 Antibody Blocks BIGH3's Antagonism. BIGH3 was pre-incubated with anti-BIGH3 antibody prior to adding to osteosarcoma cells in the MTS model system. Quantifying the average MTS area in treated and non-treated conditions demonstrated that anti-BIGH3 antibody significantly prevented the antagonistic effect of BIGH3 on the formation of large MTS. Osteosarcoma cells in medium containing exogenous BIGH3 (without BIGH3 antibody) formed small MTS only (**Figure 6**, BIGH3). A non-related control antibody did not block BIGH3's antagonistic effect on large MTS formation (**Figure 6**, BIGH3+Rabbit IgG). In contrast, an anti-BIGH3 antibody blocked BIGH3's antagonistic effect resulting in development of large MTS (**Figure 6**, BIGH3 + BIGH3 Ab). There was little if any difference in the large MTS areas that developed with BIGH3 antibody when compared to non-treated MTS (**Figure 6**, Without BIGH3). These results again indicate that BIGH3 protein itself is sufficient to suppress large MTS formation.

BIGH3 is Localized at MTS Cells and Stroma. Immunocytochemical analysis was used to investigate the spatial organization of BIGH3 in the large MTSs that formed in non-treated control conditions and in the smaller MTS that formed when the cells were treated with BIGH3. After 3 days of culture, MTS were processed for staining as described in methods section. Recombinant BIGH3 was not added to the control cultures, thus the BIGH3 protein detected represented *de novo* synthesized protein. BIGH3 in the large MTS was detected at cell bodies and within the interior stroma (**Figure 7(a)**). BIGH3 was not evident in stroma to a depth of approximately 50 μm at the MTS periphery (as opposed to detection of fibronectin within this area, **Figure 7(b)**). In contrast,

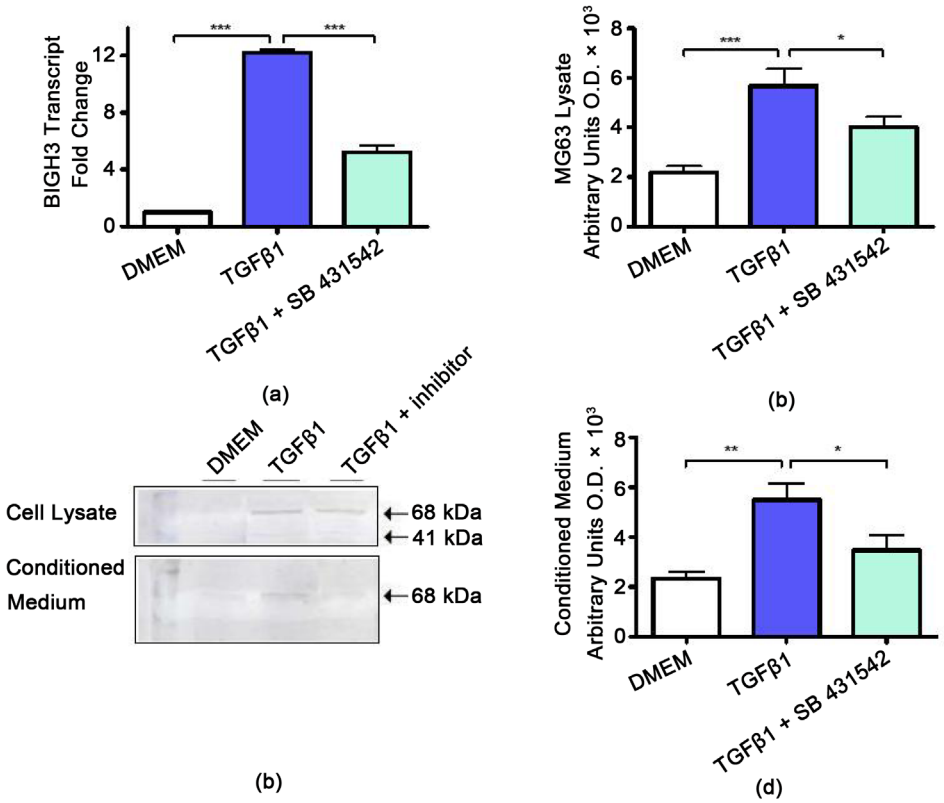

Figure 4. TGFβ1 increases the synthesis and secretion of BIGH3. (a) Shown is an increase in BIGH3 transcripts in 1.5 × 10^5 MG-63 cells cultured in DMEM with and without TGF-β1 and SB-431542 inhibitor. BIGH3 transcript levels were significantly greater in MG-63 cells cultured in 5 ng/mL TGF-β1 for 24 hours when compared to DMEM alone ($p < 0.001$). SB-431542 inhibitor (13 μM) significantly reduced BIGH3 transcripts in TGF-β1 stimulated cells [$F(2,13) = 381.4$; $p < 0.001$]. (b) Western blot of BIGH3 in the lysate and culture medium of MG-63 cells. MG-63 cells were cultured for 24 hours in DMEM alone and DMEM+ containing 5 ng/mL TGFβ1, or 5 ng/mL TGF-β1 plus 13 μM SB-4231541 inhibitor. Loading volumes were normalized by BCA and the resolved proteins in MG-63 lysates were stained for BIGH3 (68 kDa) and actin (41 kDa). (C) Densitometry of BIGH3 bands in lysate generated from MG-63 cells cultured in 5 ng/mL TGFβ1 were significantly greater than BIGH3 in lysates of MTS cultured in DMEM alone ($p < 0.001$) and 5 ng/mL TGF-β1 with 13 μM SB-431542 inhibitor [$F(2,15) = 12.92$; $p < 0.05$]. (d) Densitometry of BIGH3 bands in conditioned medium from MG-63 cells cultured in 5ng/mL TGF-β1 explains the disparity in BIGH3 transcripts (A) when compared to MG-63 cells cultured in DMEM alone ($p < 0.01$), and when cultured with 5 ng/mL TGF-β1 plus 13 μM SB-431542 inhibitor [$F(2,15) = 8.214$; $p < 0.05$]. One way ANOVA was performed with a post hoc Newman- Keuls Multiple Comparison test for significant differences. For each condition $n \geq 3$. Significance was set at $p < 0.05$.

anti-BIGH3 antibody exhibited homogenous staining throughout MTS that formed in the presence of added recombinant BIGH3 (Figure 7(d)), similar to fibronectin staining (Figure 7(e)). MTS that formed in the presence of exogenous BIGH3 likewise exhibited homogenous staining of fibronectin (Figure 7(e)). MTS that were in control medium and in medium containing BIGH3 did not stain with control antibody (Figure 7(c) and Figure 7(f), respectively). An interspersed punctum-like stain can be seen in

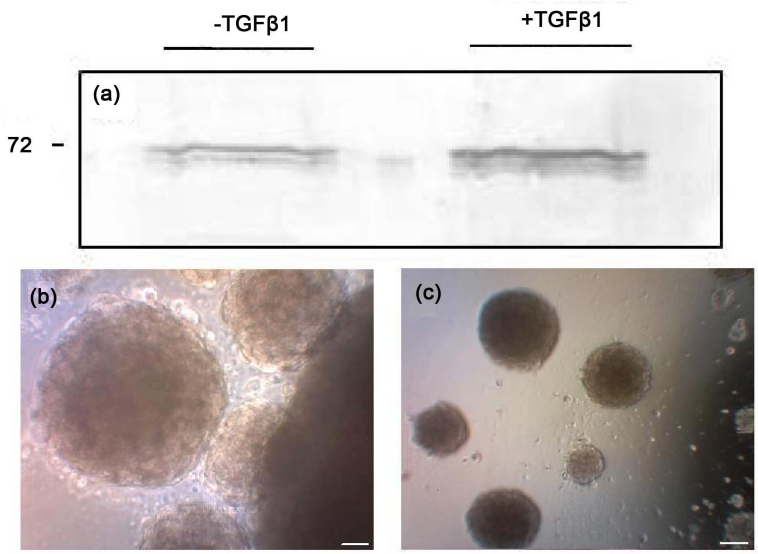

Figure 5. TGFβ1 increases BIGH3 synthesis in MTS and promotes BIGH3 antagonism toward large MTS development. Cells seeded on an agarose overlay were treated with 5 ng/ml TGFβ1. After 5 days in MTS culture, cell extracts were generated from non-treated and TGFβ1-treated MTS. Western blots show an increase in BIGH3 protein in TGFβ1-treated MTS (a); In parallel experiments the growth of MTS without TGFβ1 (b) and with TGFβ1 treatment (c) were recorded, showing TGFβ1 treatment is sufficient to increase BIGH3 synthesis to a quantity that blocks large MTS formation.

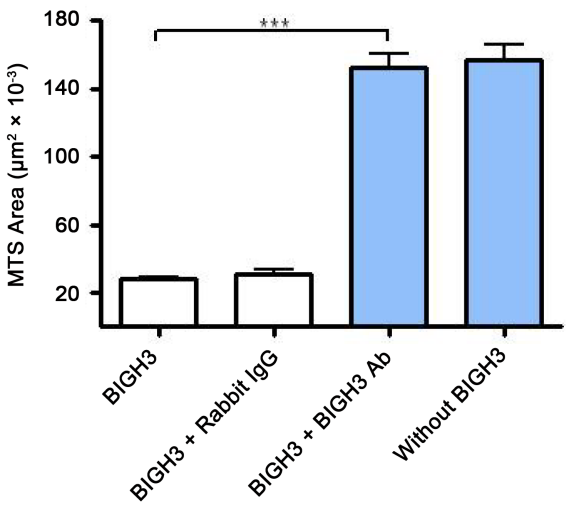

Figure 6. Anti-BIGH3 antibody blocks BIGH3 antagonism on large MTS formation. In the MTS paradigm anti-BIGH3 antibody was added at the time of cell seeding. In control conditions, exogenous BIGH3 only (BIGH3) and BIGH3 added with rabbit IgG (BIGH3 + Rabbit IgG) antagonized the formation of large MTS. In contrast, when anti-BIGH3 antibody was added with BIGH3, then the cells formed a contiguous large mass (BIGH3 + BIGH3 Ab) similar to the MTS that formed in control conditions (Without BIGH3). Differences in the averaged two-dimensional area of MTS formed in the presence of anti-BIGH3 antibody, when compared to the average area of control MTS, were not statistically significant (n = 12, Mann-Whitney U test) at either day 1 (data not shown, $P \leq 0.1873$, two-tailed) or day 3 (shown here; $P \leq 0.7916$, two-tailed). Error bars represent SEM.

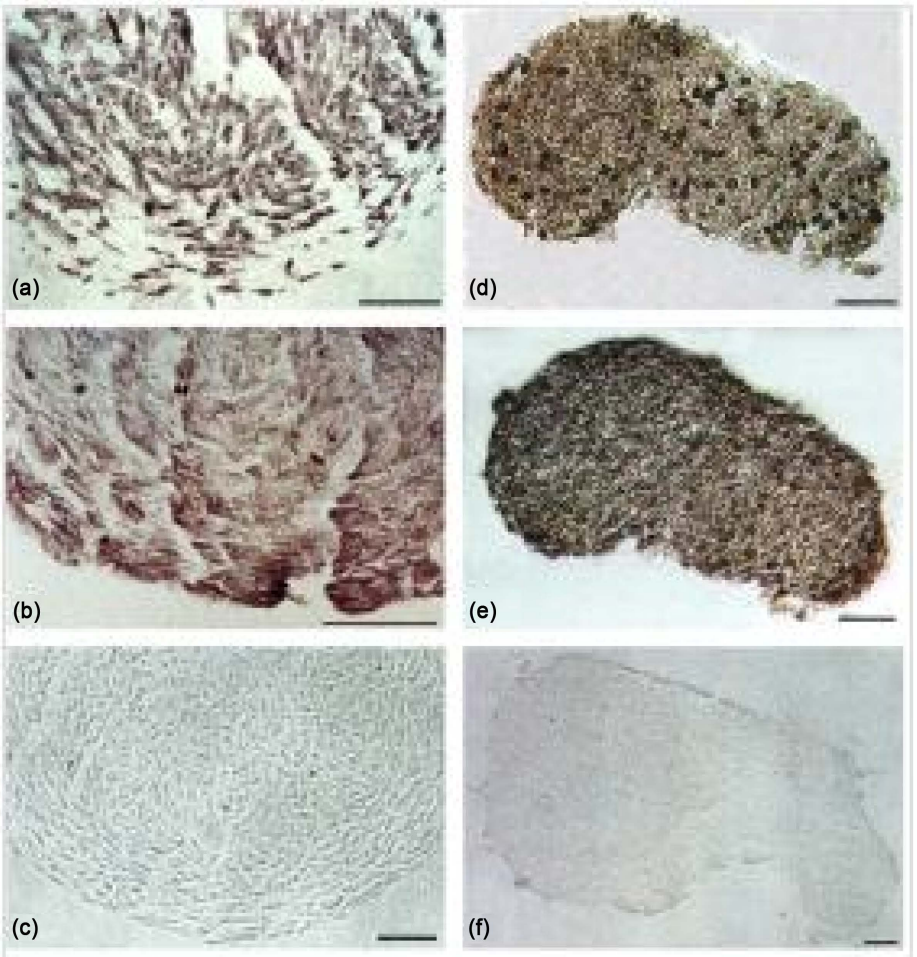

Figure 7. BIGH3 is located within MTS ECM and osteosarcoma cells. MTS formed in the MTS model in control medium ((a), (b), (c)) or medium containing 10 μg/ml BIGH3 protein ((d), (e), (f)) were stained with anti-BIGH3 antibody ((a) and (d)), anti-fibronectin antibody ((b) and (e)) and control antibody ((c) and (f)). BIGH3 deposition within MTS cultured in control medium was predominately at the interior stroma (a) when compared to the homogenous distribution observed in MTS cultured with exogenous BIGH3 (d). Conversely, fibronectin staining was homogenously distributed in both the presence and absence of exogenous BIGH3 protein ((b), (e)). Scale bars represent 50 μm.

the fibronectin stained and BIGH3 stained small spheroids. The staining was not evident in MTS prepared as frozen sections that were otherwise stained identically.

*Flow Cytometry Assays Reveal Osteosarcoma Cells Bind BIGH3.*The results of the immunolocalization experiments show that BIGH3 is a component of spheroid ECM, and indicates that BIGH3 associates with osteosarcoma cell-surface receptors. To investigate whether the osteosarcoma cells bind soluble BIGH3, we utilized flow cytometry. The results show that BIGH3 binds to cell surface (**Figure 8(a)**). As controls that indicate fluorescence intensity of known cell-surface molecules and non-specific binding, an anti-β1 integrin antibody and control antibody were utilized (**Figure 8(b)** and **Figure 8(c)**, respectively), showing that osteosarcoma cells bind fluid-phase BIGH3.

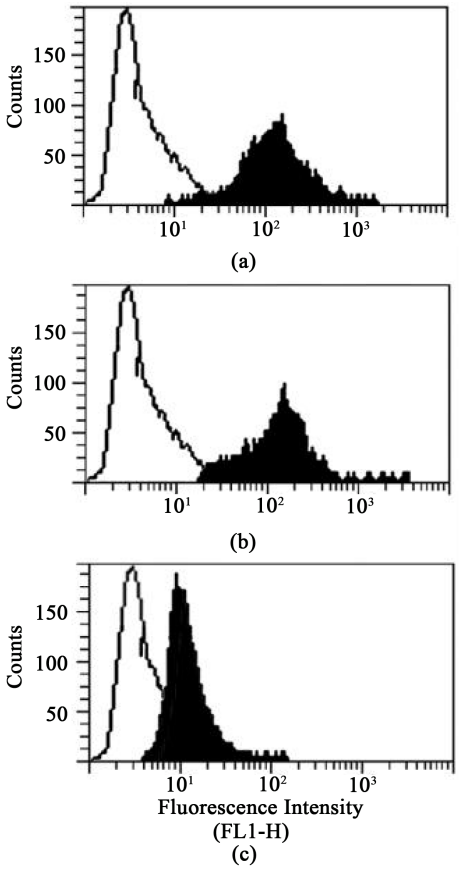

Figure 8. BIGH3 in solution binds to osteosarcoma cells. Flow cytometry shows binding of anti-BIGH3 antibody, anti-β1 integrin antibody and control antibody ((a), (b) and (c), respectively). All panels compare the chosen antiserum to a fluorescein-conjugated antibody. Counts indicate cell number. Data shown is representative of 3 experiments for each antibody.

Integrin *α4β1* Mediates Osteosarcoma Cell Adhesion onto a BIGH3 Substratum. Solid-phase cell adhesion assays were used to test directly and immunologically for integrins involved in osteosarcoma cell interaction with BIGH3. When seeded onto a BIGH3 substratum approximately 75% of the added osteosarcoma cells attached and spread within 90 minutes at 37˚C (**Figure 9** Untreated). MG-63 cells express the integrin subunits α2, α3, α4, α5, α6 and β1 [39]. To determine whether these integrin types mediate adhesion of osteosarcoma cells to BIGH3, a set of corresponding function-perturbing anti-integrin antibodies were tested. Preincubation of cells with anti-α4 antibody, and anti-β1 antibody, significantly reduced attachment of osteosarcoma cells by 92% and 93% respectively (**Figure 9** Anti-α4 and Anti-β1, $P < 0.01$). Other anti-integrin antibodies did not achieve statistical significance. These data indicate that α4β1 integrin mediates MG-63 cell adhesion onto a BIGH3 substratum. Extending this observation, we hypothesized that α4β1 integrin promotes osteosarcoma cell MTS formation. This hypothesis was tested using the exact paradigm to generate MTS in the presence of α4 and β1 antibodies. Unexpectedly, anti-α4 and anti-β1 antibodies did not block the antagonistic effect of BIGH3 on MTS formation, indicating the BIGH3-me-

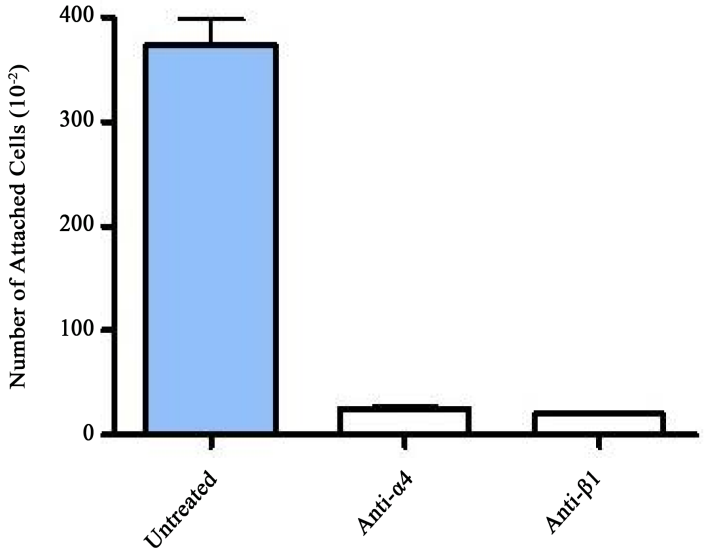

Figure 9. Anti-integrin $\alpha 4$ and $\beta 1$ antibodies reduce cell adhesion onto a BIGH3 substratum. Osteosarcoma cell attachment to a substratum comprised of 10 μg/ml BIGH3 (Untreated). Anti-$\alpha 4$ and Anti-$\beta 1$ integrin subunit antibodies blocked cell attachment. The reduction in cell attachment is significant ($P < 0.01$, n = 5) when compared to untreated conditions.

diated inhibition of large MTS formation is independent of $\alpha 4\beta 1$, while revealing that $\alpha 4\beta 1$ mediates osteosarcoma cell attachment to a BIGH3 substratum.

4. Discussion

The primary objective of this study was to document the influence of BIGH3 on osteosarcoma cell MTS development. MTS cell culture models are popular systems that support a vascular tumor growth [1] [2] [3] [4] [35] [40] [41] [42] [43]. We employed a liquid-aga- rose overlay paradigm that promoted the formation of MTS. Using TUNEL assays, we previously published findings that TGF$\beta 1$ significantly increased BIGH3 expression and apoptosis in monolayers of osteosarcoma MG-63 and Saos-2 cells [11], retinal endothelial cells [44], retinal pericytes [45], and renal tubule epithelial cells [46]. Others have shown BIGH3-mediated apoptosis in CHO and lung adenocarcinoma cells [47], HeLa and transformed corneal epithelial cells [48]. TGF-$\beta 1$ strongly upregulates expression of BIGH3 protein, which binds different integrins including $\alpha 1\beta 1$ [49], $\alpha 3\beta 1$ [50] [51], $\alpha v\beta 5$ [52], $\alpha 6\beta 4$ [53] and $\alpha 7\beta 1$ [54], implicating integrins in BIGH3 biology in disease. Thus, TGF$\beta 1$, BIGH3, integrins and apoptosis were highlighted as playing potential roles in osteosarcoma tumor biology. The results of this study demonstrate BIGH3 markedly blocked the progression of osteosarcoma cells to large MTS. When BIGH3 was added to MG-63 cells in our tumor model, small MTS formed only, when compared to the large MTS that formed without BIGH3, but otherwise cultured under identical conditions. This outcome formed the basis for three hypotheses that were tested in subsequent experiments in this study. First, a natural expectation was that BIGH3-mediated apoptosis was involved in the failure of osteosarcoma cells to form large MTS. Second, TGF$\beta 1$ was expected to increase BIGH3 synthesis and apop-

tosis. A third proposition was that integrins were involved in the mechanism underlying BIGH3's inhibition of tumor growth.

TUNEL assays were used to quantify apoptosis in BIGH3-treated small MTS. The results did not conclusively demonstrate whether the impediment of large MTS formation was related to cell apoptosis. This MTS study strongly implicates BIGH3 protein as playing a causative role in the failure of osteosarcoma cells to form large MTS. Although TUNEL-positive cells were detected, the results did not achieve significance. MTS assays were typically carried out for seven days. Little, if any MTS phenotypic differences were evident for up to 2 days following initiation of the MTS cultures. By 3 days BIGH3-treated osteosarcoma cells formed small MTS comprised of a few to approximately 30 cells. Even at day 7 the small MTS phenotype consisted of approximately 1.0 - 1.5 $\times$ 10^3 cells, which was comparatively small relative to non-treated cells that progressed to form a sheet-like mass culminating in large MTS. IHC comparisons of BIGH3 in treated and non-treated MTS indicate a homogenous distribution of BIGH3. Intense BIGH3 staining was evident in the internal stroma of non-treated MTS, and even greater intensity throughout the treated small MTS. The latter is likely explained by the BIGH3 that was added-back to the medium in order to treat the cells. The dense punctum staining observed in treated MTS that were stained with anti-fibronectin or anti-BIGH3 antibodies will require additional experiments to determine their relevance, if any. Osteosarcoma cell *de novo* synthesis of BIGH3 was not sufficient to antagonize the formation of large MTS. However, adding exogenous TGFβ1 increased BIGH3 expression in the MTS and resulted in the antagonistic effect on development of large MTS. Indeed, TGFβ1-treated MTS were indistinguishable from the MTS formed when BIGH3 protein was added to the culture medium. TGFβ1 stimulates various genes encoding for molecules of the ECM. BIGH3 binds ECM molecules including proteoglycans [55], heparin (this study and [54] [56]), collagens [57], and fibronectin [56]. Thus we cannot rule out the possibility that BIGH3 interacts with different ECM molecules to bring about antagonism of large MTS. Importantly, however, whether from osteosarcoma cell synthesis or from other cellular sources in the tumor environment, anti-BIGH3 antibody blocked the BIGH3-mediated antagonism, indicating that BIGH3 protein itself, was involved in the regulation of MTS size.

Flow cytometry confirmed that osteosarcoma cells bind to soluble BIGH3 suggesting a high-affinity interaction. Osteosarcoma cell adhesion onto a 2-dimensional planar BIGH3 substratum was dependent on the concentration of BIGH3 in the substratum, and on the time of incubation, suggesting that cell interaction with BIGH3 is receptor mediated. A set of function-inhibiting anti-integrin antibodies was used to test whether integrins mediate osteosarcoma cell adhesion on BIGH3. Function-blocking anti-α4 and anti-β1 antibodies inhibited more than 90% of the number of osteosarcoma cells binding to a BIGH3 substratum, implicating integrin $\alpha4\beta1$inosteosarcoma cell attachment onto a BIGH3 substratum. Integrin antibodies, including anti-α4 and -β1 did not affectBIGH3's antagonism toward large MTS development when added at culture initiation, or when a 30-minute pre-incubation of osteosarcoma cells with integrin antibodies was implemented in the assay paradigm.

Although the BIGH3 effect on MTS size is distinct, its physiological meaning is not yet apparent. One possibility that we examined is that BIGH3 functions as a tumor suppressor that diminishes tumor viability by induction of apoptosis. In support of this possibility *TGFBI*$^{-/-}$ mice are predisposed to form tumors when compared to wild type mice [10]. Other investigators have shown that BIGH3 is downregulated in tumors and tumor cells of mesenchyme origin, indicating a tumor suppressor role [9] [58] [59] [60] [61] [62]. We have shown that BIGH3 induces a significant increase in apoptosis in monolayers of osteosarcoma cells [11], and other different cell types [11] [44] [45] [46]. Though BIGH3-mediated apoptosis would be an attractive explanation for the antagonistic effect of BIGH3 on development of large MTS, we did not obtain convincing evidence that apoptosis was a significant influence. Additional analysis of a larger number of small spheroids and different osteosarcoma cell lines will help access the extent, if any, that BIGH3-mediated apoptosis explains, at least in part, the mechanism underlying BIGH3's antagonism on tumor growth.

An interesting alternative possibility is that BIGH3 augments tumor cell aggressiveness. The small MTS size brought about by TGFβ1 signaling and BIGH3 could provide a greater number of cells at the spheroid surfaces, and possibly increase the metastases potential. The combined surface area of small MTS would greatly exceed that of the surface area of a single large MTS of equivalent volume, where surface area $A = 4\pi r^2$. Assuming a mostly spheroid shape for tumors, a hypothetical "large" tumor with radius (r) of 2.5 mm has an $A \approx 78.5$ mm^2. If hypothetical small tumor r were limited to 0.5 mm, the volume of ≈392 small tumors would equate to the volume of the single large tumor. In this instance the small tumor combined surface area $A \approx 1227.8$ mm^2, a 15-fold increase relative to the area of the large tumor. Small tumors could offer advantageous characteristics toward tumor survival, including increased chances of avoiding detection, greater avascular diffusion of oxygen and nutrients, and a decreased chance of necrotic tumor cell death. Previous studies report hypoxic cores develop only when MTS were ≥ 400 μm diameter [63] [64] [65], however this has recently been questioned: In MG-63 osteosarcoma MTS that were ≤ 100 μm diameter, hypoxia-responsive element (HRE) activity was evident, while, interestingly, HRE activity was almost undetectable in MG-63 monolayers, indicating there is different transcriptional and translational activity in 3-dimensional MTS as opposed to cell monolayers [66]. Thus small MTS of osteosarcoma cells showing high HRE activity, as with hypoxia-inducible factor 1, would potentially give rise to exceedingly aggressive cancer cells.

In summary, BIGH3 protein has a marked influence on tumorigenesis *in vitro*. Mechanistically, at least part of the activity includes TGFB1, TGFB1 signaling, and BIGH3 protein. Potential therapeutic targets identified in this study include TGFβ1, molecules in the TGFβ1-receptor signaling pathway, and BIGH3 itself.

Acknowledgements

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How do Medical Students Learn?

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Abstract

Background & Objectives: In this paper, we try to define learning, to describe how retention of memorized material can be improved and to describe how to help students improve clinical reasoning and problem solving skills. The goal of teaching is to improve learning, but how do we know that students are learning in the proper way? Methods: We depend on our experience in medical and clinical teaching of our undergraduate and postgraduate students during implementation of the curriculum. We interview 1st year (preclinical) and 6th year medical students by certain questionnaires (VARK) in order to know their learning methods and their knowledge about the styles of learning and the techniques of learning. Learning is viewed here as developing a way of thinking and acting that is a characteristic of an expert physician. Such a way of thinking consists of three important elements: We utilize Working Memory in 3 main approaches: First, attention in a way through questions, focus attention and uses of first letter of statement of the word; Second, rule of 7 (Teach < 7 steps); Third, concentration on important relevant materials. We have to keep in our mind that even if we remember something, it doesn't mean we understand it. In addition, the case learning approach & problem solving learning approach are the methods used and we try to be cooperative, active, and experimental and the student must be self directed and the students should be competent in his learning methods, most are confident of their ability. Results: Most of the first year students have poor knowledge in regards to style & learning techniques. For final year students around 30% learn two styles and 20% trimodel, especially more in female students, 10% mention single model in their learning and the remaining learn by Quad modals. In conclusion, learning how to learn must be a standard part of the curriculum in medical school, and student must be aware of that.

Keywords

Learning Style, Learning Methods, Questionnaires, Medical Students

1. Introduction

Learning is viewed here as developing a way of thinking and acting through 3 elements: Knowledge, Thinking activities through the use of knowledge to interpret situation, Thirdly to be active in utilizing them. Elements of any educational programme depend on 3 pillars: The students, the teachers and the curriculum. The key task for a teacher is to decide what the student should learn, and this can be specified as learning outcomes, competencies and entrustable professional activities. The decision about the curriculum, teaching and learning methods and assessment is informed by the expected learning outcomes and competencies [1]. Medical students during the process of their learning must have 3 characters to establish good learning behavior: works in ethics, dedication & self confidence. Thus Learning Style is the process by which a person understands and retains information, thereby gaining knowledge or skills [2]. In the process of teaching and learning, the student's memory can be improved through increasing attention of the students by creating question and sometime we use the first letter of the statements or we can use rule of 7 (Teach < 7 steps), at the same time we have to concentrate on important relevant materials [3]. The learning revolution in higher education all over the world has resulted in a shift of paradigms from teacher-centered to learner- centered pedagogical approaches. Thus, different methods have been incorporated into classrooms to enhance student learning and replace teacher-led lectures as the predominant means of students in processing and analyzing information and making better decisions rather than merely transmit knowledge [4].

According to Pashler, Mc Daniel, Rohrer, and Bjork (2009), the term learning skill refers to the view that different people learn information in different ways, and also refers to the concept that individuals differ in regard to what mode of instruction or study is the most effective for them [5] [6]. Medical teacher should understand the style of learning that is utilized by medical students in acquiring the knowledge, and there are three learning theories:

1) Adult Learning Theory (Mal Colm) [4],

2) VARK by Neil Fleming [7],

3) Experimental Theory (Learning Style Kolb's model) [8]

This Experimental learning theory, developed by David Kolb, demonstrates the importance of individual learning styles in improving learning. This study helps elucidate the way in which medical students, surgical residents, and surgical faculty learn. The Kolb Learning Style Inventory, which divides individual learning styles into Accommodating, Diverging, Converging, and Assimilating categories [4] [5] [6] [7].

4) Inventory and Gardner's Multiple Intelligence Theory [9].

We concentrate in this study on VARK questionnaire in understanding how medical students learn depending on the preference of the students in learning by seeing, hearing, or reading/writing and by practice and interactions and performing activities. In VARK, we also assess and teach, the need of students including their knowledge, skill and attitude. Every clinical student should know and how can apply 5 important learning techniques [10]:

1) Testing & enhanced testing: Testing has been shown to more effectively improve knowledge retention compared with less active forms of studying, such as rereading information or rewatching lectures

2) Spaced-Repetition: The key concept is that spacing your studying and self-testing over time as opposed to massing, also known as "cramming", will flatten your forgetting curve and help you retain information longer.

3) Interleaved Technique: Traditional methods of learning are through massed practice, and only have short term effectiveness, as in spaced repetition, interleaving may not be as effective in the short term but is more effective in the long term. All these 3 techniques will add improvements in long-term performance and you learn more efficiently.

4) Memory associations: during my lectures to medical students I can't recall the name of all students, but I can remember their names if the group is small during role play and during bedside teaching in hospital and also I can remember all the time the name of the medical student, who helps me during the presentation of my lectures and also the name of brilliant students and the name of students with poor clinical performance. The more associations you can form to something you're trying to learn, the more likely you are to remember it in the future because there are more paths you can take to retrieve it.

5) Fogg Behavior Technique [11]: Stanford behavioral scientist B. J. Fogg reduces behavior change to three variables: motivation, ability, and trigger. If you think about any behavior, e.g. learning and studying, you need a certain level of motivation and ability, followed by a trigger, to implement the behavior. Finally in medical schools students should experience that long-term retention of factual knowledge is important and this particularly, if it is associated with procedural knowledge and learning and forgetting is the unpleasant side of learning [12]. Scientific knowledge of how to learn and acquire factual knowledge should be a standard part of the curriculum in medical school. In this paper, we present our review of how to successfully learn and retain knowledge and let medical students to know the type of learning techniques.

2. Subjects & Methods

This study performed at the department of medicine and at the college of medicine during the year 2016. An established evaluative tool, the VARK Learning Style methods was administered by questionnaire protocol paper to 2 groups of medical students: One first year pre-clinical undergraduate medical class (N = 50); and the second is 6^{th} year clinical undergraduate medical class (N = 50). The survey and explanatory materials were distributed to the groups personally. Each participant was offered to receive the result of their survey as well as an explanatory sheet on learning styles. Two third of the students are female medical students & the other are male. In addition to the questionnaire, we ask the students about their knowledge of the study techniques. We use P value to measure the difference between students.

Results: No single student from the first year class has, ever any knowledge regarding

the learning style, learning theories and the proper study techniques and they mentioned that, their main methods of learning are either visual and reading and writing and in addition around 10% they mention the auditory method as one of their learning style and in general, students are largely unaware of how to learn successfully and improve memory, meanwhile and after we explain the methods of learning to them, we get some of the following results: 10% three models of learning, 10% two modals and 80% single modal of learning mainly visual Table 1, Figure 1, and Figure 2. There is no significant difference in regards to the gender of these students, Table 2. The 6th year undergraduate Students have poor knowledge in regards to style & learning techniques and Around 30% two style including visual & kinaesthetic and 20% trimodel more seen with female students (statistically significant) and about 5% mention single model.

Table 1. Showing the representation of the VARK inventory results for learning styles among (First-year students), & (Final year students) (*VARK = V visual, A Audition, R Reading Writing and K Kinesthetic).

*VARK modals	Final year students	First year students
Quadrimodal	40%, 20 students	Nil
Trimodal	20% (V, A, K), 10 students	10% (5 students)
Bimodal	30% (V, R, K), 15 students	10% (5 students)
Unimodal	10% (V), 5 students	80% (40 Students)

Table 2. Sociodemographic features of the studied students.

Sociodemographic features	First year		Final year	
Age	19 - 20 years		24 - 25 years	
Gender	10 males	40 female	20 males	30 female
Total students	50		50	

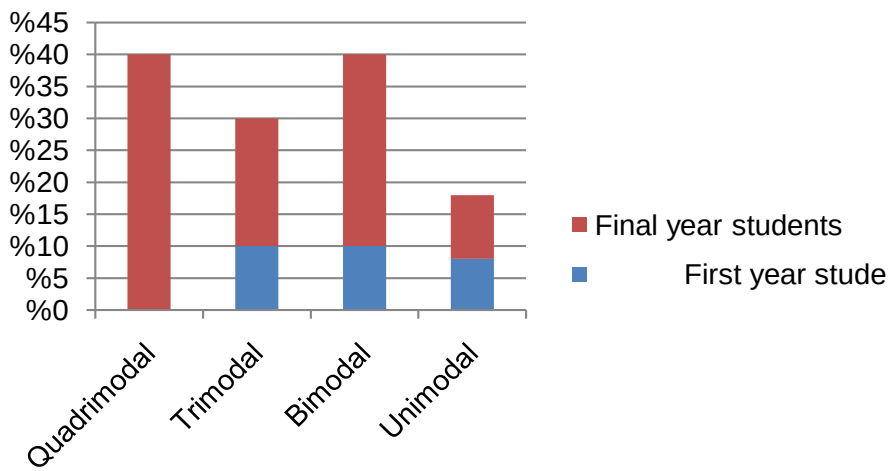

Figure 1. The representation of the VARK inventory results for learning styles among first-year students & final-year students.

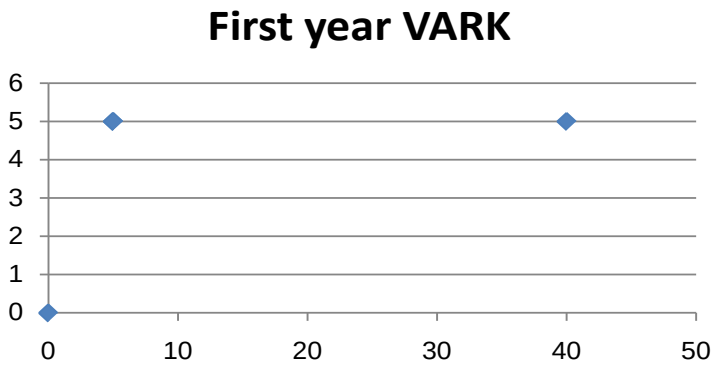

Figure 2. Scattered Diagram of First year students.

3. Discussion

The comprehension of how people learn is important in order to improve the teaching and learning methods and circumstances and will improve the student's learning [13] Our study shows that the predominate learning styles of the students of college of medicine University of Babylon is VARK style. Many models and measures of learning styles have been described in the literature, including Kolb's Learning Inventory and Gardner's Multiple Intelligence Theory Adult Learning Theory (Colm) [4] [5] [6] [7]. In present study in medical school; learning medical sciences involves two parts: one is the theoretical aspects of medical education and the second is the practical and procedural parts, which might include laboratory teaching, simulation and practice, hospital clinical medicine and bedside teaching [14]. We can say it is similar to the traditional way of medical education in medical school which include the stage of basic science, and the the stage of clinical science. In our questionnaires on how medical students learn, we noticed that most of the first year and final year medical students have no knowledge in regards to the style of learning and the techniques used in learning of medical students and we ask them any previous knowledge or experience about the following techniques: Testing effects, Active recall and spaced repetition, and this leads us to consider that these styles and techniques must be part of the curriculum in medical colleges and in previous study on medical education learning how to learn is not a standard part of the curriculum in medical school [15].

We try during the process of learning and teaching of medical students to utilize student's Working Memory in 3 main approaches.

First Attention in a way through questions, focus attention and uses of first letter of statement e.g. a patient presents with jaundice, how you are going to reach a clinical diagnosis? We ask the students to remember the words that begin with the letters from A to J: e.g.

A for appetite, acuteness, abdominal pain etc.

B for history of blood transfusion etc.

Second Rule of 7 (Teach <7 steps, & Numbers aide memory, particularly during powerpoint slide presentation of knowledge).

Third we try to concentrate on important relevant materials and especially through

bedside teaching by data interpretation and problem case discussion and we might use the brain storming techniques. The students should understand the concept that tells before students start to learn, they should be taught how to learn. This idea should become an essential part of the medical curriculum [16] [17].

Learning Style is the process by which a person understands and retains information, thereby gaining knowledge and/or skills [18]. In the present study it is clear, that the predominate learning style for undergraduate medical students is VARK, this goes with idea that A significant improvement in learning style, as evidenced by an increase in mean VARK score and percentage increase of multimodal learners in pre and post tests, and was seen especially, after introduction of PBLs (problem based learning) [19] [20].

In this study on first year medical students, they mentioned that, their main methods of learning are either visual, reading and writing and in addition around 10% they mention the auditory method as one of their learning style and in general, students are largely unaware of how to learn successfully and improve memory, meanwhile and after we explain the methods of learning to them, the students can get some knowledge of learning style, but in one study on first year medical students, they prefer Multiple learning style [21], and in this study 63.9% of the students were found to be multimodal, which means that active learning strategies have to be applied to a greater extent in the first 3 years of our educational curriculum to reach all types of learners. The lack of knowledge of the students on learning style and learning techniques is due to pre- university education system in the country, where students traditionally follow didactic lectures in primary & secondary schools and the Pre-university education is often supplemented with private tuition classes; these could be either small group classes or larger lecture based classes [22]. A similar study met the same latter criteria [23] [24] [25]. The expanding medical knowledge and the advances in the learning styles and learning techniques, raise the issue that, there is a need to review the priorities and preferences of the methods or to find out a different way to use the existing methods to increase their effectiveness, and Before students start to learn, they should be taught how to learn, this idea should become an essential part of the medical curriculum [26]. Finally, medical education needs the collaboration of several healthcare providers and improvement in education processes will be reflected on the improvement of the quality of patients and health care [27].

4. Conclusion

Medical knowledge is expanding and medical learning is also an evolving process, and this will reinforce us to look for more efficient and effective ways of medical education and learning.

Recommendations

1) Prepare faculty for new learning behavior and change.

2) Establish a new curriculum committee and working group belonging to medical

education department.

3) Design new methods of learning, e.g. learning modules and defining educational outcomes.

4) Seek advice from experts in medical education and learning and even take the experience of other medical schools.

5) We have to plan an organized learning methods.

6) Train the facilitators of the learning and defining the objectives of a facilitator.

7) Introduce students to these new learning programs & curriculums.

8) Use 3-learning to support the delivery of these modules.

9) Change the assessment methods to suit the new curriculum.

10) Encourage feedback from students and teaching staff.

11) Manage learning resources and facilities that support self-directed learning of medical students.

12) Provide continuing evaluation and make new changes.

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