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Full Length Research Paper

Malignant otitis externa: An assessment of emerging pathogens and the prognostic factors

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Although the mortalities from malignant otitis externa (MOE) have greatly reduced, it is still a potentially fatal clinical condition. This study was undertaken to review the treatment outcomes and prognostic factors in MOE and to compare the behavioral pattern of cases caused by pseudomonas and non-pseudomonas organisms. A retrospective chart review of patients diagnosed with MOE in a tertiary institution over a 13 year period was conducted. Treatment outcome was divided into survival and mortalities groups. Demographic and disease factors were analyzed regarding mortalities using univariate and multivariate analysis. Seventeen of 22 cases were analysed. Nine (53%) were diabetic while 5 were HIV positive. After average of 7 weeks of antibiotic therapy ± surgical debridement, the disease resolved in 59%. Mortality was 41%. Diagnostic delay, poor blood sugar control, and extensive disease were found to predict mortality ($P = 0.051, 0.048, \text{ and } 0.006$ respectively). Age, sex, causative organism, HIV infection, facial nerve and other cranial involvement did not significantly predict mortality. *Pseudomonas aeruginosa* was isolated in 11 patients. The rest had atypical organisms, *Staphylococcus aureus* and *Proteus* spp. There was no significant difference in the disease extension, mortality, duration of treatment and facial nerve involvement between pseudomonas and non-pseudomonas groups. However the pseudomonas group were predominantly diabetic ($p = 0.03$). It is concluded that malignant otitis externa still has a significant mortality despite aggressive therapy. Extensive temporal bone/intracranial disease, poor blood sugar control, and diagnostic delay portend a poorer prognosis. *S. aureus* is an increasingly important causative organism in MOE especially in non-diabetic patients.

Key words: Malignant otitis externa, mortality, risk factors, causative organisms.

INTRODUCTION

Malignant otitis externa (MOE) is a rapidly progressive infection of the external ear characterized by invasive inflammation of the external auditory canal, marked by necrosis of surrounding cartilage and bone tissues with

tendency to extension along sub-temporal fat planes (Walton and Coulson, 2014; Lasisi and Nwaorgu, 2001). Pathologically, MOE was divided by Benecke into necrotizing otitis externa, in which only soft tissues and

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cartilage undergo necrosis, and skull base osteomyelitis, in which temporal or skull base bones are progressively destroyed (Benecke, 1989; Peleg et al., 2007). *Pseudomonas aeruginosa* is the predominant causative organism in most reports, but increasing number of reports have implicated non-pseudomonas organisms including *Aspergillus fumigates* and *Staphylococcus aureus* as the isolated causative agents (Walton and Coulson, 2014; Hobson et al., 2014). The disease is mostly seen in elderly diabetes and immunocompromised individuals, but it has also been reported in apparently healthy non-immunosuppressed individuals (Nguyen et al., 2010). Although the mortality figures from MOE have been shown to be improving in last 2 decades, it is still a potentially fatal clinical condition with high mortalities ranging from 20 to 60%, still being reported especially from developing countries (Lasisi and Nwaorgu, 2001; Lee et al., 2011; Loh and Loh, 2013). Although studies have attempted to examine prognostic factors for survival, there still remains a lack of consensus regarding the identifiable prognostic factors to guide treatment. This is compounded by its unpredictable clinical course. The study aim to review the treatment outcomes in a tertiary institution, as well as compare the behavioral pattern of MOE caused by pseudomonas and non-pseudomonas organisms, and examines its mortality risk factors.

METHODS

A retrospective charts review of 22 consecutive patients with diagnosis of MOE at the Department of Otorhinolaryngology in a tertiary institution between 2004 and 2016 was performed. The study was approved by the ethical review committee of the institution.

The following data were assessed: Presenting symptoms and clinical signs, patients' underlying medical conditions, diagnostic delays, bacteriological culture results, radiographic features, disease extensions, cure rate, complications, and mortalities. Diagnoses were based mainly on clinical history of ear discharge, ear ache, facial nerve palsy, headache, as well as finding of necrotic debris in the ear canal, in addition to computed tomography temporal bone findings. Extensive disease was defined by infratemporal fossa involvement, temporal bone/petrous apex involvement, and intracranial involvement. The initial antibiotic therapy for most of the patients included intravenous administration of ciprofloxacin and/or ceftriaxone. The antibiotics were changed according to the antimicrobial sensitivity pattern of subsequent ear culture results. In resistant cases, gentamicin was added. Surgical debridement was carried out for removal of extensive necrotic debris and sequestrum.

Those patients with inconsistent and/or insufficient/incomplete data were excluded from analysis. Data was analyzed with the SPSS statistical software (version 16.5; IBM Corp) Chi-square or Fisher exact test were used to find the significance of study parameters on categorical scale. The potential risk factors were tested with logistic regression analysis. The criterion for statistical significance was set at P value of < 0.05 .

RESULTS

Twenty two patients with MOE during the study period

were identified. Five were excluded due to insufficient or inconsistent clinical data, and 17 were analyzed. There were 8 males and 9 females. Fifteen were adults with age range of 24 to 80 years and mean of 56.7 ± 16.3 years. The remaining 2 patients were children aged 2 and 5 years respectively. Table 1 outlined the patients' characteristics and the disease attributes. Nine (53%) were diabetic, whereas 5 of the non-diabetics were HIV positive, while the rest were neither diabetic nor HIV positive. The two children in the study were HIV positive. Among the diabetics, 5 had poorly controlled blood sugar with their fasting blood sugar exceeding 16.5 mmols per litre. The microbiological culture was positive for bacteria in 15 (88%) patients. *P. aeruginosa* was the predominant organism which was isolated in 65% of the patients. The atypical organisms were *S. aureus* (18%) and *Proteus* spp. (6%). *P. aeruginosa* was isolated in all the diabetics except one patient that had negative culture, whereas all the atypical bacteria were isolated in the non-diabetic patients (Tables 1 and 2).

Facial nerve palsy occurred in 70% of the patients with 50% of them being severe (grade IV) palsy (Table 1). Other cranial nerve palsy documented were V and VI in 2 patients respectively and IX in one patient. Table 3 outlined the pattern of disease extension from clinical and radiological assessments. The disease was limited to the external auditory canal in only 35% of the cases, with the mastoid being the most common site of disease extension.

The disease resolved in 10 (59%) after an average of 7 weeks of antibiotic therapy with surgical debridement in some. Two of the survivors had significant morbidity from extensive destruction of pinna and external auditory canal. The rest did not survive despite treatment with antibiotics and surgical debridement after varying time frames. The overall mortality rate was 41%. The mean antibiotic course was 6.9 ± 2.9 weeks. All the patients received ciprofloxacin and/or ceftriaxone either as initial antibiotic therapy or as definitive treatment following antibiotic sensitivity results. Surgical debridement was performed in 65% of the patients, and this included bedside limited debridement of soft tissue necrosis in 8 patients, mastoidectomy in 4 cases, and additional drainage of cerebella abscess in one patient.

Comparison of the pseudomonas and non-pseudomonas infected malignant otitis externa was outlined in Table 2. The pseudomonas group were predominantly diabetic than the non-pseudomonas group ($P = 0.03$). The pseudomonas group also had more propensity for extensive tissue destruction and mortality although not statistically significant ($P = 0.064$ and 0.092 respectively). The non-pseudomonas MOE were treated for an average of 2.2 more weeks of antibiotics than the pseudomonas group ($P = 0.241$). Other factors such as HIV status and rate of facial nerve palsy were not significantly different between the two groups.

Significant mortality risk factors identified for MOE in

Table 1. Summary of patients' and disease characteristics (n = 17).

Patients	Age (years)	Sex *	DM	HIV status	Symptom duration (weeks)	Ear(s) involved	Culture results	**FN palsy	Surgical treatment	Morbidity/mortality
1	2	M	-	+	4	Right	Pseud	I	Mastoidectomy	Cured***
2	5	M	-	+	5	Right	Proteus	IV	Ltd debridement	Deceased
3	24	F	-	-	2	Left	Pseud	II	None	Cured
4	28	M	-	+	3	Left	Pseud	I	Ltd debridement	Cured
5	44	M	+	-	9	Left	None	II	None	Cured
6	49	F	-	-	4	Right	None	I	Non	Cured
7	51	F	-	+	8	Right	Staph	III	Ltd debridement	Cured
8	52	M	+	-	12	Left	Pseud	IV	Mastoidectomy	Deceased
9	54	F	+	+	7	Right	Pseud	IV	Ltd debridement	Cured***
10	58	F	+	-	1	Right	Pseud	IV	None	Deceased
11	61	M	+	-	8	Left	Pseud	III	Ltd debridement	Cured
12	65	F	+	-	4	Right	Pseud	III	Ltd debridement	Deceased
13	67	F	+	-	4	Left	Pseud	IV	Mastoidectomy	Deceased
14	69	F	+	-	16	Left	Pseud	I	Ltd debridement	Deceased
15	70	M	-	-	6	Left	Staph	III	None	Cured
16	79	F	-	-	2	Both	Staph	I	Mastoidectomy/intracranial drainage	Cured
17	80	M	+	-	5	Both	Pseud	IV	Ltd debridement	Deceased

*Diabetic status, **House/Brakeman grade; ***Cured with complete necrosis of pinna and external auditory canal; (+) = positive status; (-) = negative status, Pseud = *Pseudomonas aureginosa*; Staph = *Staph aureus*; Proteus = *Proteus* species; ltd debridement = limited debridement of external ear.

univariate and logistic regression analysis included poor diabetic control with blood sugar exceeding 16.5 mmols per litre, extensive disease (defined by extensive temporal bone destruction and/or intracranial extension), and diagnosis delayed beyond 2 weeks ($P = 0.048, 0.006, \text{ and } 0.051$ respectively)(Table 4). Mortality was neither significantly related to the aetiological organism nor to HIV status. Mortality was significant for severe grade of facial nerve palsy in univariate analysis but not so in logistic regression test.

DISCUSSION

Malignant otitis external is a relatively uncommon

but severe disease of the external auditory canal which has been associated with mortality of 40 to 60% (Lasisi and Nwaorgu, 2001; Lee et al., 2011; Stevens et al., 2015; Kwon et al., 2006). The development of anti-pseudomonal antibiotics such as fluoroquinolones and ceftazidime, has reduced mortality significantly with increasing number of reports indicating better mortality figures at <10% (Franco-Vidal et al., 2007; Pulcini et al., 2012). A number of studies have examined potential prognostic factors such as clinical presentation, imaging findings, microbiology and facial nerve involvement (Lee et al., 2011; Loh and Loh, 2013; Stevens et al., 2015; Kwon et al., 2006; Soudry et al., 2011; Soudry et al., 2007). However,

conclusive prognostic factors are yet to be identified. The emergence of fluoroquinolones-resistant and multidrug resistant pseudomonas organisms have created difficulty in developing consensus on the optimal choice of antibiotics and duration of therapy (Pulcini et al., 2012; Berenholz et al., 2002).

The study found diabetes as the underlying debilitating disease in 53% of the patients, whereas 29% were immune depressed due to HIV infection similar to other reports (Franco-Vidal et al., 2007; Martel et al., 2000). However higher diabetic figures of 80 to 95% have been reported (Lee et al., 2011; Loh and Loh, 2013; Pulcini et al., 2012; Chen et al., 2011).

Table 2. Comparison of patients' attributes and biological behaviour of malignant otitis externa caused by *Pseudomonas* versus non-*Pseudomonas* organisms.

Attributes	<i>Pseudomonas</i> group (n = 11)	Non- <i>pseudomonas</i> group (n= 6)	P value*
Mean age (years)	50.9 ± 23.3	49.7 ±25.7	0.921
Male/female	5/6	3/3	0.858
Diabetes status	8	1	0.030
HIV seropositivity	3	2	0.793
Facial nerve palsy	8	3	0.349
Extensive tissue necrosis	7	1	0.064
Duration of antibiotic treatment (weeks)	4.7 ± 1.1	6.9 ± 2.4	0.241
Mortality	6	1	0.129

*Fisher's exact test.

Table 3. Disease extension pattern/radiological findings.

Disease extension	Number of patients (%)
Disease limited to the external auditory canal	6 (35%)
Disease extension to the mastoid	12 (71%)
Extension to the pinna	7 (41%)
Extension to the parotid/temporomandibular joint	5 (29)
Extension to the petrous apex	4 (24%)
Intracranial extension	5 (29%)

Table 4. Analysis of potential risk associated with mortalities among 17 patients with malignant otitis externa.

Potential risk factors	Deaths (n=7)	Survival (n= 10)	Chi square test		Logistic regression test	
			P value	OR (95% CI)	P value	OR (95% CI)
Age >50 years	6	5	0.304	0.31 (0.05- 2.0)	0.492	- -
Male/female	3/4	5/5	0.765	0.75 (0.11-5.2)	0.453	- -
HIV positivity	2	5	0.381*	1.75 (0.47-6.6)	0.563	- -
Uncontrolled diabetes	4	1	0.006*	4.33 (1.61-11.6)	0.048	2.35 (1.17-4.71)
Extensive disease	7	1	0.001*	6.0 (1.69-21.3)	0.006	3.00 (2.51-5.07)
<i>Pseudomonas</i> etiology	6	5	0.301	0.54 (0.26-1.1)	0.710	- -
Delayed diagnosis beyond 2 weeks	6	7	0.603	0.39 (0.32-4.8)	0.051	1.74 (1.01-2.82)
≥Grade IV facial nerve palsy	4	1	0.036*	3.75 (0.63-22.3)	0.162	- -
Other cranial nerves involvement	3	2	0.682*	0.79 (0.23-2.5)	0.757	- -

*Fisher's exact test.

Pathological basis of MOE propagation among diabetics has been attributed to microangiopathy which often occurs in diabetes mellitus. It was suggested that this microangiopathy may decrease local blood flow, and results in a low concentration of antibiotics in target tissue (Lee et al., 2011).

P. aeruginosa was identified as the causative organism in 65% of the patients, predominantly among diabetics, with atypical non-pseudomonal organisms constituting 24%, predominantly among non-diabetics, similar to other

studies (Loh and Loh, 2013; Franco-Vidal et al., 2007). However higher figures of 85 to 95% *pseudomonas* isolation have been reported (Pulcini et al., 2012; Gehanno, 1994). In contrast to the findings, lower rates of *pseudomonas* isolation <50% have also been reported (Hobson et al., 2014; Chen et al., 2011). In one series, *pseudomonas* was isolated in only 27% of the 19 patients studied. The increasing reports of atypical organism isolation in MOE specifically imply that *S. aureus* is an increasingly important organism leading to MOE.

Diagnosis should not be center on isolation of pseudomonas, rather a high index of suspicion for atypical organisms, should be maintained especially in patients with signs and symptoms of MOE who do not have diabetes.

Comparison of the behavioral pattern of MOE caused by pseudomonas and non- pseudomonas organisms in this study revealed that the pseudomonas infected cases were more likely to have diabetes mellitus. Although the pseudomonas group tended to develop more extensive tissue destruction and have more mortality, these observations were not significant. Hobson et al. (2014) similarly reported significant association between pseudomonas infected group and diabetes mellitus than the non-pseudomonas cases. They however reported no difference between the two groups regarding rate of bony erosion and extensive disease. This underscores the need to adopt aggressive treatment protocol for all cases of MOE regardless of organism involved.

Overall the mortality rate of the study was 41% in line with other reports (Lee et al., 2011; Stevens et al., 2015; Kwon et al., 2006). In contrast a number of reports indicated lower mortalities than the results of the study at less than 10% (Franco-Vidal et al., 2007; Pulcini et al., 2012). This apparent difference in the mortality figures between the present study and the aforementioned reports may be attributed to likely differences in the severity patterns of the MOE in this study in comparison with that in the aforementioned studies. Although the severity pattern of MOE was not specified in those studies, extensive temporal bone destruction in 47% of the patients was documented in this study. Some studies that carried out systematic stratification of the disease severity showed that the severe sub-group of MOE had more significant mortality than the less severe MOE (Peleg et al., 2007; Stevens et al., 2015; Soudry et al., 2011). However, the relatively smaller number of patients in this study compared to the aforementioned studies, which analyzed 46 and 32 patients in their series respectively, may have also contributed to poorer mortality figures.

Potential significant mortality risk factors identified in this study were extensive disease with extensive temporal bone/intracranial involvement, poorly controlled blood sugar exceeding 16 mmol/L, and diagnostic delay/commencement of definitive treatment. Quite understandably many previous reports are in agreement with the present results regarding significant association between extensive disease and poorer survival rate (Lee et al., 2011; Loh and Loh, 2013; Stevens et al., 2015; Soudry et al., 2011). Similarly, poor diabetic control with diabetic complications has been shown to lead to shorter survival in line with the study findings (Joshua et al., 2008). However, Loh and Loh (2013) found that diabetic control did not affect the prognosis in contrast to the data of this study. It is plausible that the design in Loh and Loh (2013) series and this study did not apply similar

parameter in defining the level of diabetic control. The poorer prognosis observed among the poorly controlled diabetics may be attributed to microangiopathy which may have decreased local blood flow to the diseased tissues, resulting in a low concentration of antibiotics in these tissues with resultant poor responses.

Contrary to a report in which delay in the commencement of intravenous antibiotics did not show adverse outcome (Loh and Loh, 2013), Guevara et al. (2013) demonstrated significant association of poor prognosis with diagnostic delays and delays in commencement of treatment similar to the data of this study. The average diagnostic delay in this study was 5.9 weeks, in line with other reports, which ranges from 6 to 13 weeks (Loh and Loh, 2013; Guevara et al., 2013). The reasons for diagnostic delays and commencement of definitive treatments are often related to the indistinguishable nature of the initial symptoms from simple otitis externa. It is usually only after multiple failed treatment attempts that MOE is suspected.

Although severe facial nerve palsy seemed to be significantly associated with increased mortality in the univariate analysis, it was not significant on logistic regression test. Most reports are in agreement with the data of this study on the lack of significant correlation between rate of facial nerve involvement and mortality (Lee et al., 2011; Soudry et al., 2007). Facial nerve involvement has been shown to represent a sign of progression of MEO although it does not, by itself, worsen prognosis (Soudry et al., 2007). Moreover, severer forms of facial nerve involvement also represent significant morbidity which often lingers after infection has been controlled. The statistical significance of this study was limited by the small number of cases recruited.

In conclusion the results indicate that MOE still presents as potentially fatal disease of the external auditory canal and temporal bone with significant mortality and morbidity despite aggressive antibiotic and surgical treatments. Mortality was significantly influenced by extensive temporal bone/intracranial involvement, poor blood sugar control, and diagnostic delay. The study also highlights *S. aureus* as an important causative organism in MOE especially in non-diabetic patients.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Neutrophil vacuolization in peripheral blood smear assessed with May Grünwald-Giemsa stain has direct correlation with the severity of hemorrhagic shock and serum lactate in trauma patients

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Tissue trauma induces migration and activation of neutrophils through specific mediators. Vacuolated neutrophils in peripheral blood smear of septic patients correlated with mortality. However, scarce data exist with respect to findings in hemorrhagic shock (HS) trauma patients. The aim of this work was to evaluate the number and size of cytoplasmic and nuclear vacuoles in polymorphonuclear neutrophil (PMN) obtained from a peripheral blood smear stained with the May-Grunwald-Giemsa method in trauma patients with hemorrhagic shock. Seven sequential blood samples were taken from 20 patients with severe hemorrhagic shock and 20 patients who sustained mild thoracic trauma (control group). The first sample was obtained shortly after admission to the hospital followed by new samples taken at 6, 12, 18, 24, 48 and 72 h. Blood smears from both groups were processed to assess vacuolization and vacuole morphology in one hundred PMNs at each time point. The number and the area of vacuoles in the nucleus and the cytoplasm were determined using the program Image-Pro Express version 4.0 for Windows (Media Cybernetics, Bethesda, MD, USA). The number and the area of vacuoles in the cytoplasm and nucleus were significantly different ($p < 0.05$) between shock and control groups. Moreover, serum lactate and heart rate correlated directly with the number ($r=0.634$) and the area ($r=0.624$) of cytoplasmic vacuoles as shown by multivariate analysis ($p < 0.05$). Severe hemorrhagic shock induces greater vacuolization of PMNs as compared to mild trauma. PMN vacuolization has direct correlation with serum lactate, a known marker of severe shock.

Key words: Hemorrhagic shock, trauma, lactate, inflammatory response, blood smear, neutrophils, vacuolization, apoptosis.

INTRODUCTION

Normal neutrophils are highly homogeneous cells. In a blood smear observed under light microscopy, the normal diameter of a neutrophil ranges from 12 to 15 μm . It is classically accepted that neutrophils survive for 8 to 12 h in the circulation (Gutierrez et al., 2004; Dancey et al., 1976). However, recently published data suggest that under physiologic conditions human neutrophils may remain in the circulation for up to five days (Tofts et al., 2011; Pillay et al., 2011). However, neutrophil turnover can be accelerated in inflammatory responses. The belief that neutrophils exert their function solely as pathogen killers lacks current support. Several interactions with the immune system, including macrophages, dendritic cells, cells of the adaptive immune response, and inflammatory response unrelated have been described (Mantovani et al., 2011; Amulic et al., 2012; Kolaczkowska and Kubes, 2013). Unstimulated neutrophils exhibit a smooth round cell shape with uniform cytoplasmic granularity, whereas irregular cell shape, toxic granulations, and cytoplasmic vacuolization can be observed in trauma-induced neutrophil activation (Bain, 2005). Tissue trauma induces migration and activation of neutrophils through specific mediators. Furthermore, this condition can also lead to local and systemic release of mediators capable of inducing a systemic inflammatory response syndrome (SIRS) (Bone et al., 1992; Hensler et al., 2002; Rotstein, 2003). A positive correlation between the presence of those mediators and the severity of inflammatory response has been described (Donnelly et al., 1993; Donnelly et al., 1994; Martin et al., 1997). Nevertheless, diagnostic testing to rapidly identify harbingers of SIRS is scarce. The aim of this study was to evaluate the number and size of both cytoplasmic and nuclear vacuoles in PMNs of hemorrhagic shock trauma patients, obtained from peripheral blood smears, stained with the May-Grünwald-Giemsa method, and to correlate the findings with clinical and laboratory inflammatory markers data.

MATERIALS AND METHODS

This study was approved by the Medical Ethics Committee of the Hospital Universitário Risoleta Tolentino Neves under the Protocol number 30/2011. Appropriate consent had to be signed by the patient or their next of kin prior to enrollment in the study. A preliminary analysis to determine the number of individuals to be allocated in each group was performed. Considering an expected mortality rate of 10% in severe trauma and using an alpha value of 0.05 and a beta value of 0.20, a sample size of 20 patients was determined for the study. Forty polytrauma male patients, 18 to 45 years old treated at the Hospital Universitário Risoleta Tolentino Neves (HURTN) between January 1, 2011 and June 30, 2013 were

allocated into two groups, based on the severity of trauma. Group I – control patients with mild trauma to the chest with no need for chest tube thoracostomy. Group II – patients with blunt or penetrating trauma who presented in hemorrhagic shock and a Glasgow score ≥ 14 .

Only patients brought directly to the trauma center from the scene were enrolled in the study. Inclusion criteria for patients of the group II were the presence of at least one of the following parameters: Systolic blood pressure < 90 mmHg and heart rate > 100 bpm, unresponsive to an initial fluid bolus of 1 L of crystalloid solution (0.9% sodium chloride); Massive hemothorax (≥ 1500 ml) as demonstrated by a plain x-ray or by a computed tomography scan during the initial assessment; cardiac tamponade; massive hemoperitoneum or retroperitoneal hematoma (blood in 3 or more quadrants) at abdominal ultrasound or computed tomography scan; severe pelvic fractures associated with the hemorrhagic shock; need for more than 4 L of crystalloid bolus to maintain systolic blood pressure > 90 mm Hg; need for 4 or more units of packed red blood cells in the first 6 h following the trauma. The exclusion criteria for both groups were: Patient presenting comorbidities, such as, diabetes, arterial hypertension, chronic renal, hepatic or lung failure, cardiovascular or other chronic condition; Fever or signs of infection in the first 72 h; Positive blood culture samples obtained during the first 72 h of admission to the hospital.

Procedures

Blood samples were obtained from 20 patients in each group as part of the assessment in trauma for routine laboratory tests, using 10 mL syringes, and then transferred to vials containing EDTA (Becton Dickinson). Blood smears were prepared, in duplicates, on slide glasses (Precision Glass) laid over each smear and sealed with transparent nail polish after staining with the May Grünwald-Giemsa method (Woronzoﬀ-Daskoﬀ, 2002). The first sample was obtained shortly after patient's admission followed by subsequent samples taken at 6, 12, 18, 24, 48 and 72 h as per routine assessment of trauma patients. Stained slides were stored in a slide glass box until analyzed under a light microscope. The mounted glass slides were photographed under immersion light using an ABX 35 (Olympus®) microscope with a 1000X magnification lens.

Morphometry analysis

The optical microscope images were captured with a resolution of 1392 \times 1040 pixels and transferred from a video color camera (Cool/Snap Proof Color; Media Cybernetics, Bethesda, MD, USA) to a video system attached to a computer using the program Image-Pro Express version 4.0 for Windows (Media Cybernetics, Bethesda, MD, USA). All visual analyses on image acquired using a 100 objective were performed using the freeware ImageJ 1.48. (version 1,47f, Wayne Rasband/National Institutes of Health, USA) available online from the site: <http://rsbweb.nih.gov/ij/download.html>.

Nuclear and cytoplasmic vacuoles from 100 polymorphonuclear neutrophils were counted and their areas measured in both groups using a photography camera. Images were processed with Java image processing program (Software Image J® version 1.44). The

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Table 1. Number of cytoplasmic vacuoles per neutrophil in patients with mild trauma (control) and severe hemorrhagic shock (shock).

Time (hours)	Vacuoles (control)	Vacuoles (shock)
T 0	0.033 ± 0.01	0.514 ± 0.03
T 6	0.081 ± 0.01	0.578 ± 0.03
T 12	0.081 ± 0.02	0.613 ± 0.04
T 18	0.051 ± 0.01	0.497 ± 0.04
T 24	0.047 ± 0.01	0.648 ± 0.03
T 48	0.053 ± 0.01	0.555 ± 0.03
T 72	0.076 ± 0.01	0.501 ± 0.03

T0 - Blood sample obtained on admission to the hospital. T 6 – T 72 subsequent blood sample collections, in hours, following T0. Values represent mean ± SEM of the number of vacuoles/neutrophil assessed with the May Grünwald-Giemsa method; n=20 control and 20 shock patients at each time point. A statistically significant difference was detected between the two groups in all time points ($p < 0.05$).

Table 2. Average area (μ^2) of cytoplasmic vacuoles in patients with mild trauma (control) and severe hemorrhagic shock (shock).

Time (hours)	Vacuoles (control)	Vacuoles (shock)
T 0	2.816 ± 0,46	64.018 ± 4.04
T 6	7.656 ± 0,46	60.054 ± 3.88
T 12	4.677 ± 0,69	71.699 ± 4.90
T 18	6.628 ± 1,17	58.731 ± 3.97
T 24	3.975 ± 0,61	88.538 ± 4.81
T 48	5.132 ± 0,75	55.151 ± 3.76
T 72	6.003 ± 0,83	63.056 ± 4.34

Values represent mean ± SEM of the area of cytoplasmic vacuoles assessed with the May Grünwald-Giemsa method; n=20 control and 20 shock patients at each time point. A statistically significant difference was detected between the two groups in all time points ($p < 0.05$).

remaining blood sample was used in routine laboratory examinations. Statistical analysis was performed using a statistics software (IBM SPSS statistics, Armonk, NY, USA). Mann-Whitney's U test was used to detect differences between control and the hemorrhagic shock groups. Data were expressed as Mean ± SEM of the number as well as the area (μ^2) of cytoplasmic and nuclear vacuoles/neutrophil in both groups. Spearman's rank correlation coefficient was used to detect any correlation among the variables, and multivariate linear regression analysis was performed thereafter. Statistical significant differences were set at $p < 0.05$.

RESULTS

Penetrating mechanism (gunshot wounds) was the cause of injury in 19 out of 20 patients in the hemorrhagic shock group. A single patient sustained blunt trauma secondary to motor vehicle accident. The average number of vacuoles in the PMNs of hemorrhagic shock patients (Group II) was significantly higher ($p < 0.05$) with larger vacuoles in the cytoplasm in all time points investigated (Figure1). The number and area of cytoplasmic vacuoles/

neutrophil in both control and shock groups are depicted in Tables 1 and 2, respectively.

Similarly, the average number and the area of the vacuoles in the nucleus of the PMNs of hemorrhagic shock patients were also significantly greater than in the PMNs of control group patients ($p < 0.05$) when compared with the HS group during all time points (Tables 3 and 4). More importantly however, was the direct correlation shown in multivariate linear regression analysis, between the severity of shock, assessed through serum lactate levels and heart rate, and cytoplasmic vacuolization of PMNs (Table 5).

DISCUSSION

The findings showed that severe hemorrhagic shock provokes more vacuolization of neutrophils in both the cytoplasm and the nucleus as compared to the minor trauma. Moreover, the study presented herein also

Table 3. Number of vacuoles in the nucleus per neutrophil in patients with mild trauma (control) and severe hemorrhagic shock (shock).

Time (hours)	Vacuoles (control)	Vacuoles (shock)
T 0	0.003 ± 0.001	0.032 ± 0.005
T 6	0.006 ± 0.001	0.044 ± 0.007
T 12	0.008 ± 0.001	0.075 ± 0.030
T 18	0.004 ± 0.000	0.049 ± 0.008
T 24	0.001 ± 0.001	0.078 ± 0.011
T 48	0.002 ± 0.001	0.029 ± 0.004
T 72	0.005 ± 0.002	0.091 ± 0.038

T0 - Blood sample obtained on admission to the hospital. T 6 – T 72 subsequent blood sample collections, in hours, following T0. Values represent mean ± SEM of the number of vacuoles/neutrophil assessed with the May Grünwald-Giemsa method; n=20 control and 20 shock patients at each time point. A statistically significant difference was detected between the two groups in all time points ($p < 0.05$).

Table 4. Average area (μ^2) of vacuoles in the nucleus in patients with mild trauma (control) and severe hemorrhagic shock (shock).

Time (hours)	Vacuoles (Control)	Vacuoles (Shock)
T 0	0.143 ± 0.06	2.914 ± 0.57
T 6	0.476 ± 0.21	2.003 ± 0.35
T 12	0.185 ± 0.12	1.983 ± 0.42
T 18	0.299 ± 0.11	3.209 ± 0.70
T 24	0.035 ± 0.02	5.148 ± 0.74
T 48	0.108 ± 0.06	4.242 ± 0.92
T 72	0.323 ± 0.11	4.090 ± 0.78

Values represent mean ± SEM of the area of vacuoles in the nucleus assessed with the May-Grunwald-Giemsa method; n=20 control and 20 shock patients at each time point. A statistically significant difference was detected between the two groups in all time points ($p < 0.05$).

Table 5. Multivariate linear regression analysis. Significant direct correlation between the number and area of cytoplasmic vacuoles with serum lactate and heart rate in hemorrhagic shock trauma patients.

	β value	β Coefficient	p Value	Adjusted R
Lactate (mMol/L)	18.09	0.508	<0.05	0.604
Heart rate (bpm)	0.37	0.326	<0.05	0.604

bpm = Beats per minute.

demonstrated a direct positive correlation between the severity of shock and the number and area of cytoplasmic vacuoles in the PMNs of hemorrhagic shock patients. This finding has important clinical implication, considering the current unavailability of methods to predict potential overwhelming SIRS in the setting of traumatic hemorrhagic shock.

Overwhelming SIRS triggered by severe trauma involves the activation of several mediators belonging to both humoral and cellular-mediated responses. Those

mediators are for the most part responsible for end organ damage and ultimately multiple organ failure (MOF) observed in the later stages of overwhelming SIRS (Schlag et al., 1991). PMNs have an important role in SIRS, given their activation by pro-inflammatory mediators such as $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6 , IL-8 and macrophage migration factor (MMF) (Schlag et al., 1991; Botha et al., 1995). Activation of PMNs can be detected by morphological in their resting state (Schlag et al., 1991; Fujishima and Aikawa, 1995). Accordingly, cytoplasmic

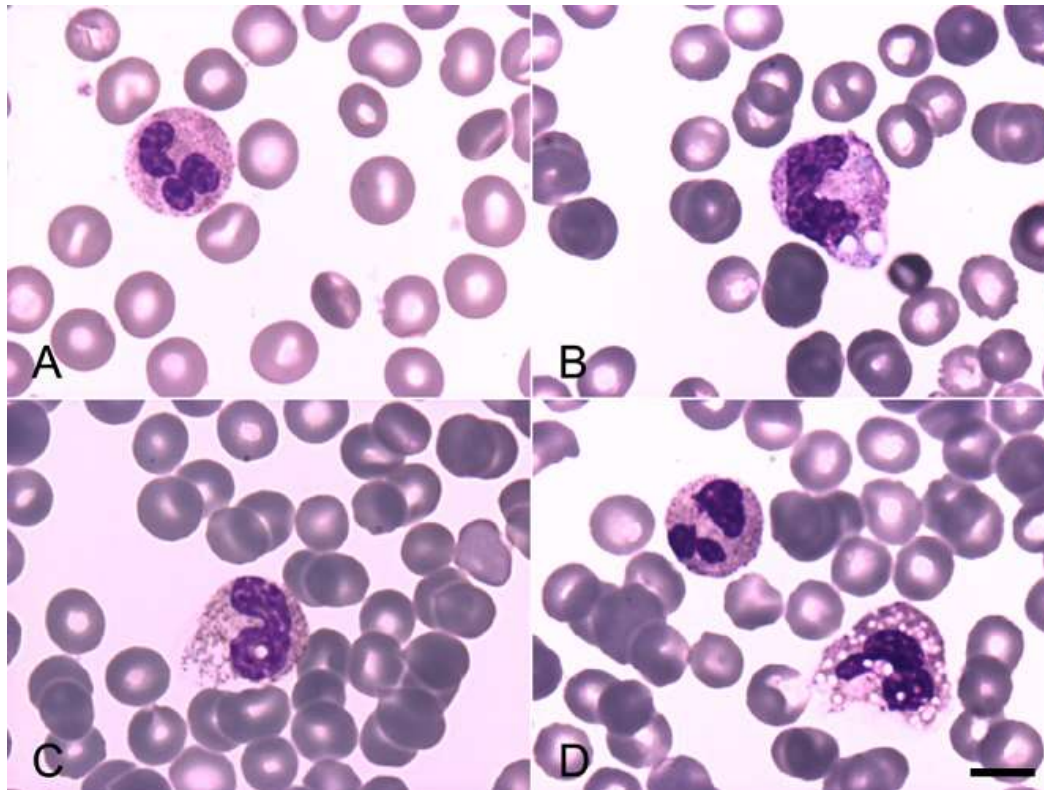


Figure 1. Microphotography of polymorphonuclear neutrophils (PMNs) on peripheral blood smears of patients who sustained mild trauma (control group) (A) and severe hemorrhagic shock trauma patients (B, C, D): A- Neutrophil with usual morphology; B, C and D- Neutrophils presenting vacuoles in the cytoplasm (B, C, D) and in the nucleus (C, D). Microphotographs show significant variation in vacuole numbers and sizes. Staining May-Grunwald-Giemsa method; Bar = 10 μm .

vacuolization is a known marker of cell degeneration and apoptosis (Fujishima and Aikawa, 1995). Previous study showed that elevated systemic release of pro-inflammatory mediators crucial to the activation of macrophages/neutrophils were detected shortly after the beginning of hemorrhagic shock (Ayala et al., 2002). Moreover, nuclear fragmentation and vacuolization have also been demonstrated in that setting and represent irreversible apoptosis (Wyllie et al., 1980). Ischemia/reperfusion and hypoxemia in septic shock patients provoke inflammatory response that leads to cytoplasmic vacuolization and lysis of cellular organelles in PMNs through a mechanism involving reactive oxygen species (Mihalache et al., 2011). Vacuoles generated in that setting seem to be the result of the fusion of endosomes containing CD44 with auto-phagosomes and secondary granules (Mihalache et al., 2011).

Considering the fact that ischemia/reperfusion process releases pro-inflammatory mediators, and excessive generation of reactive oxygen species are also present in severe hemorrhagic shock, it is hypothesized that the vacuoles observed in the PMNs of the patients described in our study could have been generated by a similar mechanism. Although, this specific hypothesis was not

investigated, it also finds support from previous investigation, wherein in vitro priming (activation) of human neutrophils with 2 μM of platelet activating factor (PAF) led to the formation of toxic granulation and cytoplasmic vacuolization similar to what was shown in the current study (Sheppard et al., 2002). Furthermore, it was recently demonstrated that PAF mediated pro-inflammatory response is in part caused by the release of reactive oxygen species (Klabunde and Anderson, 2002).

The role of apoptosis is also important to consider in this finding, given that this is the most common mechanism for neutrophil death under physiological and inflammatory conditions (Mihalache et al., 2011; Klabunde and Anderson, 2002). In both conditions, the presence of vacuoles in the cytoplasm and in the nucleus is a characteristic sign (Klabunde and Anderson, 2002). Likewise, the apoptosis pathway in PMNs also involves the generation of reactive oxygen species (Mihalache et al., 2011; Simon, 2003). Furthermore, tissue exposure to lactate can increase reactive oxygen species formation, presumably through elevations in NADH dehydrogenase produced by the lactate dehydrogenase reaction (Wolin et al., 1999). Several reports have shown that lactate clearance independently predicts mortality in trauma

patients (Odom et al., 2013). This is highly relevant to findings with respect to a prognostication potential given that hemorrhagic shock increased serum lactate levels and a positive correlation was found between lactate and neutrophil vacuolization. This observation definitely deserves further investigation.

This study has several limitations, most importantly was the lack of information relative to the outcome of the patients, particularly in the hemorrhagic shock group. Moreover, the mechanisms involved in the vacuolization of PMN's were not ascertained. Nonetheless, these findings provide an intriguing proposal for the potential use of a simple laboratory staining technique (May-Grunwald-Giemsa method) to investigate the inflammatory status of hemorrhagic shock patients in trauma as demonstrated by the increase in serum levels of lactate, as well as by the heart rate increase when the number and size of cytoplasmic vacuoles went up.

Conclusion

Peripheral blood smears of patients in severe hemorrhagic shock, stained with the May-Grunwald-Giemsa method, showed more neutrophil vacuolization as compared to samples from patients victims of minor trauma. Moreover, the number and area of cytoplasmic vacuoles had a direct positive correlation with the inflammatory clinical and laboratory markers, heart rate serum and lactate levels in severe hemorrhagic shock patients.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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ABBREVIATIONS

HS, Hemorrhagic shock; **PMN**, polymorphonuclear neutrophil; **bpm**, beats per minute.

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