JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2001, 52, 2, 293–301

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THE EVALUATION OF MURINE PLEURAL LAVAGE FLUID CELLULAR COMPOSITION IN EXPERIMENTAL HEMORRHAGIC SHOCK WITH SPECIAL REGARD TO MAST CELLS MORPHOMETRY

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In the course of a hemorrhagic shock, pathological changes occur, which result in intensifying the insufficiency of various vital organs. It can also lead to the development of the multiorgan dysfunction syndrome (MODS) that is the cause of high posthemorrhagic mortality. As a result of the ischemia in the lung there appear proinflammatory factors that mobilize and activate mast cells, inducing degranulation in them. The aim of the study was the analysis of cellular composition and cytomorphometric evaluation of mast cells present in the lavage fluid from the pleural cavity of rats in a sham operated group and in the group presenting hemorrhagic shock. The results revealed an increase of the total cell count in the lavage fluid from the pleural cavity. In the cytological smears a statistically significant accumulation of inflammatory cells was present, especially neutrophils. The increase in mast cells and eosinophils number was not statistically significant. There was not a change in the morphometric parameters of mast cells except the circularity index. A decline of the circularity index indirectly may suggest the degranulation of mast cells, which reflects an inflammatory process in the lungs.

Key words: *hemorrhagic shock, lungs, mast cells, morphometry, pleural cavity.*

INTRODUCTION

Hemorrhagic shock causes both neuroendocrine and inflammatory responses. The most important aim of the neuroendocrine response to bleeding is the maintenance of heart and brain perfusion even at the cost of other organs. The decrease in tissue oxygenation to a level not sufficient for normal metabolism and functioning can lead to microcirculation damage and to increased capillary permeability. If the bleeding is prolonged or its intensity is high, cellular dysfunction and compensatory mechanism fail. Non-treated shock inevitably leads to the mobilization of the proinflammatory mediator

cascade with the rapid progression of cardiovascular disturbances and lethal injury to other organs (1).

In the pathophysiology of injury in shock, the role of various humoral and cellular mediators was confirmed. They have both pathogenic and prognostic importance, like: the increase in protein and cytokine level in bronchoalveolar lavage fluid, the decrease in surfactant concentration, the increase in serum protease activity, the activation of the complement system, the increase of platelet aggregating factor (PAF) and arachidonic acid metabolite concentration, the activation of the coagulation system, and overproduction in toxic oxygen radicals (2).

The disturbances of microcirculation with subsequent increased vascular permeability and parenchymal edema play a crucial role in the mechanism initiating lung injury (3). Mast cells (MC) participate in this phenomenon. They occur most frequently at sites of permanent contact with the external environment, i.e. the skin, the urinary system, the alimentary tract, air tract and the lungs as they participate in immunological response and inflammatory reaction (4).

Mast cells activated due to ischemia and reperfusion are the source of various mediators like thromboxans, PAF, histamine, prostaglandins and leukotriens that affect smooth muscle contraction, the increase in vascular permeability, and neutrophil chemotaxis. The accumulation, overactivation and degranulation of leukocytes are the essence of the lung failure. These leukocytes release many toxic mediators, particularly proteases, such as elastase, collagenase, B gelatinase that directly lead to lung injury (5, 6, 7).

MATERIAL AND METHODS

The examinations were conducted on 24 young female Wistar rats divided in two groups ($n = 12$), body mass of 180—200 g (mean 190 ± 10 g).

Procedures involving the animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and Guide-lines for the Use of Animals in Biomedical Research (Thromb Haemost 1987; 58: 1078—84). Twelve hours before the experiment, the rats were not fed but they had free access to drinking water.

The experiment was carried out in a closed room at room temperature. The animals were placed on a plastic base and heated with a lamp. The rats were anesthetized with ethyl ether, by placing them in an atmosphere of ether mixed with air. Sterile instruments were used for surgical manipulations. After putting the animals on their backs and fixing their extremities, a tracheotomy was performed to keep air ducts patent. Ethyl ether was given by a vaporizer by the open method straight to the tracheotomy tube.

After exposing the cervical vessels, the left carotid artery was cannulated by a plastic cannula (0.6 mm i.d.) introduced 1 cm into the artery. The time required for the surgical manipulation was about 10 min. After 5 minutes necessary for circulatory stabilization, the measurement of systemic arterial blood pressure was initiated using a Statham P23 transducer, preamplifier, and recorder (Gould, USA).

The experimental animals were divided into two groups:

I — Sham operated (SO), which underwent anaesthesia, tracheotomy, and cannulation of the carotid artery.

II — Shocked (HS), where the initiating procedure was conducted as pointed above, and afterwards the hemorrhagic shock was evoked by the withdrawal of 25% of the circulating blood from the carotid artery during 3 min, which led to the decrease in arterial blood pressure to the mean value of 35 mmHg.

The volume of shed blood was counted according to the formula: $v = \text{body mass} \times 0.02$. After material harvesting, the rats were killed by decapitation.

Collection and fixing of the material

After 75 minutes of experiment, the abdominal cavity was opened under aseptic conditions by a medline cut in order to expose the right costophrenic angle. Pleural cavity was punctured with a 0.6 mm i.d. plastic cannula, and a volume of 7 ml of 0.15 M NaCl was given in order to perform a pleural lavage, carefully enough not to contaminate with blood. The fluid contaminated with blood was excluded from the study. An amount of 5—6 ml of fluid was obtained by washing the pleura, then a sample of 0.5 ml was centrifuged in cytospine at a speed of $250 \times g$ at temperature of 4°C for 10 min.

Cell pellets were suspended with 0.15 M NaCl up to 10^6 cells/ml. Their total number was determined in a Thom-Zeiss chamber.

Material preparation to cytological examination

Cytological smears were stained with H+E and Giemsa methods as well as alcian blue and safranin according to the Csaba's method to determine mast cells (*Fig. 1*). The percentages of cellular subpopulations were evaluated by analyzing 1000 cells in each smear. The cellular identification and measurements were provided in light microscope (Olympus), using $\times 400$ magnification simultaneously by two independent observers. Each pair of results was averaged.

Morphometric examinations

Mast cells obtained from the pleural cavity lavage and stained with Giemsa method underwent morphometric analysis using the computer program Lucia G (Nikon).

The following features of mast cells were analyzed: area in μm^2, equalized diameter in μm (Eqdia), which is the diameter of a circle of the same surface as the examined object, length in μm (the longest dimension of the object), width in μm (the biggest transverse dimension), circularity index (the feature determining the shape of the object, there is the ratio of the shortest to the longest object diameter when it is approximated to an ellipse. This ratio equals 1 for the circle while for all the other shapes it is less than 1), elongation index (the feature showing the shape of the object, it is the relation of the maximal Feret diameter to the minimal Feret diameter).

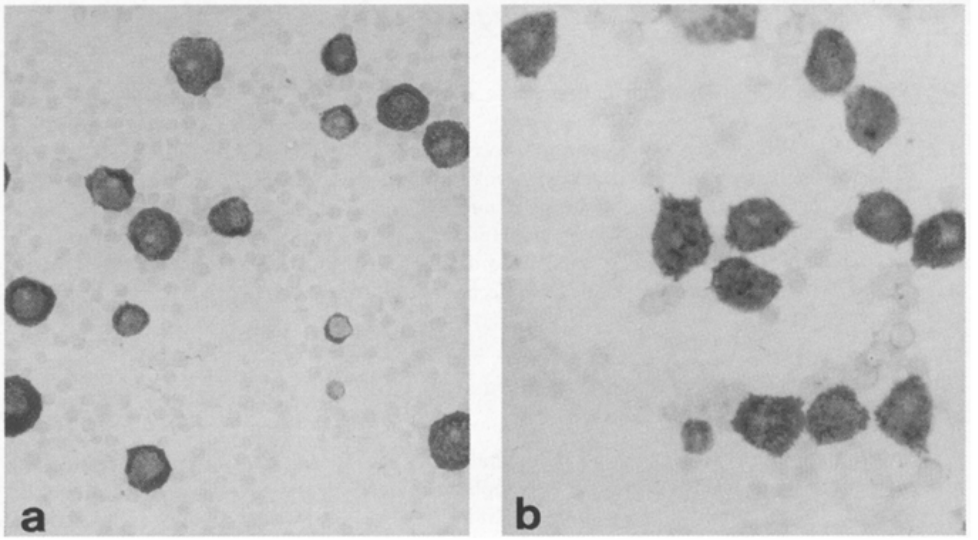

Fig. 1. Smears made from pleural lavage fluid and stained with Csaba's method.
a. Mast cells of sham operated rats. The mast cells are packed with overlapping bicolored granules (blue and red) (magn. × 400).
b. The pleural mast cells of shocked rats. An elongation of MC comparing to sham operated can be observed (magn. × 600).

Statistical analysis

The results were analyzed statistically with the calculation sheet of Microsoft Excel (Microsoft) as well as a statistical pack Statgraphics plus (Statistical Graphics Corp.).

None of the variables fitted the normal distribution, so a non-parametric test Mann-Whithey was used in the evaluation of the numerical data presented in the form of mean $\pm$ standard deviation ($\pm$SD). The level of statistical significance was $p < 0.05$.

RESULTS

Figure 1 presents the mast cells lavaged from pleural cavity and stained with Csaba's method. The granules with preponderance of biogenic amins dyed dark blue and those with preponderance of glycosaminoglicans dyed red.

The total number of cells washed from the pleural cavity in the sham operated group was $5.24 \pm 0.89 \times 10^6 \pm$SD, and after 75 min of hemorrhagic shock $7.56 \pm 0.68 \times 10^6 \pm$SD.

Hemorrhagic shock lasting for 75 min caused a statistically significant increase in the total number of cells isolated from the pleural cavity in comparison to the sham operated group ($p < 0.007$).

The cellular composition, as well as similarities and differences between the groups are shown in *Fig. 2* and *Fig. 3*. The separate changes of mast cells number in the pleural fluid are displayed on the *Fig. 4*.

group	mast cells (mean ± SD)	eosinophils (mean ± SD)	neutrophils (mean ± SD)	monocuclear cells (mean ± SD)
group I (sham operated)	7.23 ± 2.27	0.83 ± 0.55	14.06 ± 3.85	82.78 ± 3.89
group II (shock)	9.47 ± 4.14	1.41 ± 1.46	38.33 ± 5.79	60.26 ± 6.87

Fig. 2. The cellular percentages (%) in the pleural lavage fluid in the sham operated group and in the hemorrhagic shock lasting 75 minutes.

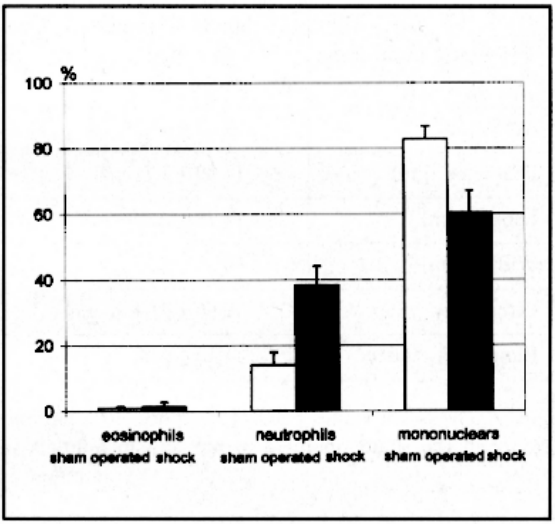

Fig. 3. Cellular percentages (%) in the pleural lavage fluid in the sham operated rats and in the hemorrhagic shock lasting 75 minutes.

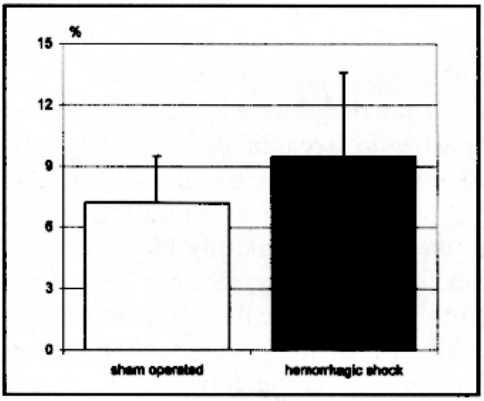

Fig. 4. The mast cell percentages (%) in the pleural lavage fluid in the sham operated animals and in the hemorrhagic shock lasting 75 minutes.

The cellular composition of the pleural lavage fluid in examined groups is displayed in *Fig. 2.* In both the sham operated and shocked rats the mononuclear cells (lymphocytes, macrophages, monocytes) dominated. They represented 82.78% and 60.26%, respectively. However, in the sham operated group, neutrophils represented 14% of the total cell number, but in the shocked group the neutrophils were rising to more then 38%. The statistical analysis showed significant differences in these examined groups. A statistically non-significant increase in eosinophils (0.83% to 1.41%) and mast cell numbers (7.23% to 9.47%) was also observed in the HS group in comparison to the SO.

The numerical results of pleural mast cell morphometric evaluation are shown in *Fig. 5.*

The analysis did not reveal any statistical significance for differences between sham operated and shock groups, except the circularity index.

Examined parameter	Group I — sham operated (mean ± SD)	Group II — shock 75 min (mean ± SD)	Statistical significance
Area (μm^2)	178.67 ± 61.39	176.29 ± 77.20	none
Diameter (μm)	14.89 ± 2.71	14.69 ± 3.36	none
Lengh (μm)	11.98 ± 2.30	12.07 ± 3.22	none
Width (μm)	14.56 ± 2.53	14.14 ± 3.18	none
Circularity index	0.96 ± 0.04	0.94 ± 0.06	$p < 0.05$
Elongation index	1.16 ± 0.08	1.17 ± 0.17	none

Fig. 5. The mean values ± SD of morphometric parameters of mast cells washed from the pleural cavity in the sham operated group and in animals subjected to hemorrhagic shock lasting 75 minutes.

DISCUSSION

In the experiments we conducted, the hemorrhagic shock caused a marked mobilization of free pleural cells with particular accumulation of neutrophil granulocytes. It also resulted in the increase in the percentages of mast cells and eosinophils. This reaction reflects a rapid recruitment of MC into the pleural cavity in response to a stimulus, that is the loss of circulating blood volume. However, the percentage of mononuclear cells was surprisingly low in the cytological smears of the lavage fluid from the pleural cavity in hemorrhagic shock. This could be effect of delayed kinetics of the cellular inflammatory response due to remarkably impaired peripheral blood flow.

In hemorrhagic shock, there is a shift of circulating blood volume to the most vital organs like heart and brain, which is known as circulatory centralization. It is also known, that the respiratory system is one of the most vulnerable effector in the inflammatory reaction to the shock.

Hypovolemia promotes the synthesis and release of many humoral mediators thus leading to clinical signs of shock. Histopathological indicators of decreased in vascular flow are the blood vessel widening and increase in their permeability, and inflammatory exudate. It can which was observed in the present experiment. The recruitment and activation of neutrophils can be initiated by degranulation of mast cells, which are significantly engaged in the inflammatory process (4, 8—13). The interactions between proinflammatory cells and endothelium play a crucial role in the mechanism of cell inflow and activation. The interactions are regulated by endothelial adhesion molecules: P- and E-selectins and intercellular adhesion molecules ICAM-1 and VCAM-1. They connect with cell receptors on the surface of the leukocytes: LFA-1 (leukocyte functional antigen-1) and VLA-1 (very late antigen-1). Mast cells present such receptors on their surface, which confirms their active participation in migration and accumulation at the inflammation site, as reported by Baghestanian *et al.* (14) and Palecanda *et al.* (15).

On the other hand, mast cell migration in the course of hemorrhagic shock has not been evidenced yet. However, examinations of the alimentary tract conducted so far, on the same experimental model, confirm the hypothesis of mast cell participation in the reaction to the hemorrhagic shock (16, 17). The authors cited above, observed an increaae in mast cell numbers in the peritoneal cavity in hemorrhagic shock, and explained the results by MC migration from the gastric and intestine walls into the peritoneal cavity (18, 19).

In our experiment, we observed a statistically non-significant increase in mast cell number in the pleural fluid in hemorrhagic shock. Although we did not observe significant changes of mast cell morphometric parameters, except the decrease in the circularity index, the results may suggest the degranulation of mast cells in the pleural cavity in HS.

A similar hypothesis concerning active participation of MC in the intestine reaction to the ischemia/reperfusion was proposed by Kanwar S *et al.* (20). Also the other authors, using the mast cell stabilizers (Lodoxamide Tromethamine or Nedocromil sodium) confirmed a reduction in organ injury, including lung, after inhibiting degranulation of mast cells in the course of reaction to ischemia/reperfusion (21—23).

There are also other authors working on animal and human models, who confirm a significant increase in the total number of cells washed from the bronchial tree. That occurs in relation to the presence and increased activity of mast cells, and their proinflammatory mediators in lung interstitial diseases (24—27) or to the response to injury factors, e.g. ozone (28, 29). According to

these authors, mast cell mediators have a direct influence on the increase in endothelial permeability, the contraction of bronchial smooth muscles, and also on increasing in the number of inflammatory cells and production of their mediators.

The results suggest that mast cells may participate in the pathogenic process of the acute lung injury in the hemorrhagic shock by means of their mobilization and activation.

Acknowledgements: We thank Dr Eric Pluygers for the kind reviewing the manuscript.

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Received: November 2000
Accepted: April 5, 2001

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