

Original Article

Neutrophilic infiltration in lungs of mice with peritonitis in acid or basic medium

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Abstract: Background: Bacterial peritonitis is associated with systemic complications such as pneumonia. Objective: To determine in an experimental model of peritonitis whether the pH of peritoneal fluid infection influences the influx of neutrophils into the lung, and whether treatment outcome would be similar in peritonitis with liquid at any pH. Materials and methods: We studied 48 mice with peritonitis induced by cecal ligation and puncture. The animals were distributed randomly into three groups: the first one had an injection into the peritoneal cavity with saline, pH 7.0; the second group was injected with saline, pH 8.0; and the third group with saline, pH 3.0. After 2 hours, half the animals in each group was treated by washing the abdominal cavity with warm saline solution and administration of ceftriaxone every 12 hours, and half of each group was killed by anesthetic overdose, and lung biopsy was done. The animals kept in treatment were killed 24 hours after treatment, and lung biopsy was also performed. The samples were stained with H&E and the number of neutrophils in 20 areas was checked. The mean number of cells in each group was compared between groups and with an untreated one. Results: The group with peritonitis associated with alkaline solution showed a higher population of neutrophils during untreated peritonitis ($P = 0.04$). The response to treatment by lavage of the peritoneal cavity and antibiotics was more effective in reducing the population of neutrophils in the group with peritonitis at pH 8.0, unlike that observed in animals with peritonitis at pH 3.0 ($P = 0.03$). Conclusion: Peritonitis associated with lower pH solution, despite the lower influx of leukocytes in the first two hours after installation of peritonitis, was not able to reduce the population of these cells in mice's lung in response to standard therapy.

Keywords: Peritonitis, neutrophils, lung

Introduction

The secondary bacterial peritonitis is an important cause of sepsis and death in surgical practice [1, 2]. The pathophysiology of this disease involves the activation of local and systemic inflammatory mechanisms in presence of intra-abdominal infectious focus. Cellular and humoral components, as well as cytokines and acute phase proteins, act together in order to contain the infection [3]. The exacerbation of these inflammatory responses, with activation of cellular elements as macrophages and mast cells, and systemic release of reactive products of oxygen and cellular mediators of the inflammation compromise the economy of the organism, leading it to death. Due to that exacerbated inflammatory response, the mortality is high

[4, 5]. In clinical practice, peritonitis resulting from perforation of chloridropeptic ulcers has been found to have a more deleterious course than peritonitis caused by more alkaline fluids such as bile, irrespective of bacterial content [6, 7]. A possible explanation is that acidity may induce the dysfunction of peritoneal macrophages, while also permitting the spread of bacteria due to increased permeability of intra-abdominal vessels.

Among the complications of the peritonitis, failure of terminal organs, like lungs, occurs in about 74% of the patients, and is one of the principal death causes in patients with peritonitis [8]. As the lung is the distant organ more frequently affected in bacterial peritonitis, pneumonia occurs frequently in these patients [9,

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10], and several studies have been done with the objective of determining the causes of lung alterations observed in abdominal sepsis. Some authors suggest that inflammatory diseases, as the peritonitis, can cause increase of the oxidative activity in the lung, resulting in peroxidation of the membrane (10), and that, in peritonitis, there are persistent migration of neutrophils to the lung mediated by action of cytokines secreted by activated macrophages, with consequent alveolocapillar lesion and liberation of oxygen free radicals [11, 12]. The clinical result of that peroxidation is edema, which may be due to an increase in microvascular permeability [13].

It is important to control the infectious focus in the handling of the peritoneal cavity as well as the mechanical removal of the gross contamination. The lavage of peritoneal cavity is made in order to increase the index of mechanical removal of particles as fibrin and septic clots and, despite largely used, there is not a consensus. It was demonstrated that the vasodilatation caused by the warm saline solution would promote cellular damage, increase the inflammatory response, and increase macrophage activation in the lesion area and cause loss of macrophage functional capacity. It is also associated with increase of absorption of bacteria by omentum. However, there was observed survival decreased of untreated rats with peritonitis, and infusion of saline into the peritoneal cavity was associated with reduced accumulation of neutrophils in the lung probably due to reduction of lung inflammatory response [14-16].

Assuming that the systemic inflammatory response may show differences in peritonitis associated with solutions at different pH, the response to treatment may also show differences according to the pH of the liquid in the peritoneal cavity. Therefore, we studied the influx of neutrophils into the lung of mice with induced peritonitis associated with liquid at acid, neutral or alkaline pH.

Material and methods

The experiment was conducted in accordance with Ethical Principles in Animal Experimentation and approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA/UFMG) under

protocol number 27/2009. Male Swiss mice were divided into three groups: Control (n = 16), cecal ligation and puncture, abdominal closure and intraperitoneal injection of pH 7.0 saline solution; Alkaline (n = 16), cecal ligation and puncture, abdominal closure and intraperitoneal injection of pH 8.0 saline solution; Acid (n = 16), cecal ligation and puncture, abdominal closure and intraperitoneal injection of pH 3.0 saline solution.

After 2 hours, half the animals from each group were killed by anesthetic overdose and lung samples were taken for histology. The other half of the animals of each group was anesthetized again. After that, relaparotomy, aspiration of the peritoneal liquid, lavage with warm (37.5°C) saline solution - three times -, and closure of the abdominal wall were performed. The animals also received a dose of ceftriaxone and analgesic, were placed into boxes and observed for 24 hours. Twelve hours after the first dose of ceftriaxone, another dosage was administered, and the animals received food and water ad libitum.

After 24 hours, the animals were killed by anesthetic overdose, withdrawal lung biopsy that was fixed in formaldehyde 10%, and forwarded to morphometric analysis. Lung fragments were dehydrated, diaphanized, and embedded in paraffin. Four-micrometer-thick tissue sections were stained with hematoxylin and eosin (H&E).

Lung parenquima of each animal was examined through 40X objective and digitized using a JVC TK-1270/RGB micro camera (Tokyo, Japan) for the achievement of 10 randomly images of each plane (upper, middle, and lower). An automatic macro recorder assembler of KS300 software (Carl Zeiss, Oberkochen, Germany) was elaborated to image processing and segmentation, definition of morphometrical conditions and counts of all the nucleus of the cells contained in each image [17]. Segmentation permitted the separation of these nuclei from the cell cytoplasm and from other structures in the section, enabling the creation of a binary image containing these two locations, nucleus and other spaces. The nucleus from the cellular types usually found in the lung e and newly recruited leukocytes were

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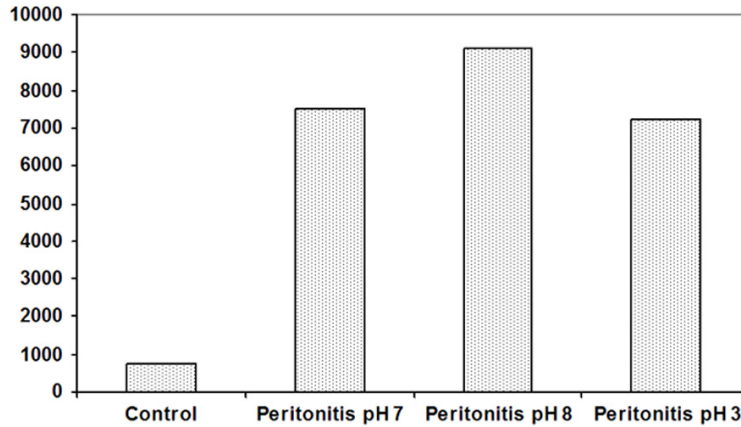


Figure 1. The leukocyte count of the negative control animals (without handling) was 765.3 ± 186.6 nuclei in 30 random areas. In the group with peritonitis and peritoneal fluid at pH 7.0, the average was 8213.7 ± 722.8 , with a significant difference when compared with the control group ($P = 0.04$). In the group with peritonitis and pH 3.0, the average was 9092.0 ± 603.9 , and in mice with peritonitis and pH 8.0, the average was 8060.8 ± 730.9 . Both groups showed significant difference compared to the control group ($P < 0.001$). There was a greater influx of neutrophils into the lungs of animals with peritonitis at pH 8.0 when compared to those of the group with peritonitis and pH 3.0 ($P = 0.04$), but not to those with peritonitis and pH 7.0.

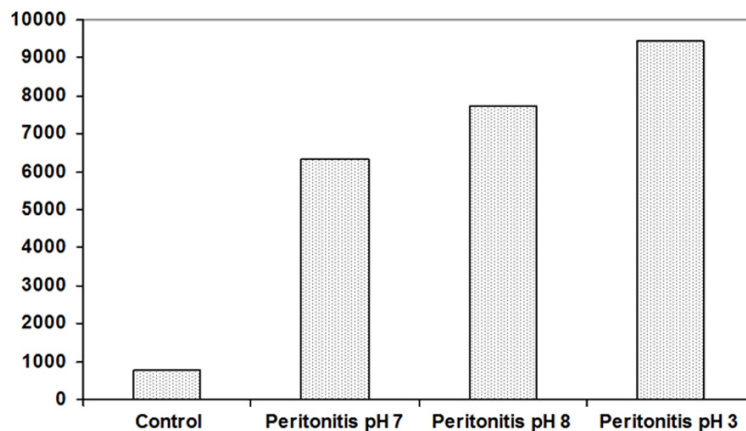


Figure 2. After 24 hours of treatment, the neutrophil count in the peritonitis group at pH 7.0 was 6342.1 ± 798.6 ; in the experimental group at pH 8.0 it was 7735.7 ± 1031.6 ; and in the experimental group at pH 3.0 it was 9464.3 ± 745.6 . In all groups there was a significant increase in neutrophil counts when compared to the control group ($P < 0.001$). We also observed a large influx of leukocytes in the group with peritonitis and pH 3.0 when compared to animals with peritonitis and pH 7.0 ($P = 0.01$).

then counted. The count obtained in the images from control animals was considered as a pattern of normal cellularity (without inflammatory infiltrate) of the lung.

The average number of cellular nucleus found in the lungs in each group was compared

between groups by Student's t test, considering differences significant at $P < 0.05$.

Results

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Response to treatment with saline lavage and systemic anti-microbials was more effective in reducing the neutrophils influx into the lung in the peritonitis group at pH 7.0 and pH 8.0. In

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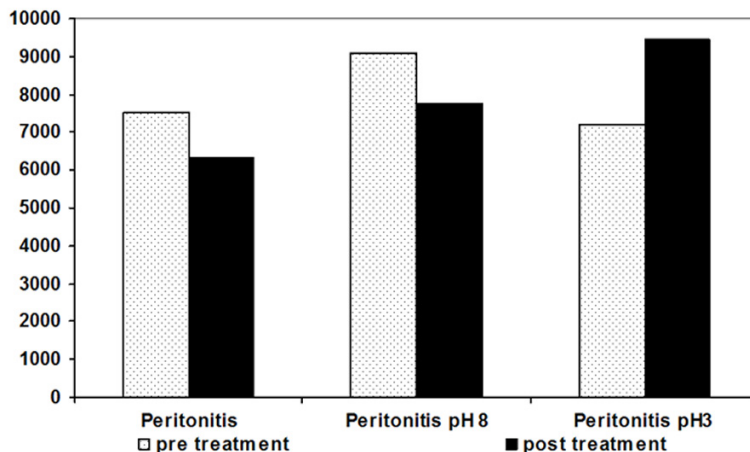


Figure 3. Response to treatment with saline lavage and systemic anti-microbiotics was more effective in reducing the neutrophils influx into the lung in the peritonitis group at pH 7.0 and pH 8.0. In animals with peritonitis at pH 3.0 influx of neutrophils into the lung was increased when pre and post treatment periods were compared, $P = 0.03$.

animals with peritonitis at pH 3.0 influx of neutrophils into the lung was increased when pre and post treatment periods were compared, $P = 0.03$ (Figure 3).

Discussion

To study the changes and response to treatment in secondary peritonitis, experimental models are used to allow good correlation with events that occur in humans. The experiments performed by Wichterman and cols [18] represented a milestone in the use of experimental models for studying the pathophysiology of infection. These authors not only emphasized the importance of rupturing the integrity of the intestinal wall in the induction of peritonitis, but also demonstrated the presence of bacteria in the peripheral blood of the animals and the initiation of sepsis within 10 h of the surgical procedure. Additionally, the evolution of the infection, leading to eventual death, could be followed through the manifestation of specific clinical signs that were used to classify eleven stages of sepsis. Some researchers have concluded that the intensity of sepsis could be modulated by the calibre of the needle used in the perforation of the cecal wall. Using needles of different caliber, Ebong and co-workers [19] demonstrated that it was possible to predict the intensity of the local inflammatory and systemic response by standardizing the size of the needle. Results of previous study showing that the use of scissors produced more intense sys-

temic inflammation, similar to the severity of sepsis seen in clinical practice [20].

Peritonitis is associated with pulmonary affections such as pneumonia and shock lung [9, 10, 21]. Approximately 74% of patients with peritonitis have respiratory insufficiency caused by pulmonary inflammation [22, 23]. This inflammation is characterized by thickening of alveolar walls, areas of atelectasis and inflammatory cells from vascular bed to the interstitium. Is not well determined as peritoneal infections cause lung damage, but is possible that high levels of circulating cyto-

kines released by immune cells from the peritoneal cavity organize local capture of neutrophils from peripheral blood by creating a chemotactic gradient that favors the capture of neutrophils through the lung, with involvement of macrophages in the process [17, 24, 25].

This study showed that peritonitis caused leukocyte infiltration in the lung, and in cases where the content was associated with alkaline peritoneal fecal contamination this influx was higher. In a previous study, we observed that in peritonitis associated with acid content the presence of bacteria in the lung was significantly higher than in animals with peritonitis in an alkaline medium. It is possible that, at first, the presence of a larger population of bacteria in the lungs of animals with peritonitis with acid content did not correlate with the greater influx of neutrophils into the area by changes in the release of inflammatory cytokines such as $\text{TNF}\alpha$ and IL-6, or changes the response of lung macrophages.

It is attributed to the neutrophils the ability to damage tissue, leading to organ failure and systemic inflammatory syndrome [4]. Some studies have also suggested that the presence of neutrophils in lung cause an increase in the oxidant activity resulting in lipid peroxidation of the membrane [26]. The clinical result of this peroxidation is edema that can also appear as a result of an increase in the microvascular per-

meability of the lung [13] and persistent neutrophil adhesion [12, 27]. This PMN influx appears to be caused by bacterial substances other than endotoxins [11].

Little has been reported in the literature about changes in the influx of neutrophils in the lung in response to therapy of abdominal sepsis. It has been shown that treatment of secondary peritonitis with washing of the cavity was associated with increased survival, attenuation of the pulmonary inflammatory response and reduction in the accumulation of leukocytes in the lung [14]. Our results showed that the standard treatment with lavage of the peritoneal cavity and broad-spectrum antimicrobial was effective in reducing the influx of leukocytes to the lung after 24 hours of treatment, except when peritonitis occurred in the presence of intracavitary fluid at pH 3, indicating that peritonitis associated with the presence of liquid acid has a lower response to therapy.

We conclude that the presence liquid than the neutral pH was associated with a greater influx of leukocytes into the lungs of rats with peritonitis. However, the response to treatment was less effective in reducing the factors responsible for leukocyte influx into the lung in animals which was associated with the acid content, suggesting that therapeutic measures were less effective in reducing inflammation in this group.

Disclosure of conflict of interest

None.

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References

- [1] Thorsen K, Søreide JA, Søreide K. Scoring systems for outcome prediction in patients with perforated peptic ulcer. *Scand J Trauma Resusc Emerg Med* 2013; 21: 25.
- [2] Samuel JC, Qureshi JS, Mulima G, Shores CG, Cairns BA, Charles AG. An Observational Study of the etiology, clinical presentation and outcomes associated with peritonitis in Lilongwe, Malawi. *World J Emerg Surg* 2011; 6: 37.
- [3] Riché F, Gayat E, Collet C, Matéo J, Laisné MJ, Launay JM, Valleur P, Payen D, Cholley BP. Local and systemic innate immune response to secondary human peritonitis. *Crit Care* 2013; 17: R201.
- [4] Shih CC, Chen SJ, Chen A, Wu JY, Liaw WJ, Wu CC. Therapeutic effects of hypertonic saline on peritonitis-induced septic shock with multiple organ dysfunction syndrome in rats. *Crit Care Med* 2008; 36: 1864-1872.
- [5] Delibegovic S. Pathophysiological changes in peritonitis. *Med Arch* 2007; 61: 109-13.
- [6] Agarwal N, Saha S, Srivastava A, Chumber S, Dhar A, Garg S. Peritonitis: 10 years' experience in a single surgical unit. *Trop Gastroenterol* 2007; 28: 117-20.
- [7] Duval-Araujo I, Sarmiento MA, Grossi GC, Simal CJ, Nascimento VC, Diniz SO. Pulmonary phagocyte depression induced by an acid or alkaline solution in a rat model of peritonitis. *Surg Infect (Larchmt)* 2007; 8: 599-604.
- [8] Rosman C, Westerveld GJ, Kooi K, Bleichrodt RP. Local treatment of generalized peritonitis in rats. Effects on bacteria, endotoxin and mortality. *Eur J Surg* 1999; 165: 1072-1079.
- [9] Kumar OS, Rao CS. Prognosis in intra-abdominal sepsis. *Indian J Gastroenterol* 1995; 14: 8-10.
- [10] Masson F, Champault G. The lung in peritonitis. Contribution of data processing to the evaluation of pulmonary risk: practical conclusions. *J Chir* 1984; 121: 229-236.
- [11] Mercer-Jones MA, Heinzelmann M, Peyton JC, Wickel DJ, Cook M, Cheadle WG. The pulmonary inflammatory response to experimental fecal peritonitis: relative roles of tumor necrosis factor-alpha and endotoxin. *Inflammation* 1997; 21: 401-417.
- [12] Fukatsu K, Saito H, Han I, Inoue T, Furukawa S, Matsuda T, Ikeda S, Yasuhara H, Muto T. Concomitant increase in neutrophil adhesion to inflammatory peritoneum and remote organs during peritonitis. *J Surg Res* 1999; 81: 156-163.
- [13] Judges D, Sharkey P, Cheung H, Craig I, Driedger AA, Sibbald WJ, Finley R. Pulmonary microvascular fluid flux in a large animal model of sepsis: evidence for increase pulmonary endothelial permeability accompanying surgically induced peritonitis in sheep. *Surgery* 1986; 99: 222-234.
- [14] van Till JW, Lamme B, van Esch TJ, van der Poll T, van Gulik TM, Boermeester MA. Surgical therapy attenuates abdominal and extra-abdominal inflammation in experimental peritonitis. *Eur Surg Res* 2006; 38: 76-82.
- [15] van Goor H. Surgical treatment of severe intra-abdominal infection. *Hepatogastroenterology* 1997; 44: 975-81.
- [16] Schein M, Saadia R, Decker G. Intraoperative peritoneal lavage. *Surg Gynecol Obstet* 1988; 166: 187-95.

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- [17] Rodrigues-Machado MG, Silva GC, Pinheiro MB, Caliari MV, Borges EL. Effects of sepsis-induced acute lung injury on glycogen content in different tissues. *Exp Lung Res* 2010; 36: 302-306.
- [18] Wichterman KA, Baue AE, Chaudry IH. Sepsis and septic shock-a review of laboratory models and a proposal. *J Surg Res* 1980; 29: 189-201.
- [19] Ebong S, Call D, Nemzek J, Bolgos G, Newcomb D, Remick D. Immunopathologic alterations in murine models of sepsis of increasing severity. *Infect Immun* 1999; 67: 6603-10.
- [20] Bicalho PR, Lima LB, Alvarenga DG, Duval-Araujo I, Nunes TA, Dos Reis FA. Clinical and histological responses to laparoscopically-induced peritonitis in rats. *Acta Cir Bras* 2008; 23: 456-61.
- [21] Von Wichert P, Wieggers U, Stephan W, Huck A, Eckert P, Riesner K. Altered metabolism of phospholipids in the lung of rats with peritonitis. *Anesteziol Reanimatol* 1978; 172: 223-229.
- [22] Vincent JL, Puri VK, Carlson RW. Acute respiratory failure in patients with generalized peritonitis. *Resuscitation* 1983; 10: 283-290.
- [23] Stamme C, Bundschuh DS, Hartung T. Temporal sequence of pulmonary and splenic inflammatory responses to graded polymicrobial peritonitis in mice. *Infect Immun* 1999; 67: 5642-5650.
- [24] Wengner AM, Pitchford SC, Furze RC, Rankin SM. The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation. *Blood* 2008; 111: 42-49.
- [25] Wang Z, Rui T, Yang M, Valiyeva F, Kvietys PR. Alveolar macrophage from septic mice promote polymorphonuclear leukocyte transendothelial migration via an endothelial cell Src Kinase/NADPH oxidase pathway. *J Immunol* 2008; 181: 8735-8744.
- [26] Demling R, Lalonde C, Ikegami K. Alpha-tocopherol attenuates lung edema and lipid peroxidation caused by zimozan-induced peritonitis. *Surgery* 1995; 117: 226-231.
- [27] Peralta JG, Barnaard ML, Turrens JF. Characteristics of neutrophil influx in rat lungs following fecal peritonitis. *Inflammation* 1993; 17: 263-271.