Peritoneal resorption capacity for the inflammatory mediators in acute experimental *Staphylococcus aureus* peritonitis

Akut deneySEL *Staphylococcus aureus* peritonitinDE enflamatuvar mediyatörlerE yönelik peritoneal rezorpsiyon kapasitesi

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**BACKGROUND**

Elevations in both endotoxin and interleukin-6 (IL-6) concentrations in peritoneal exudates are a thousand times higher than their respective concentrations in the peripheral blood in patients with gram-positive or gram-negative peritonitis. We aimed in this study to evaluate the resorption capacity of the peritoneum for endotoxin and IL-6 in a model of bacterial (gram-positive) peritonitis.

**METHODS**

Intraperitoneal (i.p.) injection of mucin-pretreated staphylococci in phosphate buffered saline (PBS) or of PBS alone was performed in 93 male Wistar rats. Studies of resorption were undertaken at time points of 4 hours (h), 8h, 12h and 24h. Endotoxin was intraperitoneally injected in 44 rats and IL-6 in 49 rats. After 0.5, 10, 15, 30 and 60 minutes (min), blood was sampled. Endotoxin and IL-6 were measured using the limulus-amoeocyte-lysate (LAL) test and ELISA technique, respectively.

**RESULTS**

No endotoxin or IL-6 was measured in the blood of controls. Plasma endotoxin and IL-6 levels were significantly high in the peritonitis groups. There was no further increase in endotoxin plasma levels after i.p. injection of endotoxin. Following i.p. injection of IL-6, there was an increase in IL-6 level over the time of sampling in the peripheral blood at 4h of peritonitis.

**CONCLUSION**

There was a clear reduction in peritoneal resorption of endotoxin and IL-6 in this acute model of gram-positive peritonitis.

**Key Words:** Acute; endotoxin; experimental; gram-positive; peritonitis; interleukin-6; peritoneal resorption.

**AMAÇ**

Gram pozitif veya gram negatif peritoniti bulunan hastaların peritoneal sıvılardında hem endotoksini hem de interlıken-6 (IL-6) konsantrasyonlarının yüksekliği, bu hastaların periferik kanlardaki endotoksini ve IL-6 konsantrasyonlarının yüksekliğinin bir katıdır. Bir bakteriyel (gram pozitif) peritonit modelinde, peritonun endotoksini ve IL-6’ya yönelik rezorpsiyon kapasitesi değerlendirildi.

**GERECH VE YÖNTEM**

Wistarchi 93 adet erkek saçana, fosfat tamponlu serum fizyolojik (FTS) içinde önceden músını ile işleme tabi tutulan stafylokoklar veya tek başına FTS intraperitoneal (IP) enjeksiyon şeklinde uygulandı. Rezorpsiyon çalışmaları, 4., 8., 12. ve 24. saatte gerçekleştirildi. Saçanların 44’üne endotoksin, kalan 49’unuyla IL-6 IP yolla enjekte edildi. 0., 5., 10., 15., 30. ve 60. dk’dan sonra kan örnekleri alındı. Endotoksin ve IL-6, rasasyla LAL testi ve ELISA teknigi kullanılarak ölçüldü.

**BULGULAR**

Kontrol grubunun kanlarında endotoksin ve IL-6 saptanmadı. Peritonit grubundaki, plazma endotoksin ve IL-6 seviyeleri anlamlı şekilde yüksek bulundu. IP endotoksin enjeksiyonundan sonra plazma endotoksin seviyelerinde ilave bir artış gerçeklemedir. IP IL-6 enjeksiyonunu takiben, peritonitin 4. saatinde periferik kandan gerçekleştirilen IL-6 örneklemesini ile ilgili olarak zamanla bir artış oluşturdu.

**SONUÇ**

Bu akut gram pozitif peritonit modelinde, endotoksin ve IL-6 ile ilgili peritoneal rezorpsiyonda belirgin bir azalma oluşmuştu.

**Anahtar Sözcükler:** akut; endotoksin; deneySEL; gram pozitif; peritonit; interlıken-6; peritoneal rezorpsiyon.
Severe peritonitis remains a feared condition that is associated with a high mortality rate attributable to gram-negative septicemia.\[[1]\] The liberation of endotoxin from the cell wall is the important pathophysiologic consequence of infection with these gram-negative organisms.\[[2]\] In septic peritonitis induced in rats, the concentration of endotoxin in the peripheral blood was significantly higher than its concentration in controls.\[[5]\] In another experiment, endotoxemia was responsible for the hemodynamic changes induced by bacterial peritonitis.\[[4]\]

A prolonged inflammatory response can lead to tissue damage by increased levels of pro-inflammatory mediators such as interleukin-6 (IL-6).\[[3]\] The concentration of inflammatory mediators in peritoneal exudates is a thousand times higher than in plasma.\[[6]\] This observation led to the hypothesis that inflammatory changes in the peritoneum clearly reduce its resorption capacity. The peritoneal resorption capacity for a molecule or particle is defined as the ability of the peritoneum to clear this molecule or particle from the peritoneal cavity into the systemic circulation.\[[7,8]\] A previous study showed this reduction in absorption of endotoxin and IL-6 in an animal model of chemical peritonitis (zymosan-induced peritonitis).\[[9]\]

It is important to study resorption in gram-positive peritonitis as this type is encountered in patients with end-stage renal failure who are treated with continuous ambulatory peritoneal dialysis (CAPD).\[[10]\] Lipopolysaccharides (LPS), the main component of endotoxin, are not a part of the cell wall of gram-positive bacteria and have nothing to do with their action and virulence. These bacteria have peptidoglycans (PepG), which are an endotoxin-like material, but cause no reaction to limulus test.\[[11,12]\] During gram-positive infections, PepG reaches the circulation by bacterial breakdown or translocation from the intestine. Administration of PepG induces all the classical features of infectious illness and endotoxemia and may cause systemic inflammation with organ failure in animal models.\[[13]\]

In spite of the fact that endotoxin is not produced by gram-positive bacteria, elevation in endotoxin in the peripheral circulation is found in peritonitis of gram-positive as well gram-negative origin. The aim of the present study was to evaluate resorption capacity of the peritoneum for endotoxin and IL-6 in an animal model of induced bacterial (gram-positive) peritonitis.

### MATERIALS AND METHODS

This model is a well-established animal model for gram-positive bacterial peritonitis.\[[14-16]\]

**Preparation of the gram-positive bacteria**

A staphylococcal inoculum was prepared by suspending colonies of a strain of *Staphylococcus aureus* (NCTC 8325) from an overnight culture on a Tryptone soya plate in Tryptone soya broth (TSB) in tube and incubated overnight at 37°C. This broth culture was diluted 1:5 in a fresh broth and incubated for 2 hours (h) in a shake stand at 37°C, then adjusted to an optical density of 1.0 in a McFarland, followed by centrifugation at 5,000 X g for 10 minutes (min). The supernatant was aspirated and resuspended in 10% glycerol in phosphate buffered saline (PBS) mix, giving a concentration of 8x10⁸ CFU/ml. Aliquots in tubes were frozen. On the experiment day, the suspension was centrifuged at 5,000 X g for 10 min and the supernatant was aspirated and resuspended in PBS mix, giving a concentration of 8x10⁶ CFU/ml. This suspension was finally diluted 1:1 in a 10% (wt/vol) mucin in PBS giving a concentration of 4x10⁶ CFU/ml. For each experiment, the size of the inoculum was determined by dilutions in sterile water up to (2x10⁶ CFU/ml and 2x10⁵ CFU/ml). 100 µl was plated on Tryptone soya agar plates, with subsequent counting of colonies after incubation overnight at 37°C.

**Experimental design**

The experimental design was approved by the Local Animal Research Ethics Committee.

**Subjects:** Young male Wistar rats (150-250 g) were used. The experiments were conducted on 93 rats, which were divided into two groups: one group for study of absorption of endotoxin (n: 44) and a second group for study of absorption of IL-6 (n: 49). The studied groups are shown in Table 1. Bacterial peritonitis was induced by a single intraperitoneal (i.p.) injection of 4x10⁶ CFU mucin-pretreated

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<th>Table 1. Number of animals in different groups</th>
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staphylococci suspended in 2 ml of PBS. Controls received PBS only. Studies of resorption were conducted at 4, 8, 12, and 24h after induction of peritonitis.

The rats were anesthetized at the beginning of the experiment through intra-muscular (i.m.) injection of ketamine (75 mg/kg) and xylazine (12 mg/kg) in the quadratus femoris muscle. Extra doses were given when needed. Internal jugular vein catheterization was then performed. Systemic heparinization was induced to facilitate blood sampling (150 IU/kg body weight [BW], half dose after 1h).

Blood was sampled at time minus five minutes (T-5). Five minutes later, E. coli O111:B4 endotoxin at a concentration of 0.1 μg/kg BW in 2 ml PBS or recombinant IL-6 rat murine B-9 at a concentration of 2 ng/kg in 2 ml of PBS was injected i.p.. Blood samples (0.5 ml) were collected immediately after the injection, and then at 5, 15, 30, 45 and 60 min. Blood was collected in pyrogen-free, heparinized tubes, and centrifuged, and plasma was collected and stored at -80°C to be used for measuring endotoxin and IL-6.

Laparotomy was performed and biopsies of diaphragmatic peritoneum were obtained. Experimental rats were euthanized by hyperpotassemic heart standstill using an intra-cardiac injection of potassium.

The plasma concentration of endotoxin was measured using the standard method of a chromogenic limulus-amoeocyte-lysate (LAL) test. Concentrations of IL-6 in plasma were determined using commercially available ELISA technique. Biopsies of the peritoneum were prepared and studied using light microscopy.

**Statistical Analysis:** Repeated measurement analysis of variance was used to study the within-group change over time, the between-group mean difference, and the interaction between groups and change over time [17,18]. An interaction effect was reported only if it was statistically significant. Data were analyzed using the Statistical Package for the Social Sciences (SPSS 13 for Windows, SPSS Inc., Chicago, IL, USA). A probability of less than 0.05 was accepted as significant.

**RESULTS**

Severe peritonitis was confirmed by operative findings that showed no macroscopic inflammatory changes in the peritoneum in the control group with minimal clear free intraperitoneal fluid. Edema of the peritoneum with an increase in the amount of free intraabdominal fluid was found in the 4h and 8h peritonitis groups. Thickening of the peritoneum with turbidity of the intraperitoneal fluid was evident in the 12h and 24h peritonitis groups. This increase in severity of peritonitis was confirmed via a histopathologic examination of a biopsy from the diaphragmatic peritoneum (Fig. 1).

![Fig. 1. Summary of the histopathologic examination of biopsies of diaphragmatic peritoneum from animals in all groups of the experiment (magnification X60, toluidine blue stain).](image-url)
**DISCUSSION**

Fig. 2. Plasma endotoxin concentration for the control and different peritonitis groups after intraperitoneal injection of endotoxin. Control group, closed squares (n=9); 4h peritonitis group, open squares (n=9); 8h peritonitis group, closed circle (n=9); 12h peritonitis group, closed diamond (n=9); and 24h peritonitis group, open circle (n=8).

Figure 2 shows the endotoxin concentration in the blood of animals after the i.p. injection of endotoxin. There was a significant difference between the five groups in endotoxin level (p=0.05). There was also a significant decrease over time in endotoxin levels (p<0.03), which was more evident in the four peritonitis groups (p=0.024). There was no significant difference in the endotoxin level between the four peritonitis groups (p=0.89, repeated measures analysis), indicating that they were significantly higher than the control group.

Fig. 3. Plasma IL-6 concentration for the control and different peritonitis groups after intraperitoneal injection of IL-6. Control group, closed squares (n=10); 4h peritonitis group, open squares (n=10); 8h peritonitis group, closed circle (n=10); 12h peritonitis group, closed diamond (n=9); and 24h peritonitis group, open circle (n=10).

Figure 3 shows the IL-6 concentration in the blood of animals after the i.p. injection of IL-6. Peritonitis of 4h duration caused a maximum increase in IL-6 level that gradually reduced at 8h of peritonitis to lower levels after 12h of peritonitis (p<0.0001). There was a trend for change over time in the IL-6 level in the studied period in the five groups (p=0.054). There was a significant difference in the IL-6 concentration between the four peritonitis groups (p<0.0001, repeated measures analysis). The five groups behaved differently in their response to IL-6 injection, as the interaction between the groups was highly significant (p=0.005).

Despite advances made in surgery and intensive care, sepsis and the systemic inflammatory response syndrome (SIRS) are the commonest causes of death in adult intensive care units. Sepsis and SIRS occurring during the course of peritonitis are associated with an exacerbated production of both pro- and anti-inflammatory mediators, including endotoxin, IL-6 and tumor necrosis factor (TNF)-alpha, which are mainly produced within tissues. The multigorgan dysfunction as a result of systemic effects of liberated inflammatory mediators leads to the high mortality rate encountered in peritonitis. In spite of the important role of TNF-alpha in sepsis due to peritonitis, we did not include the study of resorption for TNF-alpha, as the TNF molecule is an unstable molecule that is destroyed within minutes if the blood is not processed immediately, which was not possible in this study.

Peritoneal mesothelial cells play a role that extends beyond the provision of mechanisms that allow for the easy gliding of opposed peritoneal surface. Peritonitis has three consequent phases starting from a phase of rapid removal of contaminants from the peritoneal cavity into the systemic circulation, moving to a phase of synergistic interactions between aerobes and anaerobes as they encounter host defenses mounted by the complement cascade and phagocytes, and ending with the phase of attempting to localize infection (compartmentalization). All surgical maneuvers, including copious lavage, laparostomy and the use of drain tubes, are based on the simplistic notion that the abdomen is a container with impermeable walls ignoring the peritoneal defense mechanisms and the processes that support the localization of infection. Currently, efforts are being made to reduce the liberation of endotoxin and inflammatory mediators from damaged tissue into the circulation or to neutralize their
effects on organs in the hope to reduce the detrimental effects of SIRS and improve mortality. In this context, the management of peritonitis is mainly directed towards cleaning the abdominal cavity and peritoneal lining to reduce absorption of inflammatory mediators, endotoxin and bacteria from the peritoneal cavity into the systemic circulation in order to obviate organ malfunction. Although dramatic advances have been made in the pharmacological treatment of intraabdominal infections, mortality for complicated cases remains high.\(^\text{[23]}\) The management of peritonitis has not changed a great deal from the fundamental principles established by Polk in 1979 - surgical source control, fluid resuscitation, adequate nutrition, support of failing organ systems, and antibiotics.\(^\text{[24]}\) Lavage of the abdominal cavity, which constitutes an important component of the management of peritonitis and is thought to eliminate or reduce the liberation of inflammatory mediators to the systemic circulation, might be insufficient, since there is still uncertainty as to whether the peritoneal cavity is the source of the inflammatory mediators found in the systemic circulation.

There are many observations that could lead to postulation that the absorption of the inflammatory mediators from the peritoneal cavity is already reduced with the progress of peritonitis and that the source of the inflammatory mediators measured in the systemic circulation is not primarily the peritoneal cavity. One of these observations is that elevation in endotoxin levels is found in gram-positive as well as in gram-negative peritonitis.\(^\text{[25]}\) Inhibition of endotoxin absorption from the peritoneal cavity was previously observed in dogs with as compared to those without peritonitis, when endotoxin was injected i.p.\(^\text{[26]}\) Our own data in a previous similar study, but utilizing a chemical model of acute peritonitis, showed a reduction in resorption capacity of the inflamed peritoneum for both endotoxin and IL-6.\(^\text{[9]}\)

Large intermesothelial gabs (stomata) interrupt the peritoneal mesothelial cells (PMCs) lining the muscular portion of the diaphragm. Stomata can vary in size, which can increase during inflammation.\(^\text{[10]}\) Endotoxin and IL-6 have relatively small molecular mass\(^\text{[27,28]}\) with a diameter smaller than the size of the peritoneal stomata, which enables them to be cleared from the peritoneal cavity and transferred through the peritoneum to the systemic circulation. Within 10 min of exposure of the peritoneal cavity to spillage of bacteria, an increase in portal vein endotoxin could be observed.\(^\text{[29]}\) In spite of the fact that the stomata are wider during peritonitis, microvilli disappear, and mesothelial cells are covered with fibrin, leukocytes, and erythrocytes instead.\(^\text{[30]}\) This leads to a postulation that the resorption capacity of the peritoneum is reduced during the advanced stages of peritonitis.

To explore this postulation during the course of gram-positive peritonitis, we injected external endotoxin or IL-6 in the peritoneal cavity at different times in the progression of peritonitis. The increase in severity of peritonitis at various time frames was confirmed macroscopically on operative findings and histopathological examination of a peritoneal biopsy. There was a significant elevation in the basic plasma endotoxin levels in all peritonitis groups in comparison to its level in controls, and this occurred before the i.p. injection of external endotoxin. This implies that endotoxin is present in the systemic circulation even in the early stages of peritonitis and remains elevated, but to a lesser extent, in the late stages of peritonitis. Even following injection of a very high dose of endotoxin intraperitoneally, there was no significant elevation in its level in the peripheral blood in comparison to its baseline value before injection.

The maximum increase in IL-6 level was at 4h of peritonitis. This level was gradually reduced at 8h, followed by 12h and finally by 24h of peritonitis. This baseline decrease in the level of IL-6 with the progression of peritonitis could lead indirectly to the assumption that the escape of IL-6 was reducing over time with the progression of peritonitis. Another possible explanation for this reduction is that the systemic reaction to peritonitis is at its maximum in the early stages of peritonitis and this is reduced over time with the progression of peritonitis. The five groups behaved differently in their response to IL-6 injection. There was an increase over the time of sampling in IL-6 level in the peripheral blood at 4h of peritonitis. These results reflected the fact that there is a clear reduction in the resorption capacity of the peritoneum in established acute peritonitis. This could explain the observation of Berger et al.,\(^\text{[40]}\) who showed that in clinical peritonitis, the concentration of inflammatory mediators was thousand-folds higher in the peritoneal exudates than their concentrations in the peripheral blood, and this was unrelated to the type of bacteria isolated from the peritoneal cavity. Intraperitoneal cytokine levels also showed no correlation with systemic inflammatory markers in patients with inflammatory bowel
disease. These results will also support the hypothesis that systemic and peritoneal inflammatory responses occur in functionally distinct compartments. The concept of compartmentalization was also postulated by Cavaillon et al. in 2006, as the systemic pathophysiological events occurring in sepsis and SIRS differ from organ to organ, and from organ to peripheral blood.

The above-mentioned results suggest that the management of peritonitis should be extended to eliminate and reduce other possible sources of endotoxin and inflammatory mediators. The intestine is considered to be an important source of endogenous endotoxin and bacteria. A previous study demonstrated that selective decontamination of the gastrointestinal tract has been shown to prevent translocation of gram-negative bacilli to the abdominal cavity, reducing endotoxemia and mortality in rats with sterile peritonitis. Improvement in the gut microflora disturbances, increase in occluding expression, maintenance of the gut epithelial tight junction, and decrease in bacterial translocation rates were observed by supplementing probiotics in the gut in a peritonitis model of a cecal ligation and puncture. In a randomized, controlled, double-blind trial in patients with sepsis, early enteral parenteral nutrition with key pharmaceuticals in combination with an immunonutrition formula resulted in significantly faster recovery of organ function compared with controls. Clinical trials have shown that the use of intestinal lavage improved peritonitis- and toxicity-related symptoms. Experimentally, it has been demonstrated that the rate of postoperative endotoxemia is reduced using a combination of peritoneal and intestinal lavage.

Impaired absorption of inflammatory mediators from the peritoneal cavity merits further investigation with the study in a peritonitis model of gram-negative bacteria or in surgical peritonitis with mixed bacteria such as a cecal ligation and puncture model.

In conclusion, there was a clear reduction in the resorption capacity of the inflamed peritoneum for endotoxin and IL-6 in this model of acute gram-positive peritonitis. These results suggest that in peritonitis, the primary source of inflammatory mediators in the systemic circulation is not due to peritoneal resorption but is possibly due to translocation from the gut into the lymphatic or the venous circulation. Management of peritonitis should include measures to reduce or eliminate this endogenous source of inflammatory mediators. These measures could include intestinal lavage, early enteral feeding and/or selective decontamination of the gastrointestinal tract.

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