Effects of pentoxifylline in the treatment of abdominal sepsis in rats

Efeitos da pentoxifilina no tratamento da sepse abdominal em ratos

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ABSTRACT

Objective: To evaluate the effects of pentoxifylline in the treatment of abdominal sepsis in rats submitted to cecal ligation and puncture (CLP). **Methods**: We used 18 Wistar male rats three months old, randomly divided into control group C (n = 6); CLP (n = 6) for the rats submitted to CLP; Group CLP + PTX (n = 6) whose rats were treated with pentoxifylline and submitted to CLP. Pentoxifylline (40mg/Kg) was injected intraperitoneal 2 hours before and 8 hours after the induction of peritonitis. Histopathology of the lung, liver and kidney as well as analysis of renal function, markers of liver injury and cytokines were studied. The results were compared through ANOVA, and post hoc by Bonferroni test, considering $p < 0.05$ as significant. **Results**: The control group rats showed lower levels of all cytokines, compared to CLP group ($p < 0.05$). The CLP+PTX rats showed significantly lower amounts of TNF-α and IL-1 β when compared to the CLP group, but similar to group C. The levels of IL-6 in Group CLP+PTX, were higher than that found in group C and lower than that found in Group CLP ($p < 0.05$). Reduction of urea and creatinine occurred after administration of pentoxifylline. The values of AST and ALT were slightly lower in the experimental group, and no significant difference was observed comparing with the control group. The microscopic findings of kidney, liver and lung revealed less inflammatory reaction associated with the reduction of

pathological changes in animals treated with pentoxifylline. **Conclusion**: The pentoxifylline attenuated the pathophysiological events of sepsis, reducing the expression of pro-inflammatory cytokines, preserving renal function and tissue injury in an experimental model of polymicrobial sepsis.

Key words: Pentoxifylline, Sepsis, Cytokines, Pathology, Rats.

RESUMO

Objetivo: Avaliar os efeitos da pentoxifilina no tratamento da sepse abdominal em ratos submetidos à punção e ligadura do ceco. **Métodos**: Foram utilizados 18 ratos machos Wistar com três meses de idade, divididos aleatoriamente em grupo controle C (n=6); PLC (n=6) para os ratos submetidos à punção e ligadura do ceco (PLC); grupo PLC+PTX (n=6) cujos ratos foram tratados com pentoxifilina e submetidos PLC. Esse tratamento consistiu na administração de pentoxifilina (40 mg/Kg, via intraperitoneal) 2 horas antes e 8 horas após a indução da peritonite. Foram realizados estudos histopatológicos do pulmão, fígado e rim, bem como análise da função renal, dosagem de marcadores de lesão hepática e citocinas. Os resultados foram comparados através de ANOVA, seguida do teste de Bonferroni, com $p<0,05$ estatisticamente significante. **Resultados:** No que diz respeito às dosagens de citocinas plasmáticas, observou-se que os ratos do grupo C apresentaram níveis menores de todas as citocinas, quando comparado ao grupo PLC ($p< 0,05$). O grupo PLC+PTX apresentou valores significativamente menores de TNF-α e IL-1β quando comparado ao grupo PLC, porém semelhantes ao grupo C. Os níveis de IL-6 no grupo PLC+PTX, foram superiores aos encontrados no grupo C e inferiores aos do grupo PLC ($p<0,05$). A função renal foi preservada de possíveis danos decorrentes da sepse, pois ocorreu redução da uréia e creatinina após administração da pentoxifilina. Os valores de AST e ALT foram discretamente menores no grupo experimental, sem diferença significante em relação ao grupo controle. Os achados microscópicos do rim, fígado e pulmão revelaram menor reação inflamatória associada à redução de alterações patológicas nos animais que fizeram uso de pentoxifilina. **Conclusão:** A pentoxifilina influenciou positivamente as manifestações fisiopatológicas da sepse, reduzindo a produção de citocinas pró-inflamatórias, preservando a função renal e atenuando a lesão tecidual no modelo experimental de sepse poli microbiana.

Descritores: Pentoxifilina, Sepse, Citocinas, Patologia, Ratos.

Introduction

Sepsis is a syndrome involving the systemic host response to an inflammatory or infectious stimulus. Despite intensive research efforts, mortality rates in sepsis have not declined significantly[1]. In sepsis, an overwhelming inflammatory response characterized by the activation of inflammatory cells and excessive production of proinflammatory cytokines leads to tissue injury, multiple organ failure, and death[2,3]. The rationale for selecting cecal ligation and puncture (CLP) as a septic model is that it induces effects analogous to a perforated appendicitis with peritonitis[4]. The cardiovascular response to polymicrobial sepsis induced by cecal ligation and puncture (CLP) is characterized by an early hyper dynamic phase (increased cardiac output and tissue perfusion, decreased vascular resistance) followed by a late, hypo dynamic phase (reduced cardiac output and tissue perfusion)[5]. Peritonitis causes severe local damage to intra-abdominal organs due to overproduction of various proinflammatory mediators such as tumor necrosis factor (TNF-α), interleukin-1(IL-1), interleukin-6 (IL-6) and cyclooxygenase products[6]. It is now considered that these mediators bring about systemic microcirculatory injury which is thought to be the main mechanism responsible for damage in sepsis[7,8]. The host defense responses to sepsis may promote a generalized increase in leukocyte recruitment and accumulation in the tissues, which may lead to subsequent endothelial damage, leaky capillaries, and organ dysfunction and failure. The organic lesion starts in the lungs, progressing to the anguish respiratory syndrome, followed by kidney and liver failure because of damage caused in the architecture of these organs. Heart failure occurs in late stage septicemia[9,10]. Administration of various pharmacologic agents, such as the phosphodiesterase inhibitor pentoxifylline (PTX), has been shown to produce a number of beneficial effects during sepsis[11]. It has been demonstrated that PTX preserves renal and intestinal micro vascular blood flow during bacteremia, maintains hepatocellular function and improves cardiac performance in polymicrobial sepsis[12,13]. Furthermore, clinical studies have reported beneficial effects of PTX on cardiopulmonary function as well as hemodynamic performance and oxygenation during sepsis[14-16]. Sepsis is one of the leading causes of infectious death. The introduction of antibiotics and improvement in hemodynamic support resulted in a decline of sepsis-induced mortality. However, the successful treatment of sepsis remains a relevant medical challenge.

Based on this knowledge, this study aimed to evaluate the effects of this vasodilator drug in experimental abdominal sepsis, trying to ascertain possible immunomodulator effect and its repercussion on the liver, lungs and kidneys.

Methods

The experimental protocol was approved by the Research Ethics Committee of the University Hospital-UFRN, Brazil. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996.

Experimental design

We used 18 Wistar male rats with three months of age, randomly divided into 3 groups, kept in individual cages with food and water standard (Labina-Purina ®) for rodents, *ad libitum*. The groups were named as: Group C (n = 6) for the rats not operated (control); Group CLP (n = 6) for the rats submitted to the cecal ligation and puncture (CLP); Group CLP + PTX (n = 6) whose rats were initially treated with pentoxifylline and submitted to the CLP.

Surgical model

After 12 hours of fasting night, the rats were anesthetized with intraperitoneal (IP) injection of thiopental sodium (20 mg / Kg) (Thionembutal, Abbott, Sao Paulo, SP, Brazil) and ketamine (30 mg/Kg, i.m.). They breathed spontaneously throughout the procedures. After shaving, the abdominal skin was disinfected with 70% alcohol. All procedures were performed under sterile conditions. In the CLP and CLP + PTX rats, a midline laparotomy of 2 cm was performed and the cecum was exposed, ligated with silk 2-0 (Ethicon ®, Brazil), one cm distally to the ileocecal valve to avoid intestinal obstruction. Four cecal punctures were performed with an 18F needle, squeezed gently to force out a small amount of feces, and then it was returned to the abdominal cavity. The abdominal incision was closed with 4-0 nylon suture (Ethicon ®, Brazil) and, 24 hours after the procedure, reopened to the attainment of biopsies and blood collection. The peritonitis was clinically diagnosed when the animals had lethargy, frizzed hair and peripalpebral dark halo. After the procedures, the rats were killed by injection of anesthetic intracardiac overdosage.

Pentoxifylline administration

In the experimental group, the pentoxifylline (40mg/Kg) was administered by intraperitoneal (IP) way, 2h before and 8h after the of

peritonitis induction. In Group CLP, were administered 2 mL of saline solution to 0.9% (IP).

Measurement of cytokines, aminotransferases, urea and creatinine.

After 24 hours of conclusion of the procedures under anesthesia and aseptic conditions, blood was collected by cardiac puncture to measure the cytokines, transaminases, urea and creatinine. Samples of blood were treated with EDTA and the plasma was separated by centrifugation at 2000rpm and stored to -80°C for later measurement of tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and interleukin-1β(IL-1 β) by the ELISA (enzyme-linked immunoassay kits all from PeproTech® (Rocky Hill, NJ, USA) according to the manufacturer's recommended protocols. The fluorescence was measured by a Bio-Tec Instruments EL 808 ultra microplate reader, using KC4-V3.0 analysis software. The sensitivity of detection was 3 pg / mL for all cytokines. For the study of liver function, were measured aminotransferases (AST, ALT). The renal function was assessed by measuring serum creatinine and urea, using reagents Labtest (Lagoa Santa, MG-Brasil, 2007) in autoanalyser.

Histopathology

For the histopathological analysis, samples of the liver, lung and kidney were fixed in formalin to 10%. Sections made with thickness of 4μm were dehydrated, embedded in paraffin and stained with hematoxylin and eosin through conventional technique. All the slides were examined by the same pathologist, so blind. The histopathological data (swelling, congestion, inflammatory reaction, cell degeneration and necrosis) were graded according to the following scale: absent (0), lightweight (1), moderate (2) and severe (3).

Statistical analysis

For analysis of the data was used the program BioEstat® 2.0. The results were tabulated and compared by analysis of variance ANOVA, complemented by post hoc Tukey's test to compare the groups. $P < 0.05$ was considered statistically significant.

Results

Measures of cytokines, aminotransferases, urea and creatinine

Regarding the strengths of plasma cytokines, it was observed that the group C rats showed lower levels of all cytokines, compared to Group CLP (p

<0.05). The Group CLP + PTX showed significantly lower values of TNF-α and IL-1 β when compared to the group CLP, but similar to group C. The IL-6 levels in group CLP + PTX, were higher than that found in group C and lower than found in Group PLC (p <0.05) (Table 1).

Table 1 - Plasma levels of cytokines

GROUPS	TNF-α (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
Control	8.05±1.6*	6.4±1.4*	4.4±1.48*
CLP	38.0±8.8**	75.0±10.9**	20.7±2.9**
CLP + PTX	13.3±1.9	6.7±2.0	9.5±2.0§

* P <0.05 compared with CLP; ** p <0.05 compared with CLP + PTX; § p <0.05 compared with control. PTX, pentoxifylline; PLC, cecal ligation and puncture.

For biochemical analyses of aminotransferases (AST and ALT), it was noted that the group C rats had significantly lower values, when compared to groups CLP+PTX and CLP. Although the levels of AST and ALT in group CLP+PTX had minor reduction when compared to CLP group rats, the difference was not significant (Table 2). Analyzing the biochemical levels of urea and creatinine, the CLP+PTX rats showed significantly higher values when compared to group C, but statistically lower levels compared with the CLP group rats (Table 2).

Table 2 - Plasma levels of transaminase, urea and creatinine

GROUP	ALT (U/ml)	AST (U/ml)	UREA (mg/dl)	CREATININE (mg/dl)
Control	15.3±6.2*	18.5±4.8*	19.8±3.4*	0.78±0.3*
CLP	41.5±9.3	39.3±5.7	77.1±5.1**	2.38±0.6**
CLP + PTX	35.1±2.4	36.5±2.6	65.3±6.0	1.46±0.2

* P <0.05 compared with CLP and CLP + PTX; ** p <0.05 compared with CLP + PTX. PTX, pentoxifylline; CLP, cecal ligation and puncture. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Histopathological analysis

The microscopic study of kidney revealed areas of bleeding, swelling and congestion in group CLP rats, as compared to group C. However, these abnormalities found to be alleviated in group CLP+PTX, due to the use of pentoxifylline (FIGURES 1A, 1B and 1C). For the liver histopathology, it was observed liver congestion in CLP group rats, changes not found in any of the other two groups (Figures 1D, 1E and 1F). In lung, there was infiltration of

leukocytes and area of intense lung inflammation in CLP group, significantly higher when compared with the histopatologic findings in CLP+PTX rats (Figures 1G, 1H and 1I).

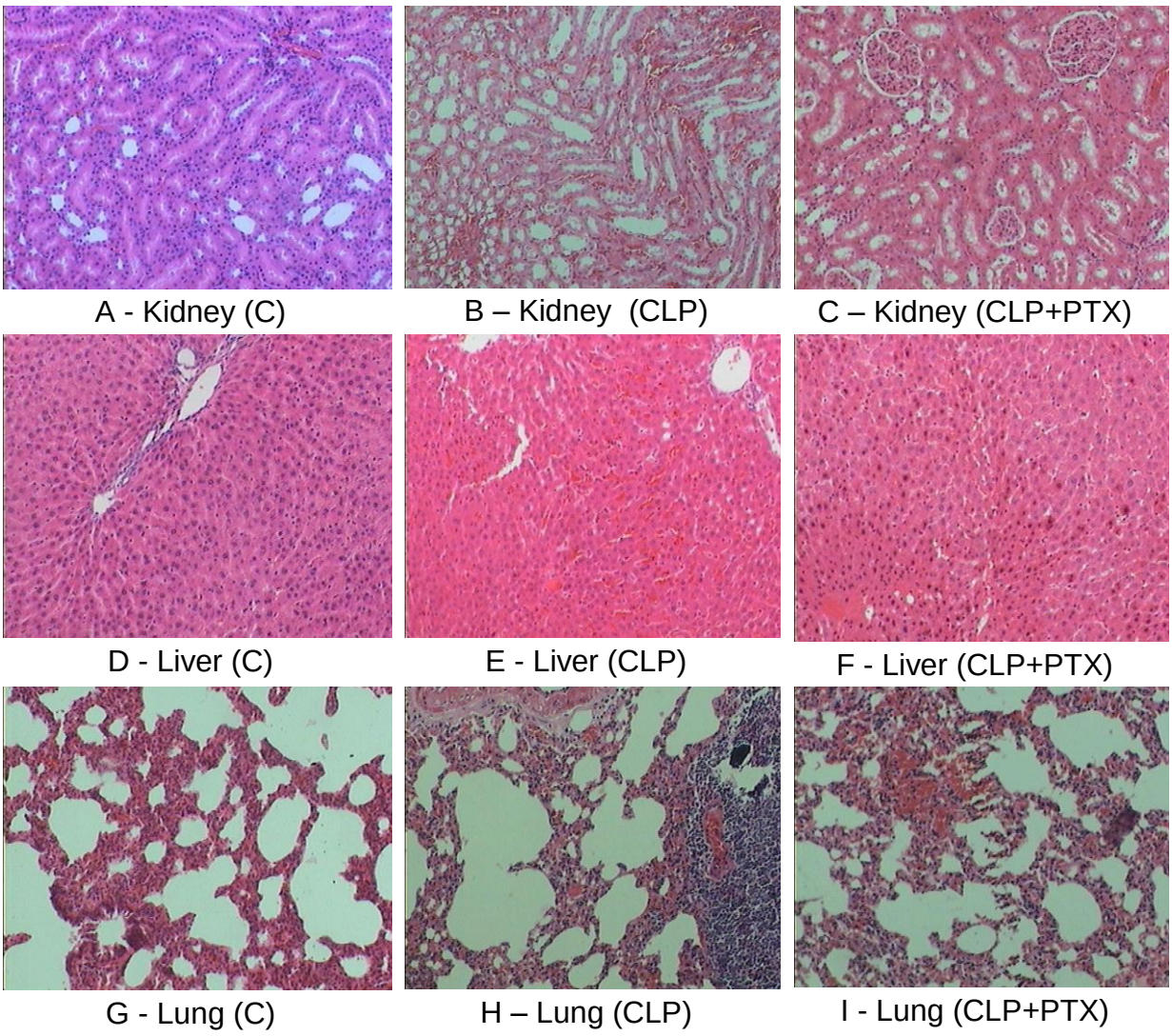

Figure 1. Microscopic aspects of the lung, liver and kidney of animals after induction of sepsis and treatment with pentoxifylline. A, D and G show the normal kidney, liver and lung (control). In B, the kidney of a septic animal shows areas of bleeding, swelling and congestion. In the liver of CLP animal inflammation and congestion is observed (E); Kupfer cells are observed and there is no congestion in F. In H, leukocytes are displayed in septa and area of intense inflammation, produced by the sepsis. In animals treated with the PTX histopathologic abnormalities are lower in the kidney (C), liver (F) and in the lung (I). (HE, 100 x)

Discussion

Pentoxifylline is a drug well known for its hemo-rheological properties. However, in recent years, studies have been performed to show potential anti-inflammatory and immunomodulatory effects of this substance and thereby increase their therapeutic approach. This study, besides evaluating the cytokines expression, has searched the impact of histological and functional use of pentoxifylline in the kidney, liver and lung by using an experimental model of abdominal sepsis induced in rats. The immunomodulatory effects of pentoxifylline are mainly attributed to its ability to inhibit the production of cytokines, especially TNF-α[17,18]. In this research, we found that PTX in rats with sepsis caused a marked reduction in TNF-α, IL-1β and to a lesser extent IL-6, compared with untreated septic rats. Voisin et al, studying the pre-treatment with PTX and its modulatory effects in sepsis induced by E. coli, found reduced levels of TNF-α, IL-1β and IL-6 in animals treated with this drug. They also reported that the effect of PTX on IL-1β was more effective than on IL-6. IL-6 is produced later than TNF-α, and simultaneously with IL-1β. Thus the remaining IL-1β can be sufficient to enhance IL-6 plasma level in PTX-treated animals[19]. Koo et al evaluated the use of PTX in rats subjected to cecal ligation and puncture and its effects on the vascular responsiveness to adrenomedullin (vasodilator peptide) and on the proinflammatory cytokines TNF-α, IL-1β and IL-6. They reported reduction in the release of cytokines, noting that the maintenance of vascular responsiveness may have been a consequence of the effect of PTX on these inflammatory mediators[20].

In the case of liver enzymes, in this study we observed significantly lower levels of AST and ALT in the control group than in the other two groups. We found no statistically significant difference between rats with sepsis, treated with pentoxifylline and that treated with saline. Yang et al, on the other hand, studied the use of PTX to prevent the changing from the hyperdynamic response to hypodinamic, during sepsis in rats subjected to PLC. They demonstrated a significant reduction in the levels of AST and lactate in septic rats treated with PTX[21] . Wang et al, evaluated the liver and heart in experimental conditions similar to Yang et al, and reported the maintenance of hepatocellular and cardiac functions at levels near the control group, due to the use of PTX[22]. A reasonable explanation for these findings observed in the literature and in our essay can be found in the different dosages administered to animals during the experiments. While in our study we used 40 mg/kg of PTX intraperitoneally, Yang et al and Wang et al administered 50 mg / kg PTX intravenously. This difference possibly means that the anti-inflammatory effects of pentoxifylline are dose-dependent and it may be more effective when

administered intravenously. In this case, a high dose of PTX could have resulted on a greater protection against liver injury than in our study.

Wang et al[23] observed that acute renal failure increases significantly the mortality (50-80%) of patients with sepsis. Sepsis induces the expression of a series of pro-inflammatory cytokines, among them, tumor necrosis factor (TNF-α), the great responsible for the pathophysiology of acute renal failure post-sepsis. It has been postulated that the use of PTX protects renal function due to the reduction of TNF-α and endothelial factors responsible for renal vasoconstriction and hypoperfusion [23]. Therefore, PTX may have prevented renal failure in our rats, both by its effects in reducing TNF-α, and by stimulating vascular relaxation. Krysztopik et al[24] reported that sepsis promotes renal hypoperfusion despite the hyperdynamic state of systemic circulation. This is due to the failure in controling the renal microcirculation. The arachidonic acid, together with TNF-α promotes microvascular vasoconstriction. Pentoxifylline reduces the release of TNF-α and modulates the action of arachidonic acid by inhibiting the phosphodiesterase enzyme, protecting renal function during sepsis[24].

According to Jaimes et al, inhibition of TNF-α expression and of NO synthesis by pentoxifylline prevents thrombosis and restores glomerulovascular tone in treated septic animals, maintaining renal perfusion[25]. This phenomenon was observed in other studies[26-28]. The renal function of our rats was assessed by measurement of serum urea and creatinine. The measurements of creatinine in the PLC and PLC+PTX rats were significantly higher than the levels of group C rats. However, although the rats with sepsis have shown high levels of urea, the PLC+ PTX group rats showed a significantly lower levels when compared with Group PLC, indicating a protective renal effect of PTX.

Structural analysis of the affected organs in sepsis (lung, liver and kidney) was made by histopathology, which showed an effective action of PTX in the control of the morphological changes caused by inflammatory injury characteristic of sepsis. It is known that during sepsis the most common injury to the kidney is acute tubular necrosis, caused by renal hypoperfusion, partly responsible for the acute renal failure. It is characterized by destruction of tubular epithelial cells, which can be functional and estrutural[29]. Among the structural damage, cellular swelling, loss of brush border, detachment of cells and lethal injury (necrosis and apoptosis) are found. The rupture of basement membrane can occur (tubulorrexis), beyond the occlusion of the tubules per cylinders. Interstitial edema and leukocyte accumulation within the vasa recta are also found[30]. Such features were observed in the present study. The septic rats which did not use PTX showed evidence of renal hemorrhage, edema and congestion. However, histopathological analysis of kidney from PLC+PTX group rats showed a decrease of these structural injuries with reduction of edema and congestion. PTX was supposed to reduce the levels of pro-inflammatory

cytokines as well. Yang et al, analyzing the renal histopathology after 20h of PLC in rats, found tubular swelling, degeneration of epithelial cells and restriction of the Bowman's space. They observed that treatment with PTX in these animals with sepsis decreased renal injury in small tubular edema, with maintenance of the integrity of the Bowman's capsule [21].

In the lung, histological pattern exhibited by PLC group rats was according to the literature by presenting diffuse leukocyte infiltrate, edema of alveolar septum and hemorrhagic areas. However, there were no changes considered as typical of severe alveolar collapse and abscess formation. On PTX+PLC group rats, the changes in the lung parenchyma were less severe with lower leukocyte infiltrate, less edema and hemorrhage than in PLC group rats. Considering the classification adopted by Guidudli et al[31], we may postulate that animals treated with pentoxifylline showed less pronounced pattern of lung injury than the untreated ones. That same study showed the relationship between the histological severity of inflammatory lung and abdominal sepsis: the more intense and widespread abdominal infection, the greater is the lung injury[31]. This suggests that PTX atenuated the lung injuries in the PTX+PLC rats.

Oliveira-Junior et al. found that pretreatment with PTX could attenuate most of the sepsis-induced functional lung alterations. For them, the involvement of inflammatory mediators in lung dysfunction was assessed by the measurement of TNF-α in bronchoalveolar lavage. Levels of TNF-α were higher in septic animals than in controls. Alternatively, pentoxifylline could afford protection by its effects on inflammatory cells[32]. Studies evaluating the effects of pentoxifylline on adhesion molecules expression have shown decreased expression of these molecules after hemorrhagic shock and decreased proinflammatory cytokines expression in human pulmonary epithelial cells and polymorphonuclear leukocytes[33,34].

The histological analysis from PLC group rats showed liver congestion. In PLC+PTX group rats, pentoxifylline was able to attenuate this abnormality; consequently, their liver microarchitecture was comparable to the control group rats. This finding is supported by Yang et al. research with regard to liver protection by PTX. In their study, the hepatic histopathology of rats subjected to sepsis by 20h showed areas of necrosis, eosinophilic cytoplasm and nuclear condensation. They observed that the hepatocytes injury was significantly attenuated by the treatment with PTX[21]. The PLC dependent liver injury can be explained by a significant decrease of microvascular perfusion[35]. The protective effect of PTX in all organs studied was probably due to its ability to inhibit pro-inflammatory cytokines, especially TNF-α, IL-1β and IL-6. In fact, several studies suggest that the beneficial effects of PTX in septic situations are due to their ability to modulate cytokines expression[36-38].

Conclusion

The pentoxifylline was revealed able to positively modulate the pathophysiological events of sepsis by reducing the expression of pro-inflammatory cytokines, preserving the renal function and mitigating tissue injury in a model of abdominal polymicrobial sepsis in rats.

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