Sildenafil as a vasodilatatory madiator in the treatment of abdominal sepsis

Sildenafil como um mediador vasodilatador no tratamento de sepse abdominal

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ABSTRACT

Objective: To analyse the effects of previous treatment with sildenafil in rats with abdominal sepsis induced by cecal ligation and puncture (CLP). **Methods:** Wistar rats were randomly allocated in 3 groups of 6 each. Sham group (SG): rats were subjected to laparotomy and no induction of sepsis; CLP/sil: rats subjected to cecal ligation and puncture, treated with sildenafil 1mg/Kg via gavage, 60 min before sepsis induction; CLP group: rats with sepsis, no sildenafil. After anesthesia under aseptic technique, we underwent a laparotomy and CLP. Postoperative pain was controlled with tenoxicam 3mg/Kg, i.m. The rats were observed for 24 hs, and examined on the late stage of polymicrobial abdominal sepsis. Body weight, leukogram, C-reactive protein, TNF-α, IL-1β, IL-6, IL-10. Statistical analysis was done by ANOVA and Tukey test, with significance $p<0.05$. **Results:** Group CLP rats had their weight reduced in 12% when compared with *sham* rats ($p<0.05$). However, comparing the *sham* and CLP/sil rats the diference on weight loss was not significant. Leukocytes and neutrophils counts were significantly elevated in group CLP rats compared with *sham* ($p<0.05$). In CLP/sil group rats a decreased total leukocyte and neutrophil counts were detected, compared with the CLP group ($p<0.05$). Reduced levels of C-reactive protein in the LPC/sil group were observed, compared with CLP rats ($p<0.05$). Serum levels of TNF-α, IL-1β and IL-6 were lower in the CLP/sil rats, compared with untreated CLP animals ($p<0.05$). Increased IL-10 in the CLP/sil rats, compared with CLP rats, demonstrated a protective effect on the vasodilatory sepsis. **Conclusion:** The data revealed that the pretreatment of abdominal sepsis with the vasodilator sildenafil favorably influenced the evolution of inflammation and immune response in rats.
Keywords: Abdominal Sepsis. Treatment. Sildenafil. Vasodilation. Cytokines. Rats.

RESUMO

Objetivo: Analisar os efeitos do tratamento prévio com sildenafil em ratos com sepse abdominal induzida por ligadura e punção do ceco. **Métodos:** Ratos Wistar foram distribuídos aleatoriamente em 3 grupos de 6 animais cada. Grupo *Sham* (GS): Animais submetidos a laparotomia sem indução da sepse; grupo LPC/sil: Ratos submetidos à ligadura e punção do ceco (LPC) tratados com sildenafil 1mg/kg via oral por gavagem, 60 minutos antes da indução da sepse; grupo LPC: indução de sepse, sem uso do sildenafil. Após anestesia e com técnica asséptica, foi realizada laparotomia mediana e LPC. Dor pós-operatória controlada tenoxicam i.m. 3 mg/Kg. Os animais foram observados até completar 24 horas, estágio tardio e hipodinâmico da sepse abdominal polimicrobiana. Foram analisados: Peso dos animais, leucograma, dosagem sérica de proteína C reativa, TNF-α, IL-1β, IL-6 e IL-10. Análise estatística pelo ANOVA e teste de Tukey, significância $p<0,05$. **Resultados:** Redução de 12% do peso dos animais do grupo LPC quando comparado com grupo *sham* ($p<0,05$). Comparação entre o grupo *sham* e LPC/sil não revelou diferença significante. A quantificação de leucócitos totais e neutrófilos revelou aumento significante nos animais do grupo LPC quando comparado com o grupo *sham* ($p<0,05$). No grupo LPC/sil houve redução de leucócitos totais e neutrófilos, comparando-se com o grupo LPC ($p<0,05$). Houve redução dos níveis de proteína C reativa no grupo LPC/sil, em comparação com o grupo LPC ($p<0,05$). Redução significante da expressão de TNF-α, IL-1β e IL-6 no grupo LPC/sil em comparação com os animais não tratados ($p<0,05$). O aumento de IL-10 no grupo LPC/sil, comparado com o grupo LPC sugeriu efeito protetor do vasodilatador sobre o quadro séptico. **Conclusão:** Os dados permitem afirmar que o pré-tratamento da sepse abdominal com o vasodilatador sildenafil influenciou a evolução favorável da inflamação e da resposta imune em ratos.

Descritores: Sepse abdominal. Tratamento. Sildenafil. Vasodilatação. Citocinas. Ratos.

INTRODUCTION

Sepsis is a disease whose pathophysiology is linked to an imbalance between anti-inflammatory and pro-inflammatory substances that will mediate a response to damage to organs caused by infection. The infection process may be initiated by gram-negative, gram-positive and anaerobic bacteria, fungi, protozoa and/or virus, the latter being in smaller proportions. Gram-negative bacteria are the main cause of sepsis[1]. In spite of increasing advance on the treatment of sepsis, this is still a major cause of death in intensive care units, has a high cost, as diagnosed in the last two decades, especially in polytraumatized patients in use of catheters, mechanical ventilation, steroid-dependent patients and those with pneumonia[2].

By using video-microscopy in vivo, investigators have demonstrated in rats models that sepsis is characterized by decreased rate of flow in the microcirculation,

increased heterogeneity of flow, increased vascular stasis and decreased capillary perfusion[3].

The ideal therapy for improving the microcirculation must modulate endothelial function prematurely and cause vasodilation of low flow units. Treatment with vasodilators (prostacyclin) and a mixture of inotropes/vasodilators (dobutamine) have been used for this purpose and have demonstrated an increase in systemic oxygen consumption, suggesting improvement in microcirculation[4-6].

Recently there has been considerable interest in investigating the role of sildenafil in protecting ischemic and reperfusion injury in animals[7]. This is a new class of vasoactive drugs that have been developed for the treatment of erectile dysfunction. Its mechanism of action involves active inhibition of type 5 phosphodiesterase enzyme that increases cGMP, nitric oxide (NO) and improves endothelial dysfunction[8].

Based on these aspects of interaction between microcirculation and its effects on sepsis and in the vasoactive effects of sildenafil, the present experimental model aims to analyze the effects of previous use of sildenafil in abdominal sepsis induced by cecum ligation and puncture in rats.

METHODS

Rats sample

We used 18 Wistar rats weighing 280±18g, from the Center for Health Sciences, Federal University of Rio Grande do Norte, Brazil. The experimental protocol and the care of the animals were in accordance with the Animal Welfare Act and Brazilian Law 11,794/2009. This project was approved by the Institutional Animal Care and Use Committee of UFRN, Brazil. The animals were kept in an adaptive period of seven days and throughout the experiment they were observed at the Nucleus for Experimental Surgery in individual cages with water and standard food for rodents (Labina-Purina ®) *ad libitum*, subjected to light-dark cycle of 12 hours with humidity and noise control.

Experimental design

The animals were randomly distributed into 3 groups of 6 animals each, with the following characteristics: Sham group (SG) – Sham-operated rats (control) underwent laparotomy and the cecum was neither ligated nor punctured. Group CPL /sil – The rats underwent cecal ligation and puncture (CLP) and were treated with sildenafil. CPL Group - Induction of sepsis, without sildenafil treatment.

Sildenafil treatment

In groups SG and CLP/sil the rats were treated with sildenafil (Pfizer, Brazil) suspension 1mg/kg by gavage, 60 minutes before sepsis induction.

Operative procedures

Rats were fasted overnight (16 h) before the induction of sepsis but allowed water *adlibitum.* They were anesthetized with ketamine 50mg/kg body weight and xilazine 20 mg/kg (intramuscular), their abdomens were shaved, and a 4-cm ventral midline incision was made. The cecum was exposed and isolated by ligation with a 3-0 cotton ligature just distally to the ileocecal valve to avoid intestinal obstruction. The cecum was punctured twice at opposite ends with an 18-gauge needle and confirmation of the punctures was established by forcing a small amount of the cecal contents out of the cecum. The ligated and punctured cecum was then returned into the abdominal cavity. The abdominal incision was then closed in two layers using a 4-0 nylon suture and the animals received 3 mL per 100 g body wt of normal saline solution subcutaneously (i.e., fluid resuscitation).

Postoperative examinations

The animals were observed for 24 hours, considering that 24 hours after CPL represents the late stage of hypodinamic polymicrobial abdominal sepsis[1]. The following parameters were analyzed: 1) Weighing of the animals at the beginning and end of the experiment on a digital scale. 2) Collection of blood through cardiac puncture at the end of the observation period for leukocytes and neutrophils count in automatic cell counter (AbbottCell-Dyn 3500R-CD 3500 5L, USA). Blood serum was separated by centrifugation at 2000rpm and stored at -40°C for subsequent dosage. C-reactive protein was analyzed by autoanalyzer (BT Plus WeinerLab 3000). TNF-α, IL-1β, IL-6 and IL-10 were assayed by ELISA (enzyme-linked-immunosorbent-assay), an analytical methods based on antigen-antibody interaction used to determine specific amounts of protein in tissue samples and body fluids. The reagents were from PeproTech, NJ, USA. Observation of animal behavior (presence or absence of hair bristling, lethargy and dark circles around the eyes), morbidity, mortality and weight control were done.

Data were statistically analyzed by ANOVA and the Tukey test, considering differences significant when $p < 0.05$.

RESULTS

There was a 12% weight loss on the CLP animals compared with sham group ($p<0.05$). When the comparison was made between the sham group and the CLP/sil group, no significant difference was observed. All animals in LPC group showed lethargy, hair bristling and dark circles around the eyes after 24 hours. These findings were not observed in animals from other groups.

The leukocytes and neutrophils counts showed significant increase in CLP group animals when compared to the sham group ($p<0.05$). Treatment with sildenafil (CLP sil group) reduced the levels of leukocytes and neutrophils, compared with the

CLP group (p<0.05). We observed a decrease in the levels of C-reactive protein in the LPC/sil group rats, indicating a lower inflammatory response in this group compared with CLP group (p <0.05), whose numerical data are shown in Table 1.

Table 1 - Results of leukocytes, neutrophils counts and measurement of C-reactive protein.

Groups	Leukocytes/µL	Neutrophils (%)	C-reative protein (mg/L)
Sham	4.67±0.54*	54.8±2.6*	0.4±0.08*
CLP	9.63±1.29	80.8±10.4**	17.5±2.7
CLP/sil	6.75±3.2**	75.5±6.5	11.7±4.4**

*p<0.05 vs. grupos CLP, CLP/Sil; ** p<0.05 vs. grupos Sham, CLP /sil.

Inflammatory cytokines

The serum levels of proinflammatory cytokines TNF-α, IL-1β and IL-6 showed a significant reduction (p<0.05) in the group previously treated with sildenafil (CLP/sil) compared with untreated animals. The serum level of the anti-inflammatory cytokine L-10 was significantly higher in CLP rats than in CLP/sil animals, meaning a protective effect on the vasodilator sepsis. Numerical data are summarized in Table 2.

Table 2 – Values of serum cytokines on each group rats.

Groups	n	TNF-α (pg/mL)	IL-1β (pg/ml)	IL'-6 (pg/mL)	IL-10 (pg/mL)
Sham	6	21.7±3.5*	20.9±3.8*	22.1±4.4*	34.2±4.3*
CLP	6	149±12	170.2±14	209±32	123±11
CLP/Sil	6	81.7±9.1**	58.4±8.6**	154±15	195±16**

*p<0.05 vs. CLP, CLP/Sil groups; ** p<0.05 vs. Sham, CLP groups.

DISCUSSION

Sepsis has a high mortality rate, with estimated values between 20 and 50% and several studies have been developed in the search for more effective therapies to reverse sepsis[2,9-11]. In the present study, we verified the effectiveness of sildenafil as a protector against the effects of sepsis caused by CLP. The pathophysiology of this disease is related to an inflammatory response to infection. There is an exacerbation in the release of inflammatory modulators and excessive activation of inflammatory cells, meaning that the patients own defense can't control the disease. This may evolve into a severe sepsis until the failure of multiple organs, leading to death[1].

The presence of gram-negative bacteria in the peritoneal cavity triggers the release of endotoxins and exotoxins that stimulate the expression of primary modulators through activation of macrophages and monocytes. TNF-α and IL-1β are

some of these modulators, particularly TNF-α has been shown to be the most involved in the development of septic shock. In the current study the pretreatment with the vasodilator sildenafil interfered on the level of these pro-inflammatory cytokines by reducing them in significant amounts in comparison with the untreated group. This reduction was important to demonstrate that pretreatment with sildenafil may act in the attenuation of the inflammatory response during sepsis. Cadirci et al demonstrated that sildenafil attenuated exacerbated release of the pro-inflammatory cytokine TNF-α in a study in which sildenafil was also used in rats subjected to CLP[12]. IL-6 is another cytokine that appears to be the an efficient promoter on hepatic production of acute phase proteins as the C-reactive protein, a very sensitive marker of systemic inflammation. Damas et al[13] demonstrated that IL-6 acts as a second messenger released by macrophages, endothelial cells, fibroblasts and other cells in response to sepsis. As seen in the quantitative evaluation of the present study, pretreatment with sildenafil resulted in a significant reduction in IL-6 and C-reactive protein levels, suggesting that these two mediators are correlated during development of the sepis.

The exaggerated inflammatory response that occurs in sepsis is counterbalanced by the early and sustained expression of potent anti-inflammatory cytokines such as IL-10. In sepsis, IL-10 has been identified as a modulator of the production of frequently lethal proinflammatory cytokines. Neutralization of IL-10 results in increased expression of proinflammatory cytokines and death, while the administration of its recombinant form provides therapeutic protection[14]. Thus, the increase in IL-10 in rats of CLP/sil group was certainly an important moderating and neutralizing factor on the intense inflammatory process induced by sepsis.

Happening pro-inflammatory stimuli, such as IL-1, IL-6 and TNF-α, or oxidative stress[15], endothelial activation occurs, leading to a procoagulant environment, pro-adhesive cell surfaces, dysregulation of vasomotor tone, and compromised barrier function. This inflammatory environment is further propagated by the release of additional cytokines directly from endothelial cells, which leads to local microvascular damage, disrupted tight junctions, edema, and tissue hypoxia[1,15].

In the presence of infectious focus, endotoxins and cytokines will activate cellular and humoral immune response. The first line of defense against infections after natural barriers are the monocyte-macrophages. Furthermore, the presence of TNF-α, IL-1β and IL-6 also activates other cells or blood components such as the polymorphonuclear cells[1]. In the present study we detected an increased migration of leukocytes to circulation and to the infectious focus in the CLP rats, showing that there was an intense attraction to the infected site by chemotactic factors. The use of sildenafil, however, by reducing the intensity of the infectious process, required a smaller amount of immune cells during sepsis. This was validated by the reduction of total leukocytes and neutrophils in the rats of CLP/sil group compared to the CLP group.

Kukreja et al demonstrated that sildenafil induces cardioprotective effect against ischemia and reperfusion in dogs, rabbits, rats and mice. The effect was attributed to the pharmacological preconditioning mechanism[16]. Lledo-Garcia et al

demonstrated a beneficial effect of sildenafil after renal ischemia. The aim of their experimental study was to determine the hemodynamic, biochemical, and histological effects of sildenafil as a preconditioning vasodilator before a period of warm ischemia[17]. A similar effect was demonstrated in our laboratory[7].

Tissue hypoperfusion can be present even in the normal blood pressure and adequate cardiac output, a state sometimes referred to as cryptic shock. This hypoperfusion may be related to preferential maldistribution of blood flow at the regional or microvascular level[18,19]. Derangements of small vessel perfusion are largely a function of intrinsic events in the microcirculation. The causes of microcirculatory flow alterations in sepsis are multifactorial and include endothelial cell dysfunction, increased leukocyte and platelet adhesion, fibrin deposition, erythrocyte stiffness, altered local perfusion pressures due to regional redistribution of blood flow, and functional shunting[20,21]. Although research on septic shock is classically focused on macrocirculatory hemodynamics that reflect the distribution of blood flow globally throughout the body, a functioning microcirculation is another critical component of the cardiovascular system that is necessary for effective oxygen delivery to tissues.

In the present study we used the pretreatment with sildenafil, a potent vasodilator, in rats with abdominal sepsis, with significant changing in leukocytes, cytokines and reactive C protein. Regardless of the cause, it seems that an early and aggressive hemodynamic intervention can impart the best opportunity to limit the damage caused by tissue hypoperfusion, including attenuating the inflammatory response and endothelial injury[22].

An ideal therapy to improve microcirculation would modulate endothelial function and vasodilate low-flow units. Pharmacotherapies such as vasodilators (prostacyclin) and mixed inotropes/vasodilators (dobutamine) have been used to this end and have demonstrated an increase in systemic oxygen consumption suggesting microcirculatory recruitment[23]. De Backer et al demonstrated increases in capillary perfusion independent of systemic hemodynamic effects with dobutamine and recombinant human activated protein C (rhAPC). These effects are likely to be due to the vasodilatory and rheologic action of dobutamine and perhaps to modulation of the leukocyte-endothelial cell interaction by rhAPC[6,24].

Seen through the lens of the microcirculation, sepsis-induced increases in nitric oxide (NO) may actually be an adaptive response that is an attempt to restore blood flow at the level of capillaries. Contrary to previous lines of investigation, exogenous NO may be viewed as an attractive therapeutic agent in sepsis if it is able to augment microcirculatory flow. NO fulfills the requirement of being a potent vasodilator and modulator of leukocyte-endothelial reactions, as seems to be the sildenafil used in the present experimental model[25].

Administration of exogenous vasodilator sildenafil, prior to the induction of sepsis, demonstrated important protection against inflammation. One obvious concern about the use of exogenous sildenafil in sepsis is the potential for exacerbation of arterial hypotension, which could attenuate its positive effect on the microcirculation. Studies about this are lacking. Therefore, at present, whether or not

exogenous sildenafil administration to patients with sepsis is efficacious and safe is unknown and the subject of ongoing clinical investigation. We are sure that, as promising as targeting the microcirculation in sepsis may appear, the microcirculation is only one of many pathophysiologic factors that contribute to the overall picture of circulatory failure and, ultimately, cellular dysfunction.

In conclusion, the data of our study support the statement that pretreatment of abdominal sepsis with the vasodilator sildenafil influenced the favorable evolution of inflammation and immune response in rats.

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