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ORIGINAL ARTICLE

Antioxidant protection of statins in acute kidney injury induced by sepsis

PROTEÇÃO ANTIOXIDANTE DA ESTATINA NA LESÃO RENAL AGUDA INDUZIDA PELA SEPSE

PROTECCIÓN ANTIOXIDANTE DE LA ESTATINA EN LA INSUFICIENCIA RENAL AGUDA INDUCIDA POR LA SEPSIS

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ABSTRACT

Objective: Evaluating the effect of preconditioning with simvastatin in acute kidney injury induced by sepsis. **Method**: Male adult Wistar rats were divided into the following groups: SHAM (control); SHAM+Statin (0.5 mg/kg simvastatin, orally); Sepsis (cecal puncture ligation – CPL); Sepsis+Statin. Physiological parameters, peritoneal fluid culture, renal function, oxidative metabolites, severity of acute kidney injury and animal survival were evaluated. **Results**: The treatment with simvastatin in induced sepsis showed elevation of creatinine clearance with attenuation of generation of oxidative metabolites, lower severity of acute kidney injury and reduced mortality. **Conclusion**: This investigation confirmed the renoprotection with antioxidant principle of the simvastatin in acute kidney injury induced by sepsis in an experimental model.

DESCRIPTORS

Antioxidants
Acute kidney injury
Sepsis

RESUMO

Objetivo: Avaliar o efeito do pré-condicionamento com sinvastatina na lesão renal aguda induzida por sepse. **Método**: Ratos Wistar, adultos, machos foram distribuídos nos grupos: SHAM (controle); SHAM+Estatina (0,5 mg/kg sinvastatina, via oral); Sepse (ligadura punção de cécum – LPC); Sepse+Estatina. Foram avaliados parâmetros fisiológicos, cultura líquido peritoneal, função renal, metabólitos oxidativos, gravidade da lesão renal aguda e sobrevida dos animais. **Resultados**: O tratamento com sinvastatina na sepse induzida demonstrou elevação do clearance de creatinina com atenuação da geração dos metabólitos oxidativos, menor gravidade da lesão renal aguda e redução da taxa de mortalidade. **Conclusão**: Esta investigação confirmou a renoproteção com princípio antioxidante da sinvastatina na lesão renal aguda induzida pela sepse em modelo experimental.

DESCRITORES

Antioxidantes
Lesão renal aguda
Sepse

RESUMEN

Objetivo: Evaluar el efecto del pre condicionamiento con sinvastatina en la insuficiencia renal aguda inducida por sepsis. **Método**: Ratas Wistar, adultas, machos, fueron distribuidos en los grupos: SHAM (control); SHAM+Estatina (0,5 mg/kg sinvastatina, vía oral); Sepsis (ligadura y punción cecal – LPC); Sepsis+Estatina. Fueron evaluados los parámetros fisiológicos, la cultura de líquido peritoneal, la función renal, los metabolitos oxidativos, la severidad de la insuficiencia renal aguda y la supervivencia de los animales. **Resultados**: El tratamiento con sinvastatina en la sepsis inducida demostró elevación del aclaramiento de creatinina con atenuación de la generación de los metabolitos oxidativos, menor severidad del fallo renal agudo y reducción del índice de mortalidad. **Conclusión**: Esta investigación confirmó la renoprotección con principio antioxidante de la sinvastatina en el fallo renal agudo inducido por la sepsis en modelo experimental.

DESCRIPTORES

Antioxidantes
Lesión renal aguda
Sepsis

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INTRODUCTION

Sepsis is the leading cause of death in the intensive care unit (ICU)[1]. In Brazil, a multicenter study carried out in ICUs showed that 16.7% of patients were diagnosed with sepsis, severe sepsis or septic shock, and the described mortality rate was 46.6%[2].

In sepsis, the first line of host defense, the innate immunity, is developed by phagocytic cells (macrophages, monocytes, granulocytes, polymorphonuclear cells), followed by endotoxins of gram-negative bacteria and mainly the lipid A and teichoic acid from gram-positive bacteria that activate immunoglobulins and induce a specific or acquired immune response, with the release of primary inflammatory mediators. This cellular reaction triggers an excessive inflammatory response with release of secondary mediators, including cytokines, complement factors, the prostanoids, the activation of platelet aggregating factor (PAF) and the generation of reactive oxygen species (ROS)[3]. These functional changes induce thrombosis of the microvasculature with hypercoagulability by fibrin deposits, evolving to a condition of disseminated intravascular coagulation (DIC), especially in cases of severe sepsis and septic shock[4].

In kidneys, the induction of inflammatory processes and the generation of ROS activate the coagulation cascade in the renal microvasculature, resulting in a state of hypoxia and hypotension. The increased ROS generation is observed, which directly interferes with the cell signaling cascade and exerts deleterious effects on renal tubule cells, highlighting the lipid peroxidation of the cell membrane, protein oxidation and DNA damage[5-6]. The vasoconstrictor predominance results in decreased renal blood flow (RBF) hence, reduces the glomerular filtration rate (GFR)[5] and unbalances the excretion of electrolytes and water, with accumulation of nitrogenous metabolites such as urea and creatinine[7]. The systemic increase of these compounds establishes the clinical diagnosis of acute kidney injury (AKI)[8].

In the clinic, the AKI is characterized as abrupt reduction in kidney function, with increase in the absolute value of serum creatinine, equal to or higher than 0.3 mg/dl or increased 1.5 times or more, compared with baseline creatinine or yet, the urinary volume lower than 0.5 ml/kg/h for six hours or more[8]. The occurrence of AKI drastically increases the mortality profile of the patient with sepsis.

Considering the particularities of sepsis-induced AKI, it is extremely important to analyze all its variables, and seek possibilities to interfere in complications. In this context, the pharmacological interventions that reduce the mechanism of oxidative damage in sepsis stand out. Among the alternatives, statins have been investigated for their pleiotropic actions, i.e., not lipid-lowering. Statins are anti-inflammatory[9], antioxidant[10-11], immunomodulatory[12], antiproliferative[9], antithrombotic and with action of endothelial protection[11]. Data from experimental models in vitro, in vivo and clinical studies suggest that statins are potentially beneficial agents in the condition of sepsis-induced AKI.

Thus, the objective of this study was to evaluate the effect of preconditioning with simvastatin in animals with sepsis-induced AKI.

METHOD

The procedures for this study are in accordance with the ethical principles for animal experimentation adopted by the Colégio Brasileiro de Experimentação Animal (COBEA). The study was approved by the Ethics Committee in Animal Experiments of the Faculty of Medicine, Universidade de São Paulo (CEUA-FMUSP), under protocol number 378/13.

Animals: Male Wistar rats, weighing between 250 and 350 g. The animals were kept in collective cages with free access to water and food in thermal conditions, with alternating cycles of day and night.

The animals were divided into the following groups: **SHAM** (sepsis control) - the animals underwent laparotomy with cecal manipulation and received fluid resuscitation of 25 ml/kg of sodium chloride 0.9%, which was administered intraperitoneally (i.p.) at the end of the CPL (cecal puncture ligation) procedure in the sixth, eighth, and 24th hours of the postoperative period[13]; **SHAM + Statin** – the animals were preconditioned with 0.5 mg/kg of simvastatin by gavage (p.o.) for five days, once a day[14] and underwent laparotomy (cecal manipulation), with start of fluid resuscitation; **Sepsis** - the animals were anesthetized with ketamine / xylazine (75 mg/kg / 10mg/kg) intraperitoneally and underwent laparotomy for completing the CPL procedure with fluid resuscitation; **Sepsis + Statin** - the animals were pretreated with simvastatin for five days and submitted to CPL and fluid resuscitation.

Metabolic cage: A day (24 hours) after surgery, the animals were placed in individual metabolic cages for 24-hour urine collection for evaluation of renal function and oxidative metabolites.

Physiological parameters: At the end of the 24-hour period, the animals were anesthetized with thiopental sodium (100 mg/kg) i.p., for evaluation of rectal temperature with a Premium Oval® clinical thermometer of mercury column. Then, a small sample of tail blood of the animal was collected to evaluate blood glucose with the Accu-Chek Active®.

Culture of peritoneal fluid: 2 ml of peritoneal fluid were collected in laminar flow cabinet using aseptic technique, transferred to a test tube with culture medium supplemented with TSB (Tryptic Soy Broth, Bacto™ BD, Lot. 2206180), and remained incubated in an oven for 14 days at approximate temperature of 37 ° C. Readings were done in the periods of 72 hours, seven days and 14 days for checking the turbidity of extract[15].

Whole-blood collection: The whole blood collection was carried out by puncture of the abdominal aorta and subsequent evaluation of renal function.

Renal function: It was evaluated by creatinine clearance. The colorimetric Jaffé method was used to determine the values of serum and urinary creatinine. Creatinine clearance was calculated by the formula: creatinine clearance = urine creatinine x urine output 24h / serum creatinine[16].

Urinary peroxides: It was carried out with the FOX-2 method, by the use of orange iron-xylenol, which oxidizes Fe2+ and produces a purplish-blue color complex (α= 4.3 x 10^4 M^{-1} cm^{-1})[17].

Urinary TBARS: Allows the evaluation of the cascade end products of lipid peroxidation which react in the presence of thiobarbituric acid in body fluids (α= 1.56 x 10^5 M^{-1} cm^{-1})[18].

Severity of AKI and survival curve: The severity of AKI was classified according to serum creatinine and the AKIN criteria (Acute Kidney Injury Network). By this criterion, the AKI can be classified into three stages: the AKIN stage 1, in which is observed the increase of 0.3 mg/dl, or of one to two times the baseline serum creatinine value, or urine output less than 0.5 ml/kg/h for 6 hours; AKIN stage 2, where there is an increase of two to three times the baseline serum creatinine value, and urine output less than 0.5 mL/kg/h for 12 hours; the AKIN stage 3, which represents an increase of more than three times the baseline serum creatinine value, or urine output less than 0.3 mL/kg/h for 24 hours[8]. The survival rate was analyzed by the adaptation of the Kaplan-Meier curve[19].

Statistics: The variance between groups was analyzed by One Way ANOVA test, followed by the Newman-Keuls multiple comparison post-test of the *Graph-Pad Prism version-3 for Windows®*. Values of p<0.05 were considered significant.

RESULTS

Body temperature and blood glucose

Table 1 shows that Sepsis and Sepsis + Statin groups showed significant reduction in body temperature and elevated blood glucose compared to the SHAM and SHAM + Statin groups (p<0.001).

Table 1 - Results related to body temperature and blood glucose of various groups - São Paulo, 2013.

Grupos	N	Temperature (ºC)	Glycemia (mg/dl)
SHAM	8	36.7±0.6	106.9±8.4
SHAM + Statin	8	36.4±0.3	107.9±8.3
Sepse	8	34.8±0.6[ab]	299.0±7.2[ab]
Sepse + Statin	8	35.1±0.4[ab]	300.3±9.3[ab]

[a]p < 0,001 vs SHAM.
[b]p < 0,001 vs SHAM + Statin.
Data show mean ± standard deviation

Culture of peritoneal fluid

O grupo SHAM apresentou resultado negativo para o crescimento de micro-organismo, verificado pela ausência de alterações no extrato em 72 horas, sete dias e 14 dias de incubação. Por outro lado, o grupo Sepse apresentou resultado positivo para cultura do líquido peritoneal com turvação do extrato em 72 horas, sete dias e 14 dias de incubação.

Renal function

A Table 2 shows that animals which suffered induction of sepsis showed a significant reduction in urinary output, elevated serum creatinine, and consequent reduction in creatinine clearance (p<0.05). In contrast, it was found that preconditioning with simvastatin in septic animals significantly reduced the level of serum creatinine, resulting in elevation of creatinine clearance in relation to the Sepsis group (p<0.05), with maintenance of urine output when compared with Sepsis animals.

Urinary peroxides and TBARS

Table 3, shows that the Sepsis group showed an increase in the excretion of urinary peroxides when compared to the SHAM and SHAM+Statin groups (p<0.001). The Sepsis+Statin group demonstrated significant reduction of urinary peroxides in relation to the Sepsis group (p<0.001).

Regarding urinary TBARS, it was found that the Sepsis group had higher levels of this metabolite compared with the SHAM and SHAM+Statin groups (p<0.001). The preconditioning with simvastatin significantly reduced the TBARS levels in septic animals (p< 0.001).

Table 2 - Results related to global renal function of various groups - São Paulo, 2013

Grupos	N	Urinary Output (ml/ min)	Urine Cr (mg/dl)	Serum Cr (mg/dl)	Crcl/ 100g (ml/ min)
SHAM	8	0.015 ± 0.003	57,66 ± 17.5	0.32 ± 0.08	0.85 ± 0.08
SHAM + Statin	8	0.014 ± 0.002	58,06 ± 12.5	0.35 ± 0.08	0.83 ±0.09
Sepse	8	0.007 ± 0.003[ab]	99,52 ± 37.3	0.93 ± 0.11[ab]	0.22 ± 0.07[ab]
Sepse + Statin	8	0.009 ± 0.002[ab]	49,82 ± 16.1	0.41 ± 0.10[ac]	0.46 ± 0.08[abc]

Note: Cr – creatinine, Crcl –creatinine clearance.
[a]p < 0.05 vs SHAM.
[b]p < 0.05 vs SHAM + Statin.
[c]p < 0.05 vs Sepse.
Data show mean ± standard deviation.

Table 3- Results related to the values of urinary peroxides and TBARS of various groups - São Paulo, 2013.

Groups	N	Urinary peroxides (nmol/g of UCr)	Urinary TBARS (nmol/g of UCr)
SHAM	8	5.3 ± 1.3	219.5 ± 22.7
SHAM + Statin	8	6.2 ± 2.7	200.6 ± 26.3
Sepse	8	20.3 ± 5.8[ab]	945.6 ± 26.9[ab]
Sepse + Statin	8	11.6 ± 3.8[abc]	616.8 ± 25.8[abc]

Note: UCr – urinary creatinine, TBARS – thiobarbituric acid reactive substances.
[a]p < 0,001 vs SHAM.
[b]p < 0,001 vs SHAM + Statin.
[c]p < 0,001 vs Sepse.
Data show mean ± standard deviation.

Severity of AKI and survival curve

Figure 1 shows the classification of the severity of AKI using the serum creatinine variation (Figure 1A) for the various groups. In the study, it was found that the SHAM and SHAM + Statin groups had serum creatinine values that were considered reference of normality, so these animals were classified as *no AKI* and showed a survival rate of 100%.

According to the AKIN criteria for classification of severity of AKI, the animals of the Sepsis group were stratified in stages AKIN 2 and AKIN 3. It was found that 75% of the animals in the Sepsis +Statin group were classified as AKIN stage 1, and 15% as AKIN stage 2, while 85% of animals with sepsis were classified as AKIN 2 and 15% as AKIN 3.

The adapted survival curve of Kaplan-Meier (Figure 1B) showed a survival rate of 100% for animals in the SHAM and SHAM + Statin groups. The animals submitted to CPL showed a survival rate of 62% and the preconditioning with simvastatin determined the rise of 80% in the survival rate of animals with sepsis.

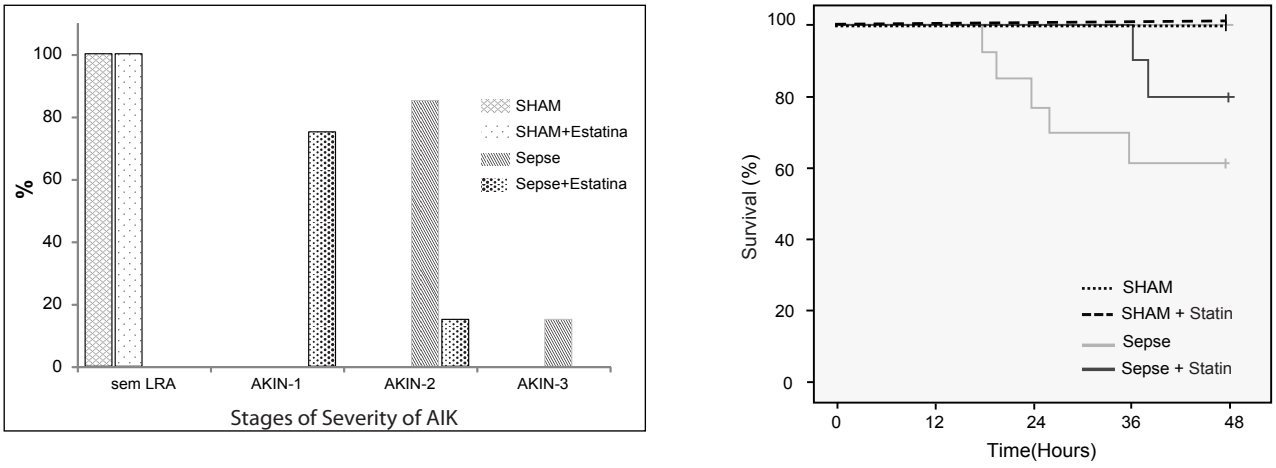

Figure 1– Stages of severity of AKI and survival curve - São Paulo, 2013

DISCUSSION

The present study demonstrated that preconditioning with simvastatin in rats with sepsis-induced AKI attenuated the renal dysfunction, increased GFR and decreased the release of oxidative metabolites, showing favorable impact on the severity of AKI and survival of animals.

The results of this study reaffirmed the accuracy of the AKI model, since animals evolved with physiological parameters characteristic of sepsis. They showed a reduction in body temperature, increased rate of blood glucose and the presence of microorganisms in the culture of peritoneal fluid. According to the definition of the *International Sepsis Definitions Conference*, held in 2001, the diagnosis of sepsis is suggested by primary nonspecific clinical findings, such as fever or hypothermia, tachycardia, tachypnea, and hypotension. In clinical, the laboratory findings revealed leukocytosis or leukopenia, hyperglycemia and hyperlactatemia. Subsequently, the syndrome is confirmed by isolation of the etiologic agent in cultures of different biological materials[20].

According to the current guidelines of the Surviving Sepsis Campaign, in face of the diagnosis, the intravenous fluid resuscitation is recommended as an immediate intervention, as well as vasoactive drugs administration, oxygen delivery and, when necessary, mechanical ventilation. These measures positively affect the mortality rates in patients with sepsis[21].

In this study, all animals received fluid resuscitation with sodium chloride 0.9%. The hyperhydration was essential for keeping the animals subjected to CPL, since the model reproduces a serious medical condition, which explains the high mortality of animals[8].

The first manifestation in response to the severe sepsis is the reduction of oxygen to the tissues due to microvascular thrombosis and hypotension. In parallel, hyperglycemia occurs with high prevalence in patients with severe sepsis[21]. The stress hyperglycemia, or the hyperglycemia condition displayed by the animals subjected to CPL is caused by excessive glucose production by liver cells and increased insulin resistance. There are evidences that hyperglycemia is a physiological and beneficial response of cells, and frequently it is well tolerated by the organism. Therefore, hyperglycemia must be treated only when the renal excretion of glucose results in osmotic diuresis and consequent hypovolemia of the patient[22].

In kidneys, the dysfunction of the microvasculature increases capillary permeability and reduces the RBF, with disproportionate increase of the vasodilatory action on the afferent arteriole compared to the efferent. The reduction in pressure in the afferent arteriole reduces the intraglomerular pressure gradient, decreasing the GFR[7], with consequent oliguria and avid sodium absorption by the tubuloglomerular feedback mechanism[7-8]. In this study, the decline in GFR, with reduced creatinine clearance and decreased urine flow characterized the oliguric AKI in animals in the Sepsis group.

Additionally, the Sepsis group showed increased oxidative metabolites in urine in the group submitted to the CPL, confirming the generation of ROS through analysis of urinary peroxides and TBARS. The cellular hypoxia and the interaction between the microorganism and the host immune system contribute to the formation of ROS, with subsequent oxidative and kidney damage[5-7].

The AKI is a disorder that involves clinical manifestations, which may present with minimal change in serum creatinine, progressing to anuria and subsequent renal failure. Epidemiological studies have shown an association between AKI and increased mortality rates, particularly for patients in need of renal replacement therapy (dialysis)[1].

In a current approach, the diagnostic criteria involving the evaluation of urinary flow and serum creatinine are used to identify the stage of renal impairment[1,8].

In the present study, the severity of AKI was classified according to the AKIN criteria, using the value of serum creatinine. The results showed that the Sepsis group had greater severity of AKI, considering that 85% of the animals were stratified in AKIN stage 2 and 15% in AKIN stage 3. The mortality rate among the Sepsis animals was 38%. The same results were observed in a study[9] of animal model, in which the mortality increase in the groups was related to the severity of AKI, similar to what was observed in this study[19].

One factor that positively interferes in the treatment of sepsis is the prevention of multiple organ dysfunction, i.e., the investigations focused on secondary reactions for reducing ROS generation, or inhibiting the anticoagulation cascade, show promising results for protection of organs, particularly the kidney and lungs[4-5].

Among these possibilities, there are the statins, which belong to the class of drugs with lipid-lowering action, with the ability to reduce the action of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, that reflects in the reduction of serum levels of total cholesterol, low density lipid cholesterol (LDL), apolipoprotein B and triglyceride levels[23].

In this study, the preconditioning with simvastatin demonstrated a protective effect on renal function of animals subjected to CPL. Despite not observing improvement in urine flow, simvastatin increased creatinine clearance, which confirmed the recovery of renal function. This probably occurred largely in response to its antioxidant action, which became evident due to the reduction of urinary peroxides and TBARS. Other experimental studies confirm these results, since the administration of simvastatin in a cisplatin-induced nephrotoxicity model resulted in improved renal function, with an increase in the level of antioxidant enzymes and reduction of oxidative metabolites[24]. An *in vivo* study in ischemic AKI model associated with statin therapy showed a reduction of ROS and consequent improvement in renal function[14].

The animals induced to sepsis and treated with simvastatin (75%) were classified as AKIN stage 1 for AKI severity, with improvement in survival rate and mortality reduction for 20%. These results confirm a direct relationship between serum creatinine values, stage of severity of AKI and the mortality rate of animals with sepsis.

The results of this experimental study highlight and complement the experiences in clinical sepsis, and show advantages for its understanding because they enable the isolation of variables. It is worth noting that the sepsis-induced AKI has important differences, such as responses to interventions and clinical outcomes compared to AKI not associated with sepsis[25].

CONCLUSION

The technique of CPL induced a condition of sepsis, evidenced by hypothermia and the increase in blood glucose and oliguric AKI with increased urinary excretion of peroxides and TBARS in the animals. The induction of sepsis consisted in greater severity of AKI, with high mortality rate. The preconditioning with simvastatin improved the renal function and reduced the release of oxidative metabolites in the urine of animals, confirming its action as an antioxidant.

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