Effect of pre-treatment with simvastatin in aged rats with abdominal sepsis

Efeito do pré-tratamento com sinvastatina em ratos idosos com sepse abdominal

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ABSTRACT

Background/purpose: Sepsis is a severe inflammatory disease, high-risk, representing the immune response to infection with high morbidity and mortality. It is a particularly serious problem in the geriatric population. Advanced age is a risk factor for worsening of the infection, and the inhibition of inflammation is one of the many pleiotropic effects of statins. The objective of this study was to assess whether pretreatment with simvastatin alters the response of the elderly rat with abdominal sepsis model. **Methods:** We examined: 1- The effect of simvastatin on survival and the prevention and treatment of abdominal sepsis induced by cecal ligation and puncture (CLP). 2- Effect of simvastatin in the expression of proinflammatory cytokines and quantification of peripheral blood leukocytes. Twenty four Wistar rats were used: 12 young (3 months old). Six rats were treated with simvastatin v.o. and 6 with 0.9% saline solution; Six aged animals (24 months old; n=6) were treated with simvastatin and 6 with 0.9% saline solution. **Results:** In the aged rats treated with simvastatin 2 deaths occurred. One held 6 hours after CLP and the other one after 16 hours. In the young rats with sepsis treated with saline (n = 6), there was 1 death. In aged animals treated with simvastatin (n = 6) the number of total leukocytes and neutrophils was significantly lower in those treated with saline, the same occurring in young animals ($p<0.05$). Comparing the old and young animals treated with saline, no significant differences were observed in total leukocyte count ($p> 0.05$). As for lymphocytes, no significant differences between groups ($p> 0.05$) were observed. It was found that the % eosinophils was significantly higher in young animals treated with simvastatin compared to aged animals ($p <0.05$). Overall animals treated with simvastatin showed percentage of eosinophils significantly higher than those treated with saline. Serum

levels of TNFα, IL-1β and IL-2 showed significantly higher values in older animals treated with simvastatin than in young rats treated with the same drug (p<0.05). Comparing the old and young saline-treated animals, the values in the measurement of cytokines were consistently higher in the elderly, with no statistically significant differences (p> 0.05). **Conclusion:** This study demonstrated that *in vivo* administration of simvastatin, an inhibitor of HMG-CoA reductase inhibitor, decreased mortality, the leukocyte counts in peripheral blood and the expression of proinflammatory cytokines, attenuating abdominal sepsis in aged rats, in which the response to sepsis was more intense than in young animals. These observations may help to extend a new field in the therapeutic use of statins with respect to the attenuation of inflammation in cases of abdominal sepsis in the elderly.

Key words: Abdominal Sepsis, Elderly, Rat, Simvastatin, Cytokines.

RESUMO

Objetivo: Sepse é uma doença inflamatória grave, de alto risco, que representa a resposta imune a uma infecção, com alta morbidade e mortalidade. É um problema particularmente grave na população geriátrica. A idade avançada é um fator de risco para agravamento da infecção, e a inibição da inflamação é um dos muitos efeitos pleiotrópicos das estatinas. O objetivo do presente trabalho foi avaliar se o pré-tratamento com a sinvastatina altera a resposta do rato idoso à sepse abdominal. **Métodos:** Foram examinados: 1- A ação da sinvastatina na sobrevida e na prevenção e tratamento da sepse abdominal induzida pela ligadura e punção do ceco (LPC) de ratos. 2- Repercussão do uso da sinvastatina na expressão de citocinas pró-inflamatórias e na quantificação de leucócitos periféricos. 24 ratos Wistar foram usados: 12 jovens (3 meses de idade), sendo 6 tratados com simvastatina v.o. e 6 com solução salina 0,9%; 12 animais idosos (24 meses de idade), sendo 6 tratados com simvastatina e 6 com solução salina 0,9%. **Resultados:** Nos grupos de ratos tratados com sinvastatina foi observado que, entre os animais idosos, não tratados com sinvastatina (n=6), ocorreram 2 óbitos. Um deles após 6 horas de realizada a PLC e outro após 16 horas. No grupo dos ratos jovens com sepse, tratado com solução salina (n=6), houve 1 óbito antes de completadas 24 horas da PLC. Nos animais idosos tratados com sinvastatina (n=6) o número de leucócitos totais e de neutrófilos foi significativamente menor que nos tratados com salina, o mesmo ocorrendo no grupo de animais jovens (n=6). (P<0,05). Entre os animais idosos e jovens tratados com salina não foram observadas diferenças significantes nos leucócitos totais (p>0,05). Quanto aos linfócitos, não foram observadas diferenças significantes entre os grupos (p>0,05). Verificou-se que o % de eosinófilos foi significativamente maior no grupo de animais jovens tratados com sinvastatina, comparados aos animais idosos tratatos (p<0,05). O global de animais tratados com sinvastatina apresentaram percentuais de eosinófilos significativamente mais elevados do que os tratados com salina. As dosagens de TNFα, IL-1β e IL-2 mostraram valores significativamente mais elevados nos animais idosos tratados com sinvastatina, do que nos ratos jovens tratados com a mesma droga (p<0,05). Comparando-se os animais idosos e jovens tratados com solução salina, os valores nas dosagens de citocinas foram sistematicamente mais elevadas nos idosos, porém sem diferenças estatisticamente significantes (p>0,05). **Conclusão**: Este estudo demonstrou *in vivo* que a

administração de sinvastatina, um inibidor da HMG-CoA redutase, diminuiu a mortalidade, a contagem de leucócitos no sangue periférico e a expressão de citocinas pró-inflamatórias, atenuando a sepse abdominal induzida em ratos idosos, nos quais a reação à sepse foi mais intensa que nos animais jovens. Essas observações podem contribuir para expandir um novo campo no uso terapêutico de estatinas, no que diz respeito à atenuação da inflamação em casos de sepse abdominal em indivíduos idosos.

Descritores: Sepse abdominal, Idoso, Rato, Sinvastatina, Citocinas.

INTRODUCTION

In 1976, Endo et al. and Brown et al isolated mevastatin, the first discovered family of statin drugs, which are able to inhibit the HMG-CoA reductase (enzyme responsible for cholesterol biosynthesis)[1-3]. This group of drugs has attracted the attention of numerous research centers because of its ability to reduce serum cholesterol levels and soon emerged several other statins[3]. Several studies now report that the use of statins was responsible for a significant decrease in the levels of serum lipids, with encouraging results (reduction of ischemic attacks) in patients with atherosclerosis and coronary disease, regardless of presenting symptoms of these diseases [4-6]. Subsequent studies began to reveal other actions (pleiotropic), cholesterol independent, such as anti-inflammatory action, thrombogenesis inhibiting and even immunomodulatory[5,7,8,9]. Among the various lines of research aimed at explaining his actions on inflammation highlights the modulation (stimulation) of nitric oxide synthase-derived endothelial (eNOS), where statins would increase the concentrations of nitric oxide (NO) [10,11].

Pruefer et al demonstrated anti-inflammatory properties of simvastatin in the presence of infection by *Staphylococcus aureus* toxin. Starting from the principle that entoxemia is a potent stimulus for vascular inflammation, they demonstrated that pre-treatment with clinical doses of simvastatin 18 hours before injection of endotoxin attenuated leukocytes moving induced by endotoxin and their migration to the mesentery[12]. In 1997, Laufs et al demonstrated that statins acted directly on the pathway of NO, increasing its regulation. This work, which was an initial step in the study of the functions of these statins, simvastatin showed increased half life of the mRNA encoding eNOS[11]. Following the discovery that statins regulate the function of eNOS, some studies have reported potent anti-inflammatory actions of these drugs, which are dependent eNOS. Lefer et al were the first to report anti-inflammatory effects of statins in an experimental model of isquernia/reperfusion injury[13]. They showed a significant inhibition of endothelial cell-leukocyte interaction, regardless of their anti-cholesterol actions[14,15]. Significant anti-inflammatory effects have been demonstrated in situations like myocardial infarction in experimental models of normocholesterolemic, hypercholesterolemic and diabetic patients [13,16]. It was shown that the treatment with statins inhibits leukocyte accumulation in tissue subjected to ischemia and reperfusion, which is highly dependent on eNOS [13,15,16] action. Concerning this, there is a prominent

role to the activated monocytes in the inflammatory reaction, which release a number of proinflammatory factors, including tissue factor and various cytokines, including tumor necrosis factor (TNF) and interleukin-6 (IL -6) [17,18].

Therapy with these drugs attenuates vascular inflammation in patients, as evidenced by the significant reduction of inflammatory markers such as C-reactive protein[19-2]]. Ando et al in 2000 revealed that sepsis in mice injected with LPS (lipopolysaccharide) can be prevented with the use of statin (cerivastatin) with significant improvement in survival[22]. Stefanec[23] stated that apoptosis is the central event of sepsis-related phenomena (bacteremia, systemic inflammatory response syndrome, disseminated intravascular coagulation). It is argued that LPS, tumor necrosis factor and CLP trigger endothelial apoptosis *in vivo*. The endothelium activated by these factors tends to spread indiscriminately, making systemic an initially localized condition. Drugs with anti-inflammatory action have not a satisfactory effect on sepsis because they not act on the central event (apoptosis). Nevertheless, statins have shown anti-apoptosis effect through stimulation of eNOS [23]. Liappis et al in 2001 performed a retrospective study where the effect of statins was evaluated in patients with bacteremia and showed that mortality rates were lower in patients receiving the drugs in question. They disagree that apoptosis has a central role and prefer to understand the effect of statins as resulting from multiple actions such as inhibition of the expression of adhesion molecules, important in leukocyte/endothelial integration, regulation of chemotactic proteins, among others[24]. The mortality from sepsis is highly dependent on the inflammatory response and cytokine expression[25-27]. Sepsis is a severe high-risk inflammatory disease, representing the immune response to infection, a common disorder that affects about 750,000 people per year in the United States[28]. It is a particularly serious problem in the elderly population, with a relative risk of 13.1 times higher compared to young patients. Case-fatality rates increase linearly with age, being an independent mortality predictor[29]. While this issue is increasingly recognized, the mechanisms responsible for this vulnerability associated with age are unknown.

Assuming that advanced age is a risk factor for worsening the infection, and that inhibition of inflammation is one of the many pleiotropic effects of statins, the objective of this study is to test this hypothesis: simvastatin alters the host response of the elderly to abdominal sepsis induced by cecal ligation and puncture in rats, as well as prevents and treats established sepsis. In literature review we did not find clinical trials and preclinical experimental studies investigating this line of research related to statin therapy in elderly with sepsis. Depending on the results of this research new horizons can opened in the treatment of sepsis with statins as anti-inflammatory and immunomodulatory drugs, particularly in elderly.

OBJECTIVES

This study aimed to examine the effects of simvastatin, which are not related to their lipid-lowering action, in an experimental model of abdominal sepsis in aged rats. Were focused:

1. The effect of simvastatin on survival and treatment of abdominal sepsis induced by cecal ligation and puncture in rats.
2. Effect of simvastatin in the expression of proinflammatory cytokines.
3. Effects of simvastatin on the peripheral leukocytes count.

METHODS

Twenty four Wistar rats were randomly divided into 2 groups. Twelve young rats (3 months aged) and 12 elderly rats (24 months of aged) were kept in individual cages with water and standard rodent food *ad libitum.* They were previously acclimated in the Lab for 7 days, kept under controlled temperature (21ºC), humidified air, with 12 hours clear/dark cycles, and handled in accordance with the standards of the Ethics Committee on Animal Use (CEUA) and the Brazilian College of Animal Experimentation (COBEA).

All of them were anaesthetized with xylazine 20 mg/kg and ketamine 50 mg/kg intraperitoneally (IP). The abdominal wall was shaved and antisepsis with 70% ethyl alcohol was performed and the surgical procedures were performed under aseptic technique. A 4 cm laparotomy was followed by cecum ligation and puncture (LPC). Then the organ was replaced in the abdominal cavity, which was closed with 4-0 nylon suture. Postoperative pain was controlled with meperidine 3 mg / kg, s.c. The animals were handled in the Nucleus of Experimental Surgery, Dapartment of Surgery, UFRN, Brazil.

Experimental design

Twelve young rats (3 months old) were treated as follows: 6 rats received simvastatin and 6 were treated with 0.9% saline solution; Twelve aged rats (24 months old) were olso treated with simvastatin (n=6) and with 0.9% saline solution (n=6). The treated rats were injected once daily 10 mg/kg/day of simvastatin (suspension) by gavage for 3 days before induction of peritonitis and 2 hours before surgery (LPC). In the others, the animals received oral injection of 1 ml of saline 0.9% during the same intervals.

Survival

The animals were kept under surveillance for 24 hours and survival was recorded at hourly intervals.

Laboratory measurements

In rats that survived for 24 hours, we collected a sample of whole blood by cardiac puncture for the following dosages: tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-2 (IL-2). Plasma was separated by centrifugation at 2000 rpm and stored at -40°C for subsequent dosing by ELISA (enzyme-linked immunosorbent-assay-) analytical method based on antigen-antibody interaction that determines specific amounts of proteins in samples. The kits were purchased from PeproTech, USA. In addition, peripheral blood leukocytes were counted.

Statistical analysis

All data were presented as mean±standard deviation and compared using BioStat 5.0 software. Analysis of variance ANOVA was performed, using pot-hoc

Bonferoni test. The difference between means was considered statistically significant when $p<0.05$.

RESULTS

We observed that among the older animals, not treated with simvastatin, two deaths occurred. One held after 6 hours of the PLC and the other after 16 hours. Meanwhile, in group of young rats with sepsis treated with saline, only one died before completed 24 hours of PLC (Table 1).

Table 1 – Mortality occurred in animals in the various groups.

	CPL+ simvastatin	CPL+saline
Old rats	0	2
Young rats	0	1

There was a significant difference comparing the mortality between groups of young and old animals with sepsis treated with saline.

The mean values of the different samples submitted to white blood cell count (WBC) are summarized on table 2.

Table 2 – Values of total leukocytes count and percentage of neutrophils, lymphocytes and eosinophils in CPL young and old animals, with and without treatment with simvastatin.

Leukocytes Groups	***Leuk/µL)**(1)**	***Neutrophils (%)**(1)**	***Linfocyts (%)**(2)**	***Eosinophys(%)**(2)**
Aged/saline	14.36 ± 1.16a	79.35 ± 6.51ab	34.14 ± 5.58a	0.37 ± 0.42a
Aged/simvastatin	8.42 ± 0.41ab	60.47 ± 6.19ac	29.12 ± 9.54a	0.56 ± 0.15b
Young/saline	13.78 ± 0.52bc	76.14 ± 4.60c	32.13 ± 6.21a	0.34 ± 0.14a
Young/Simvastatin	6.62 ± 0.31ac	51.03 ± 3.55bc	30.24 ± 5.45a	0.68 ± 0.10c

*Mean ± Standard deviation; (CPL, cecal ligation and puncture)
(1) Measures followed by the same letter differ significantly at the level of $p<0.05$ by the Bonferroni test.
(2) Measures followed by the same letter do not differ significantly at the level of $p<0.05$ by the Bonferroni test.

When comparing the values of Table 2, for animals with sepsis, the Bonferroni test accused significant differences ($p<0.05$) in several parameters. In the animals of the elderly subgroup treated with simvastatin the number of total leukocytes and neutrophils was significantly lower than in those treated with saline, the same occurring in young animals ($p<0.05$). Total leukocytes, and neutrophils were significantly higher in treated elderly when compared with young rats ($p<0.05$). Among old and young animals treated with saline, no significant differences were observed in total leukocyte count ($p>0.05$). Comparative the measures of lymphocytes, no significant differences were observed among groups ($p>0.05$). We observed significantly higher percentage of eosinophils in young animals treated with simvastatin compared to treated aged animals ($p<0.05$). The animals treated with simvastatin showed percentage of eosinophils significantly higher than those treated with saline (Table 2).

Table 3 – Serum values of TNF, IL-1β e IL-2 in all rat groups.

GROUPS	TNF α (pg/ml)	IL-1β (pg/ml)	IL-2 (pg/ml)
Aged/saline	778.5±56[a]	231.9±44[a]	133.6±21[a]
Aged/simvastatin	564.8±44	126.3±14	98.4±8
Young/saline	711.3±43.1[a]	215.1±11[a]	122.8±14[a]
Young/simvastatin	410.7±24.5	87.3±8	83.1±11

Measures followed by the same letter do not differ significantly at the level of p<0.05 by the Bonferroni test.

The data in Table 3 show that serum TNFα, IL-1β e IL-2 levels are significantly higher in older animals treated with simvastatin than in young rats treated with the same drug (p <0.05). Comparing the old and young saline-treated animals, the values in the measurement of cytokines are consistently higher in the elderly, although no statistically significant differences are observed (p>0.05).

DISCUSSION

It has been clearly demonstrated that simvastatin, administered 18 hours before endotoxin infusion in pharmacological doses, is able to attenuate the interaction of leukocytes to cells, most likely via selectin-dependent mechanism[12]. Because of the important role of leukocytes during sepsis, this study was designed to investigate the protective role of simvastatin in counting these cells in peripheral blood, through experimental model of abdominal sepsis by cecal ligation and puncture (CLP), comparing young and old animals.

We have chosed this experimental model of CLP because it repeats widely and with relative accuracy the pathophysiology of multibacterial sepsis that occurs in humans. The CLP involves a complex pathophysiological scenario, with activation of multiple inflammatory mediators, simulating disease found in septic patients[30].

The present study demonstrated that simvastatin, administered prior to sepsis induction, was able to attenuate the number of leukocytes in peripheral blood most likely explained not by the cholesterol effects of simvastatin, but likely by inhibitors mechanisms of HMG-CoA. Furthermore, the anti-inflammatory effect of simvastatin in aggression caused by toxins may have caused the reduction in the count of peripheral blood leukocytes. Kruger et al [31] analyzed a cohort of 438 patients with bacteremia. Approximately 66 (15%) of patientes received statins orally in the hospital admissions and 56 (85%) continued statin therapy during hospitalization. Mortality was significantly lower in patients treated with statins than in untreated (0.6% vs. 23.1%). The difference persisted when deaths were related to bacteraemia, immunosuppression and infection caused by Escherichia coli, differing in significantly between groups. The authors observed that the difference in mortality was primarily related to the maintenance statins treatment, ie, mortality was higher in patients who had discontinued treatment with the drug. Liappis et al reported a retrospective study of 388 bacteremic patients in intensive care unit (UCI) [24]. Demonsraram that patients using statins at the time of ICU admission with bacteremia due to Gram-negative aerobic bacilli and bacilli type Staphylococcus aureus, had a significant reduction in hospital mortality (6% versus

28%, P = 0.002), comparing with controls not using statins. These patients had high incidence of diabetes mellitus, hypertension, and cardiovascular disease in the statin group. Similarly, in the present study, a significant reduction was observed in the mortality of animals with sepsis and treated with simvastatin, particularly in young rats.

The action of statins in reducing serum cholesterol levels is well described and understood [4,5], but their pleiotropic effects are yet to be defined, and several lines of research are trying to explain them [10,11]. Its anti-atherosclerotic action also seems to depend on several effects that not only reduction in cholesterol; the anti-inflammatory role seems to be a key point in understanding the stabilization of atherosclerotic plaque and combating the progression of the disease. Some studies state that patients undergoing cardiac transplantation, using statins, have better results because there is a possible immunomodulatory effect that beneficit them[28]. The cascade of inflammation and the immune system are mainly involved in the complex system of sepsis. Research about this therapeutic are promising and have publish important results [26,27]. The use of statins in sepsis has no well-defined mechanisms, but it has scientific basis due to its immunomodulatory and anti-inflammatory effects [32-34].

Experimental research and clinical trials that confront statins and sepsis are scarce, the topic is recent, but the few published studies have reported encouraging results [35]. In our literature review did not find any work to investigate this line of research concerning elderly. Were did not found in the international literature, published studies that tested the response to statin therapy in experimental models of abdominal sepsis in the elderly.

Ando et al revealed that sepsis in rats triggered by injection of lipopolysaccharide (LPS) can be prevented with the use of statin (cerivastatin) with significant improvement in survival[35]. Some studies suggest that apoptosis plays an important role in immune dysfunction and multiple organ failure seen in sepsis and endotoxemia[36,37]. It is argued that LPS, and tumor necrosis factor after CLP trigger endothelial apoptosis in vivo. The endothelium activated by these factors and local conditions tends to spread the process indiscriminately, making an initially localized systemic condition. Drugs with anti-inflammatory action have not satisfactory effect on sepsis because they do not act on the central event (apoptosis). However, statins have shown anti-apoptosis effect through stimulation of eNOS, which could partly explain the beneficial effects of simvastatin in this study.

It has been shown that the elderly (> or = 65 years old) represent 12% of the North American population but they have 65% of sepsis cases, yielding a much higher level of risk compared to younger patients[38]. Aging is characterized by alterations in immune function and stress responses. It is associated with increased susceptibility to infections, as well as the increased incidence of auto-immune and chronic inflammatory diseases[39]. The elderly have a higher incidence of complications and mortality after bacterial infection[40]. Our data showed that the levels of proinflammatory cytokines were significantly higher in older animals after induction of sepsis, resulting in more severe organ damage and increased lethality, findings corroborated by other authors[41]. Thus, it appears that the alteration of the immune response in the aged population can contribute to a higher mortality rate after bacterial infection, which was observed in the present work.

In summary, simvastatin positively influenced the effectiveness of the treatment of sepsis, as demonstrated in this study in elderly and young animals. We believe that further investigations are mandatory to clarify the effects of simvastatin on sepsis and its mechanisms of action.

CONCLUSION

In conclusion, this study demonstrated that in vivo administration of simvastatin, an inhibitor of HMG-CoA reductase, contributed to reducing mortality, the leukocyte counts in peripheral blood and the expression of proinflammatory cytokines, attenuating abdominal sepsis in aged rats, in which the response to sepsis was more intense than in young animals. These observations may help to extend a new field in the therapeutic use of statins with respect to the attenuation of inflammation in cases of abdominal sepsis in the elderly.

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