Bacterial translocation in rats treated with simvastatin undergoing intestinal ischemia and reperfusion.

Translocação bacteriana em Ratos tratados com sinvastatina submetidos a isquemia intestinal e reperfusão.

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Abstract

Purpose: To evaluate the anti-inflammatory effect of simvastatin in an experimental model of intestinal ischemia/reperfusion, as well as in the prevention of bacterial translocation. **Methods:** We used Wistar rats, randomly allocated in 5 groups: C (n=10) controls; S (N=10) Shan-operated; I/R (n=10) intestinal ischemia and reperfusion; S+Sim (n=7) sham treated with simvastatin and I/R+Sim (n=7) ischemia/reperfusion treated with simvastatin. In the group S, a laparotomy and manipulation of intestinal loops were performed. In the groups I/R and I/R+Sim, the superior mesenteric artery was occluded with a vascular microclamp, the laparotomy was closed and reopened 60 minutes after for pull back the clamp. The reperfusion was confirmed by the return of the pulsation of the mesenteric arcade. The animals were sacrificed after 120 minutes of reperfusion. Simvastatin microemulsion (10mg/kg) was administered (gavage) 18 hs and 2 hours before the surgical procedure. Blood was collected by cardiac puncture for measurement of TNF-α, IL-1β, IL-6 and IL-10. One gram of spleen, liver and mesenteric lymph nodes was harvested for culture in selective means for Gram (-) and Gram (+) bacteria. A sample of terminal ileum of each animal was harvested, fixed in formalin 10% and included in paraffin. Slices were stained with hematoxilin-eosin for morphometric measurement. The damages of the intestinal samples were examined in a blind way by an experienced pathologist, in agreement with microscopic criteria for levels of aggressions based previously on a grade system. ANOVA and the post-hoc Tukey test were used, considering $p<0,05$ as significant. **Results:** We observed bacterial translocation to mesenteric lymph nodes, spleen, liver and blood in all animals submitted to I/R, being smaller in the group I/R treated with simvastatin than in controls. In the I/R group rats the values of

pro-inflammatory cytokines were significantly higher, when compared to the I/R+Sim group rats. The I/R+Sim group rats showed higher levels of IL-10, when compared with the other groups ($p<0.05$). The ileal segments presented macroscopic dilation and intramural hemorrhage. The microscopy revealed intense mucosa lesion in the group I/R compared to the other groups. The histopathologic findings of the I/R+Sim group rats were similar to that found in the groups C and S. **Conclusion:** The simvastatin contributed to reduce the bacterial translocation, the values of pro-inflammatory cytokine and to increase the levels of anti-inflammatory cytokine, preserving the integrity of the intestinal epithelium in an experimental model of ischemia/reperfusion.

Key words: Simvastatin. Ischemia/Reperfusion. Bacterial Translocation. Sepsis. Rat.

Resumo

Objetivo: Avaliar o efeito anti-inflamatório da sinvastatina em modelo experimental de isquemia/reperfusão intestinal, assim como na prevenção da translocação bacteriana. **Métodos:** Ratos Wistar foram distribuídos aleatoriamente em 5 grupos: C (n=10) controle; S (n=10) simulação (*sham)*; I/R (n=10) isquemia intestinal e reperfusão; S+Sin (n=7) *sham* tratado com sinvastatina e I/R+Sin (n=7) isquemia/reperfusão tratado com sinvastatina. No grupo S, procedeu-se laparotomia e manipulação atraumática de alças intestinais. Nos grupos I/R e I/R+Sin, a artéria mesentérica superior foi ocluída com microclamp vascular por 60 minutos. A laparotomia foi fechada e reaberta após 60 minutos para retirada do clamp e a reperfusão foi confirmada pelo retorno da pulsação da arcada mesentérica. Os animais foram sacrificados após 120 minutos de reperfusão. Microemulsão de sinvastatina (10mg/kg) foi administrada por gavagem 18 horas e 2 horas antes do procedimento cirúrgico. Sangue total foi coletado por punção cardíaca, para dosagem de TNFα, IL-1β, IL-6 e IL-10. Um grama de baço, fígado e linfonodos mesentéricos foram removidos para cultura bacteriana em meios seletivos para Gram (-) e Gram (+). Segmento de íleo terminal de cada animal foi fixado em formalina a 10% e embebido em parafina. Secções coradas com hematoxilina-eosina para usadas para medidas morfométricas. Os danos intestinais foram avaliados de acordo com critérios microscópicos para níveis de lesões baseados em um sistema de graduação previamente descrito. Foram utilizados os testes ANOVA e Tukey, considerando $p<0,05$ estatisticamente significante. **Resultados:** Observou-se translocação bacteriana para linfonodos mesentéricos, baço, fígado e sangue em todos os animais submetidos a I/R, sendo menor no grupo I/R+ Sin, tratado com sinvastatina. No grupo I/R, os valores de citocinas pró-inflamatórias foram significativamente maiores, quando comparados ao grupo I/R+Sin. Os animais do grupo I/R+Sin apresentaram os maiores níveis de IL-10, em relação aos demais grupos ($p<0,05$). Os segmentos de íleo apresentaram dilatação e hemorragia intramural macroscópicas. A microscopia revelou lesão intensa de mucosa nos ratos do grupo I/R comparado aos demais grupos. Os achados histopatológicos do grupo I/R+Sin foram semelhantes aos encontrados nos grupos C e S. **Conclusão:** A sinvastatina contribuiu para a redução da translocação bacteriana, dos valores de citocinas pró-inflamatórias e elevou os níveis de citocina anti-

inflamatória, preservando a integridade do epitélio intestinal em modelo experimental de isquemia e reperfusão.

Descritores: Sinvastatina; Isquemia/Reperfusão; Translocação Bacteriana; Sepse; Rato.

Introduction

Intestinal ischemia-reperfusion (I-R) injury is a severe condition resulting from acute mesenteric ischemia, small bowel transplantation, abdominal aortic aneurysm, hemorrhage, trauma, septic shock, or severe burns[1,2]. Various chemical and cellular mediators have been implicated in the pathogenesis of intestinal ischemia/reperfusion, such as reactive oxygen, cytokines, endotoxins, and neutrophils. Following adhesive interactions among neutrophils and endothelial cells, neutrophil accumulation in the intestinal mucosa contributes to intestinal ischemia/reperfusion injury via production of reactive oxygen metabolites and proteases[3]. Leukocyte accumulation is a complex phenomenon that also involves endothelium-based adhesion molecules as well as leukocyte chemotaxis factors such as interleukin-8 (IL-8). Intercellular adhesion molecules are normally expressed at a low basal level, but their expression can be enhanced by several inflammatory cytokines such as IL-1β and tumor necrosis factor-α (TNF-α). A variety of cytokines, including TNF-α, interferon-γ and IL-1β, are released from post-ischemic tissues[4]

On 1976, in independent publications, Endo et al. e Brown et al, described the first statin able to inhibit the HMG-CoA redutase. These drugs attracted the attention of researchers because of their capacity to reduce serum cholesterol. Then, many other statins were isolated[5-7]. Increasing number of evidence suggests that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, exert pleiotropic effects which are independent from their cholesterol-lowering action[8]. One of these effects appears to be protection against ischemia/reperfusion injury. Several studies dealing with ischemia/reperfusion have shown that statins significantly reduce infarct size not only in heart but also in brain[9,10]. Statins have been shown to elevate the expression of endothelial nitric oxide synthase (eNOS); hence enhancing the basal and stimulated production of NO and improving endothelium dependent vasorelaxation besides promoting antiinflammatory processes[11]. They are involved in diverse cellular functions, including actin cytoskeleton organization, cell adhesion and motility, gene expression, and inflammation. Thus, inhibition of Rho kinases may contribute to some of the cholesterol-independent beneficial effects of statin therapy. It has been postulated that their antiinflammatory effects may be associated with modulation of both adhesion molecule and cytokine production[12,13].

Maintenance of bacteria and their products in the intestinal lumen are made by mucin and epithelial cells, essential for survival. The enterocytes in constant division, form an impermeable barrier to the intestinal contents. Because they are metabolically active, they are susceptible to hypoxia, reducing the absorptive function with loss of basement membrane integrity, leading to the bacterial translocation [14,15].

Berg and Garlington (1979) described the translocation as the passage of viable bacteria through the intestinal mucosa to mesenteric lymph nodes, tissues and órgãos[16]. It is postulated that ischemia and reperfusion promote translocation, bacteremia and toxemia, initiating an inflammatory response and activating inflammatory mediators, including cytokines[17]. In sepsis, the mortality ratio is directly proportional to the intensity of the inflammatory response[18-20]. Based on these principles, the objective of this study was to evaluate the anti-inflammatory effect of simvastatin in an experimental ischemia/reperfusion model, as well as bacterial translocation.

Methods

Animals

Fifty male Wistar rats weighing 265±32g (from Nucleus of Experimental Surgery, Federal University of Rio Grande do Norte-UFRN, Brazil) were used. Rats were housed in polypropilene cages and maintained under controlled temperature conditions on a 12h light-dark cycle and allowed *ad libitum* access to commercially available rat chow and water. The experimental protocol was approved by the Research Ethics Committee of the Federal University of Rio Grande do Norte, Brazil, and adhered to the Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996.

Experimental design

The rats were randomly assigned to 5 groups (n=7 in each group) as described below. The control group (C) received only chow and water; Group sham (S) served as a normal control, and a laparotomy was performed and the rats received only chow and water. The ischemia/reperfusion rats (I/R) were submitted to mesenteric ischemia/reperfusion; The sham/simvastatin (S+Sim) and the Ischemia/reperfusion (I/R+Sim) group rats were treated with simvastatin. Treatment with simvastatin or normal saline (0.9%) was done according to the protocol. Simvastatin rats received 20 mg/Kg of simvastatin microemulsion via gavage for 5 days before surgery.

Animals were fasted 12 hr before the experiment and anesthetized with an intramuscular injection of 50mg of ketamine/Kg of body weight and

thiopental 20mg/Kg IP. In groups I/R and I/R+Sim, under sterile conditions, a laparotomy was performed and the superior mesenteric artery (SMA) was occluded with a microvascular clamp for 60 minutes. In order to block any collateral blood supply, the right colic and proximal jejunal arteries were also clamped. The laparotomy incision was then closed, to be opened later for removal of the clamps after 60 minutes of ischemia. Reperfusion was confirmed by the return of the mesenteric arcade pulsation. The incision was closed again and the animals were killed by anesthetic overdose (thiopental 100mg/Kg) after 120 minutes of reperfusion. The sham-operated rats received the same surgical procedure as the other groups without being subjected to the ischemia-reperfusion protocol.

Measurement of bacterial translocation

At the end of the procedures (time = 180 minutes), a midline laparotomy was performed under aseptic conditions and biopsies were aseptically obtained for bacterial colony counts. One gram of MLN complex, liver and lung was removed for culture. Tissues were homogenized and aseptically solubilized after addition of 0.5 mL of 0.9% saline. Aliquots of 0.2mL were processed and cultured on selective MacConkey's agar and blood agar for detection of gram-negative and gram-positive bacteria, respectively. The agar plates were incubated at 37 ^{o}C and examined for growth after 24 and 48 hours. Any growth in the plates of bacteria of the same biotype as cultured was considered positive and expressed as colony-forming units per gram of tissue (CFU/g).

Histological study

Ileum specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections cut at a thickness of 4μm were stained with hematoxylin and eosin for morphometric measurements using an image analyzer (Image-Pro Plus, Media Cyber®). The damage of the intestinal specimens was assessed in a blinded manner by an experienced pathologist according to microscopic criteria for degree of damage based on a grading system previously described [17]: normal mucosa, 0; subepithelial space at the viluus tip, 1; more extended subepithelial space, 2; epithelial lifting along villus, 3; denuded villi, 4; loss of villus tissue, 5; crypt layer infarction, 6; transmucosal infarction, 7; transmural infarction, 8.

Cytokine assays

Portal blood samples were collected and used for measurement of tumor necrosis factor-alfa (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-10 (IL-10), determined using 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. Sensitivity of detection was 30 pg/ml for cytokines.

Statistics

Data analysis was performed using the BioEstat 2.0 program. Differences between the microbiological samples as measured by positive cultures were evaluated by a test for differences between proportions. The results were tabulated and compared by ANOVA using post hoc analysis with Tukey test. $P<0.05$ was considered significant.

Results

We observed bacterial translocation to mesenteric lymph nodes, spleen, liver and blood in all animals subjected to I / R. However, in I/R group rats treated with simvastatin, translocation to these organs was significantly lower than in I/R untreated (Table 1). In the control group, there was no bacterial translocation as well as in the sham group rats, except in the mesenteric lymph nodes. In group S+Sin, paradoxically, there was bacteremia at the expense of Gram-positive (*Staphylococcus aureus*).

Table 1- Bacterial Translocation in groups treated and not treated with simvastatin (colony-forming units per gram of tissue - CFU/g).

Groups	n	LNM	Spleen	Liver	Blood
C	10	0	0	0	0
S	10	2±0.2	0	0	0
I/R	10	253±32*	112±12	178±18	166.5±32
S+Sim	7	0	0	0	18±12.2
I/R+Sim	7	6.0±5.4	12±4.4	7±4.3	22.3±15.9

*p < 0.01 compared with groups C, S, IR/Sim.

Cytokines were not detected in group C rats. In group S+Sin, we observed lower levels of proinflammatory cytokines (TNF-α, IL-1 β, IL-6) and a higher level of antiinflammatory-cytokine (IL-10), when compared with group S (Table 2). In I/R group rats, the levels of pro-inflammatory cytokines were

significantly higher when compared to I/R+Sim. This group had the highest values of IL-10 when compared with all other groups ($p<0.05$).

Table 2- Serum levels of cytokines in groups with and without simvastatin treatment.

Groups	n	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)
C	10	0	0	0	0
S	10	41.7±9.4	34±11	144±17	94±21
I/R	10	753.7±91	588.7±100	422.1±56	311±52
S+Sim	8	19.6±3.3	13.6±3.5	45.2±14.8	576.25±86
I/R+Sim	8	383.3±76*	282.8±71*	179±73*	781±108*

*p<0.01 compared with I/R, S, S+Sim

Macroscopically, the ileum segments studied showed dilation and intramural hemorrhage, with higher intensity in I/R group rats, compared with the other groups. The microscopic findings revealed marked mucosal injury after ischemia and reperfusion injury; we observed most intense injury in I/R group rats compared with the other groups. The lesions most often found were: thinning of the mucosa, transmural infarction, infiltration of leukocytes in the lamina propria and mucosa. (Figures 1,2,3). Histopathological findings of I/R+Sim were comparable to those found in groups C and S.

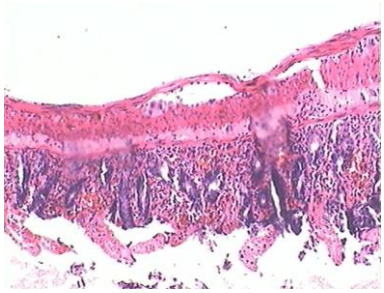

Fig 1. The mucosa is injuried, and leukocite infiltration of lamina propria and mucosa are shown (group I/R), 100x.

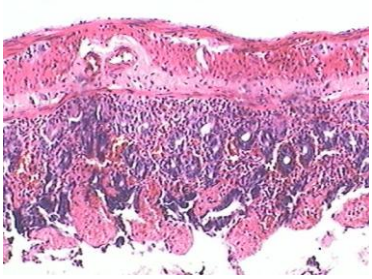

Fig.2. Hemorrhage and inflammation of mucosa (group I/R), 100x.

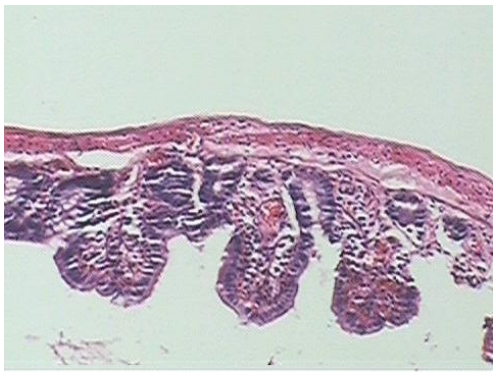

Fig 3. Thinning of mucosa (group I/R), 100x.

Discussion

The HMG-CoA reductase inhibitors, including simvastatin, are used in the treatment of hypercholesterolemia, because of their effects on cholesterol biosynthesis, mevalonate pathway. Some studies have reported that statins preserve endothelial function in the absence of hypercholesterolemia, raising eNOS.[22,23]. The activity of eNOS (nitric oxide synthase) inhibits leukocyte-endothelial interactions in microcirculation[24], reducing injury during ischemia and reperfusion of myocardium[25] indeed "stroke protection" [26]. Nitric oxide suppresses the expression of several endothelial adhesion molecules, including P-selectin, VCAM-1 and ICAM-127 [28].

In consequence, the present study used an experimental model of ischemia and reperfusion, to verify the effect of simvastatin on intestinal injury and bacterial translocation. Some authors consider the bacterial factor, crucial in the pathogenesis of sepsis and multiple organ failure. In surgery and intensive care, intestinal obstruction and intestinal ischemia are the most associated pathological conditions[29-31]. This study showed that pretreatment with simvastatin attenuated the translocation of bacteria to the liver, spleen and lymph nodes of rats. We used a simvastatin dose of 10 mg/kg via gavage 18 hours and 2 hours before ischemia and reperfusion, based on findings of Pruefer et al, who concluded that these are the periods with the best results of the antiinfective effects of simvastatin[32]. Naito et al found that pretreatment with rosuvastatin inhibited bacterial translocation, reducing the levels of cytokines in maintaining the integrity of epithelial mucosa[33]. A similar phenomenon occurred in the trial of Ozacmak et al. They demonstrated that atorvastatin not only preserved the ileal mucosa but also the muscle contractility after ischemia and reperfusion[34].

OuvirLer In the sham group without simvastatin treatment it was observed bacterial translocation only in mesenteric lymph nodes, but in Group S + Sin, some animals showed *Staphylococcus aureus* in the blood. As we observed growing exclusively of Gram positive bacteria, we interpreted it as a probable contamination in the isolate. The I/R+Sim group rats showed levels of bacterial translocation significantly lower in lymph nodes and liver, compared with group I/R. In the study of Pirat et al, simvastatin reduced the neutrophil infiltration and severity of injury in lung tissue in similar experimental model[35]. Other research found that treatment with simvastatin reversed inflammatory alterations in mice subjected to sepsis by cecal ligation and puncture, attributing these effects to the reduction of monocyte adhesion to endotelium[36,37].

It is known that the balance between anti-and pro-inflammatory cytokines is important in the development of bacterial translocation and septic shock. Activated leukocytes produce TNF-α and IL-6, which activate additional neutrophils to propagate inflammation[32]. In our study the levels of proinflammatory cytokines (TNF-α, IL-1 β, IL-6) were significantly lower in I/R+Sin rats than in other groups; however, the levels of the anti-inflammatory

IL-10 were higher in I/R+Sim rats than in other groups. It is attributed to IL-10 the inhibition of tissue factors released by activated monocytes via endotoxins, and reduction of expression of TNF-α by monocytes[38,39]. Ando et al argued that cerivastatin was able to reduce serum levels of TNF-α, IL-1 β in rats with sepsis induced by endotoxin, improving survival[41]. Finally, Waehre et al researching the effect of statins in patients with coronary artery disease, demonstrated significantly reduced serum levels of IL-1 in treated patients compared to controls[42]. In this regard, we observed that simvastatin exerted marked anti-inflammatory effects, a phenomenon found in other studies[38,40].

Conclusion

The use of simvastatin in rats subjected to intestinal ischemia and reperfusion reduced bacterial translocation and the levels of pro-inflammatory cytokines and increased levels of anti-inflammatory cytokine, influencing the integrity of the intestinal epithelium.

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