Effect of simvastatin in attenuation of mucositis induced by methotrexate in rats

Efeito da sinvastatina na atenuação da mucosite induzida pelo metotrexato em ratos

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ABSTRACT

Purpose: Based on studies that have attributed anti-inflammatory properties to statins, the aim of this work was to observe the effect of simvastatin in the attenuation of mucositis induced by methotrexate in the gastrointestinal tract in rats and its effects on cytokines. **Methods:** Twelve Wistar rats weighing 270±18 g were randomly distributed into two groups: methotrexate/saline (MTX/S n = 6) and methotrexate/simvastatin (MTX /SV n=6). In all animals, 3 mg / kg of methotrexate was injected subcutaneously for 3 consecutive days. In the MTX / SV simvastatin was administered orally one week before and during treatment with methotrexate. In the MTX/S, saline was administered at the same doses and schedules. We determined the plasma levels of TNF-α, IL-1β and IL-6 and the histological analysis by HE staining in segments of esophagus, stomach, duodenum, jejunum and colon. **Results:** The expression of TNF-α, IL-1β and IL-6 (14±91.7, 119.3±4 and 83.1±4, respectively) was lower in the MTX/SV group rats than in the MTX/S (171.3±16, 218. ±15 and 114.8±3, respectively). Histopathology showed that simvastatin reduced significantly ($p<0.05$) the damage induced by methotrexate in the mucosa of the esophagus, stomach, jejunum and colon. **Conclusion:** Simvastatin showed anti-inflammatory action

in rats, suggesting potential clinical implication in the prevention or attenuation of mucositis induced by methotrexate.

Key words: Estatins, Inflammation, Methotrexate, Mucositis, Rat.

RESUMO

Objetivo: Com base em estudos que atribuem propriedades anti-inflamatórias às estatinas, o objetivo foi observar a ação da sinvastatina na atenuação da mucosite induzida por metotrexato no trato gastrointestinal em ratos e sua repercussão na expressão de citocinas. **Métodos:** Foram utilizados 12 ratos Wistar pesando 270±18 g, aleatoriamente alocados em dois grupos: metotrexato/salina (MTX/S n=6) e metotrexato/sinvastatina (MTX/SV n=6). Em todos os animais foi administrado metotrexato na dose de 3mg/Kg por via subcutânea, por 3 dias consecutivos. No grupo MTX/SV foi administrado sinvastatina por via oral uma semana antes e durante o tratamento com metotrexato. No grupo MTX/S foi administrada solução salina 0,9% nas mesmas doses e prazos. Foram realizadas as dosagens plasmáticas das citocinas TNF-α, IL-1β e IL-6 e feita a análise histológica pela coloração HE de segmentos do esôfago, estômago, duodeno, jejuno e cólon. **Resultados:** A expressão das citocinas TNF-α, IL-1β e IL-6 (91,7±14, 119,3±4 e 83,1±4, respectivamente) foi menor no grupo MTX/SV que no grupo MTX/S (171,3±16, 218,1±15 e 114,8±3, respectivamente). Na análise histopatológica houve redução significante ($p<0,05$) nos danos induzidos pelo metotrexato na mucosa do esôfago, estômago, jejuno e cólon. **Conclusão:** A sinvastatina mostrou ação anti-inflamatória em ratos *Wistar*, sugerindo potencial inplicação clínica na prevenção ou atenuação da mucosite induzida pelo metotrexato.

Descritores: Estatinas, Inflamação, Metotrexato, Mucosite, Rato.

Introduction

In 1976, the mevastatina was discovered, the first drug of the statins family, which are capable of inhibiting HMG-CoA reductase (enzyme responsible for cholesterol biosynthesis)[1]. Several studies now report that the use of statins was responsible for a significant drop in levels of serum lipids, with encouraging results (decrease in ischemic heart disease) in patients with atherosclerosis and coronary heart disease, regardless of presenting symptoms

of these diseases[2-4]. Subsequent studies have revealed various actions (pleiotropic), cholesterol independent, as anti-inflammatory action, thrombogenesis inhibition and immunomodulators effects [4-7]. Several lines of research aimed at explaining actions on inflammation, such as modulation (stimulation) of nitric oxide synthase-derived endothelial (eNOS) [8,9]. In 1997, Laufs et al[9] showed that statins acted directly on the pathway of NO, increasing its regulation. This work, which was an initial step in the study of these functions of statins, showed that simvastatin increased from 13 to 38 hours the average life of the mRNA that encodes eNOS[9]. It was demonstrated that statins significantly inhibit endothelial cell-leukocyte interaction, regardless of their anticolesterolemic actions[6,8]. Significant anti-inflammatory effects have been demonstrated in situations such as myocardial infarction in experimental models simulating normocholesterolemic, hypercholesterolemic and diabetes situations10-12. There is a relevant role for activated monocytes in the inflammatory reaction, releasing a series of pro-inflammatory factors, including tissue factor and various cytokines, tumor necrosis factor (TNF-α) and interleukin 6 (IL- 6) [13,14].

Treatment with these drugs reduces vascular inflammation in patients, as evidenced by the significant reduction of inflammatory markers such as C-reactive protein[15-18]. Cytokines such as tumor necrosis factor-alpha (TNF-alpha), interleukin 1 (IL-1) and interleukin 6 (IL-6) are involved in the pathogenesis of oral mucositis after using quimioterapics[19]. One of the most widely used chemotherapy drugs in medical practice is methotrexate (MTX), an inhibitor of dihydrofolate reductase and DNA synthesis. This drug has documented activity against leukemia, breast cancer, head and neck, lymphoma and others. MTX causes acute damage to the intestinal epithelium, characterized by a reduction in mitosis in the crypts and shortening of vilosities[20]. Treatment with MTX induces changes in intestinal passive absorption of drugs, as well as active absorption of glucose and nutrientes[21], for interfering with the transporter PEPT1 di/tripeptides[20]. MTX also induces apoptosis in intestinal epithelial cells by a mechanism dependent on activation of caspases[22]. Such cytotoxic effects of chemotherapeutic agents such as MTX cause mucositis in the gut. Oral mucositis, best studied so far, is manifested clinically with pain, erythema and ulceration in addition to symptoms such as bloating, abdominal pain and diarrhea[19]. The gastrointestinal tract is particularly vulnerable because of its high rate of celular proliferation[20]. Mucositis is a complex biological process and occurs in four stages: inflammatory / vascular stage, epithelial stage; ulcerative/ bacteriological, and resolution stage. These are interdependent phases, resulting respectively in: actions mediated by TNF-α, lL-1 and IL-6; direct effect of chemotherapy in the epithelium; action of bacterial flora and the integrity of the patient spinal[19].

Although the many efforts in the prophylaxis or therapy of oral mucositis induced by chemotherapy or radiotherapy, the process of therapy is largely

palliative and preventive. More effective measures in treating this complication are still being investigated[19]. Currently there are very few or no knowledge about the use of statins for the prevention and treatment of damage to the digestive tract caused by MTX. In the present study we tested the effect of simvastatin on the mucosa of the esophagus, stomach, small intestine and colon and cytokine expression.

Methods

We used 12 Wistar rats, weighing 270±18g. The rats were randomly distributed into 2 groups of 6 animals each, a control group named methotrexate / saline (MTX/S) and an experimental group methotrexate / simvastatin (MTX/SV). They were kept in individual polypropilene cages with water and of standard food for rodents (Labina-Purina) *ad libitum* in an environment of 24°C with 12h cycle of light and dark throughout the experimental study. The procedures involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996 and the protocol was approved by the Ethics in Research of Hospital Universitário Onofre Lopes-UFRN.

In all animals, MTX was administered at a dose of 3 mg / kg subcutaneously for 3 consecutive days. In the MTX / SV (n = 6) we injected 10 mg / Kg of simvastatin microemulsion by gavage, beginning one week prior to treatment with MTX, and during treatment with this drug. In the MTX/S (n = 6) saline solution 0.9% was administered at the same doses and schedules. On the second day after completed treatment with MTX, blood was collected by cardiac puncture and then the rats were killed with an overdose of anesthetic (Tiopental 100mg/Kg i.v.). The blood was treated with EDTA, centrifuged and plasma was separated for determination of TNF-α, IL-1 β and IL-6 by ELISA, using kits Peprotec, USA. A laparotomy was performed and samples of the esophagus, stomach, duodenum, jejunum, and colon were harvested for histopathological examination. For histological analysis, the specimens were embedded in paraffin, 4µm sections were stained with hematoxylin and eosin, and examined by light microscopy. Histological evaluation of organ damage was done by semiquantitative analysis of gastrointestinal damage, according to the criteria described by Howarth et al[23].

Five parameters were considered for the examination of esophagus: epithelial thinning, surface epithelial injury, neutrophil infiltration, edema, stenosis, and changes in the height of the basal cell layer of squamous epithelium. For the histopathology of the stomach we considered: infiltration of mononuclear cells, polymorphonuclear infiltration, atrophy of gastric glands,

surface epithelial lesion, intestinal metaplasia. Each histological parameter of the esophagus and stomach was scored on a scale ranging from 0 (normal), 1 (mild), 2 (moderate) to 3 (maximum) damage. The scores analyzed in the duodenum, jejunum and colon were obtained from the sum of scores for the following histological criteria: fusion of villi, villous atrophy, rupture of enterocytes in the mucosal surface, reducing the number of goblet cells, reduction in the number of mitosis figures, loss of crypts or distortion of their cells, abscesses, infiltration of polymorphonuclear cells and lymphocytes; dilation of lymphatics and capillary vessels, thickening and edema of submucosa and muscular layer. Each histologic variable was scored from 0 (normal), 1 (mild), 2 (moderate) to 3 (maximum damage).

Data analysis was performed using the Statistica software. The results were tabulated and compared using analysis of variance (ANOVA). When differences were detected, means were compared using the Bonferroni test, considering significant when $p<0.05$.

Results

Histopathology

Oral mucositis was evaluated according to the scores mentioned in the methodology. The animals in the MTX/SV showed damage in the mucosa significantly lower than in the MTX/S rats ($p <0.05$). These histopathological changes were obtained with the use of simvastatin a week before and during treatment with MTX. Table 1 and Figures 1 and 2 show differences in histological damage in the digestive tract between the groups MTX/MTX and S/SV. The results correspond to the average sum of scores for each organ of six animals per group. The analysis was performed by a pathologist without knowledge of which groups they belonged to the histological slides.

Table 1 - Histopathological data representing the damages induced by methotrexate in different regions of the digestive tract after treatment with saline and simvastatin.

Groups / *Organs*	MTX/saline	MTX/simvastatin
Esophagus	133±3.4*	10.3±2.2
Stomach	12.6±4.1*	9.5±2.1
Duodenum	22.1±4.8	20.7±3.9
Jejunum	21.6±2.7*	15.5±3.6
Colon	17.6±2.9*	14.1±2.7

The values represent the sum of histological criteria, as described on methods.
*A significant difference was observed in esophagus, stomach, jejunum and colon, comparing the two groups ($p<0.05$).

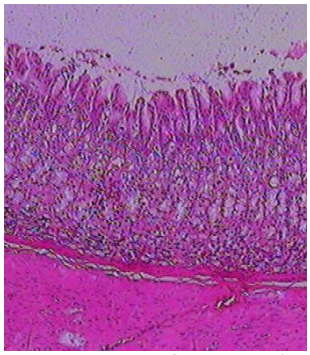
Table 2 – Values of the levels of cytokines determined in plasma.

Cytokines / *Groups*	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)
MTX/S	171.3±16	218.1±15	114.8±8
MTX/SV	91.7±14*	119.3±10*	83.1±9*

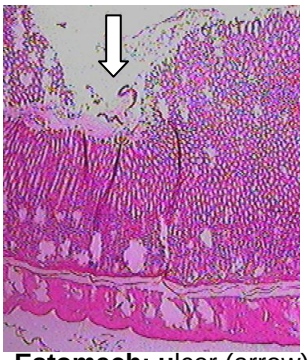
*$p<0.05$ compared with the values from group MTX/S.

Cytokines expression

The plasma expression of TNF-α was demonstrated to a lesser degree in mice treated with simvastatin group (91.7 ± 14 pg / ml) compared with the group treated with saline (171.3±16). The expression of IL-1 β and IL-6 was significantly higher in the saline group (MTX/S) than in the rats which used simvastatin (MTX/SV) after the use of methotrexate. The data are summarized in Table 2.

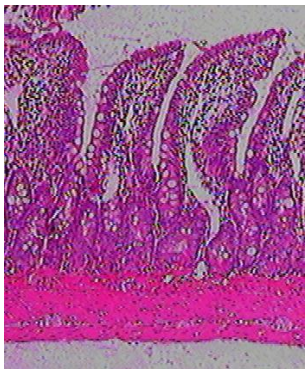


Estomach: normal
(Group MTX/simvastatin)

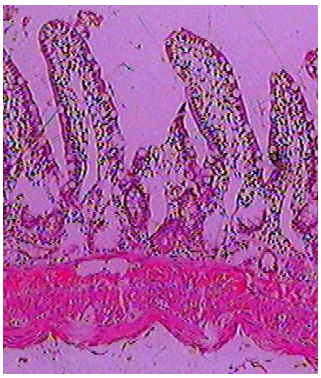


Estomach: ulcer (arrow)
(Group MTX/saline)

Figure 1 - Comparing the gastric mucosa of rats from MTX/simvastatin and MTX/saline grupos. HE 100X.



Jejunum: normal
(Group MTX/simvastatin)



Jejunum: epithelial damage
(Group MTX/saline)

Figure 2 – Jejunal vilosities from MTX/simvastatin and MTX/saline groups. HE 100X.

Discussion

Chemotherapy is often associated with side effects such as mucositis because of the anti-mitotic properties of drugs. Their actions are not specific to tumor tissues, and they also induce deleterious effects on rapidly proliferating cells such as bone marrow and gut mucosa cells[24]. The severity of mucositis depends on the type of chemotherapy used, the dosage, frequency of administration, patient age and the time of diagnosis of neoplasia[20].

The present study was designed to evaluate the protective effect of oral simvastatin before and during MTX-induced intestinal mucosal injury and to determine the expression of cytokines after treatment with simvastatin. Our data demonstrate that MTX has obvious damaging effects on esophago-gastro-intestinal mucosa. In this study, we showed esophageal injury secondary to subcutaneous MTX administration. We also established the beneficial effects of exogenous simvastatin in the prevention of tissue damage, which was observed

by less histopathologic damage. These results are in agreement with other recent experimental model[25]. Esophageal mucositis is a common toxic side effect of MTX in childhood period and can be dose limiting in therapeutic regimens, and treatment reduction or withdrawal[26]. In this study we found that histopathologic changes in esophagus of MTX given rats were more serious than those MTX and simvastatin given rats. We believe that these findings were related to its antiinflammatory effects. Damage such as edema, epithelial thinning, and luminal narrowing was observed in the esophagus mucosal epithelium in microscopic examination.

The pathogenesis of mucositis can be attributed to the direct mucosal toxicity of high-dose chemotherapy and to indirect mucosal damage caused by concomitant local bacterial, viral, and mycotic infections[27]. Owing to mucosal desquamations, esophageal mucosal barriers are exposed to injury by proinflammatory factors, such as inflammatory cytokines, leukocytes, and oxidative stress. The evidence specifically indicates that the inflammatory response to chemotherapy plays an important role in the pathogenesis of mucositis[28].

The magnitude of the structural intestinal damage induced by MTX was clearly substantiated by the Howarth injury score[23]. Indeed, histological data obtained in MTX animals showed gastric and intestinal increased cellularity of subepithelial tissue, vascular dilation, significant epithelial atrophy, and signs of crypt remodeling. In addition, MTX injection led to intestinal mucosal hypoplasia. The observed decrease in bowel and mucosal weight, and decrease in villus height and crypt depth in this model support this conclusion. Our observations are consistent with the data of other investigators[29,30].

It has been suggested that proinflammatory cytokines, e.g., TNF- α and IL-1 β, may be involved in the amplification phase of intestinal mucositis[31], but that inflammation may be the functional consequence of the weakened barrier function, i.e., weakened epithelial integrity (atrophy and altered protein metabolism) and altered mucus protection. In addition, malnutrition that is associated with cancer and chemotherapy is able to induce by itself cytokine expression in intestine[32]. Gene up-regulation as a result of the activation of transcription factors induced by chemotherapy results in the production of pro-inflammatory cytokines, such as tumour necrosis factor-a (TNF-a), interleukin (IL)-1b, and IL-6, leading to tissue injury and apoptosis. The up-regulation of other genes causes adhesion molecule expression, followed by activation of the cyclooxygenase-2 pathway and angiogenesis[33]. Pentoxifylline has antiinflammatory effects like simvastatin and has been found to reduce TNF-a synthesis by inhibiting transcription of the TNF-a gene and to modulate the synthesis of other cytokines, such as IL-1b,IL-6, IL-8, and TNF-b[34]. In agreement with our investigation, previous experimental studies have shown a great association between cytokine-mediated biological manipulation of the oral mucosa and the course of mucositis. Alamir et al found taht intramucosal

concentration of proinflammatory cytokines, interleukin-1β and cytokine-induced neutrophil chemoattractant, was markedly increased in methotrexate-treated rats[35]. Finally, there is no doubt that chemotherapy may exert cell-damaging or a cell-destroying effect through the generation of reactive oxygen species[36], or through enzymatic or transcription factors that leads to upregulation of genes responsible for production of proinflammatory cytokines TNFα, IL-1β, IL-6 [37]. This leads to tissue injury and apoptosis[38]. In addition, growing evidence suggests that some proteins play an important role in the regulation of apoptotic cell death in chemotherapy induced mucositis[39]. The present data clearly indicate that simvastatin has a strong attenuating effect on cytokines expression, important for recovery from intestinal mucosal injury caused by MTX.

Conclusion

In conclusion, we found that MTX-induced esophageal, gastric and intestinal damage in a rat model. Simvastatin had significant preventive effects on damage, histopathologically, and biochemically. We speculate that it may be possible to reduce MTX-induced esophago-gastrointestinal damage by adding sinvastatin to the treatment protocol as an antiinflammatory agent. Further additional investigations using other statins are needed to confirm our results. These preliminary observations suggest that oral simvastatin may have clinical implication and value in preventing or reducing the severity of chemotherapy-induced mucositis.

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