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ORIGINAL ARTICLE

Effect of atorvastatin on 5-fluorouracil-induced experimental oral mucositis

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Abstract
Purpose Oral mucositis (OM) is a frequent side effect in patients with cancer. We investigate the effect of atorvastatin (ATV), a cholesterol-lowering drug, on OM induced by 5-fluorouracil (5-FU) in hamsters.
Methods OM was induced by the i.p. administration of 5-FU, with excoriations of the cheek pouch mucosa. The animals were pretreated with i.p. ATV 1, 5 or 10 mg/kg or vehicle (saline and 5% (vol/vol) ethanol) 30 min before 5-FU injection and daily for 5 or 10 days. Samples of cheek pouches and main organs were removed for histopathological analysis, determination of TNF-α, IL-1β, nitrite, non-protein sulfhydryl group (NP-SH) levels, myeloperoxidase (MPO) assay and immunohistochemistry for induced nitric oxide synthase (iNOS). Blood was collected for a leukogram analysis of biochemical parameters and analysis of bacteremia.
Results ATV at doses of 1 and 5 mg/kg reduced mucosal damage and inflammation, as well as the levels of cytokines, nitrite and myeloperoxidase activity on the 5th and 10th day of OM and immunostaining for iNOS on the 5th day of OM.ATV at 1 mg/kg increased cheek pouch NP-SH when compared to 5-FU groups on the 10th day of OM. The association between ATV 5 mg/kg and 5-FU decreased the survival rate, amplified the leukopenia of animals, increased transaminase serum levels and caused liver lesions. We also detected the presence of Gram-negative bacillus in the blood of 100% of the animals treated with ATV 5 mg/kg + 5-FU.
Conclusions Atorvastatin prevented mucosal damage and inflammation associated with 5-FU-induced OM, but the association of a higher dose of ATV with 5-FU induced hepatotoxicity and amplified leukopenia.

Keywords Oral mucositis · 5-Fluorouracil · Atorvastatin · Statin

Abbreviations

5-FU	5-fluorouracil
ATV	Atorvastatin
HMG-CoA	3-hydroxyl-3-methylglutaryl coenzyme A
NP-SH	Non-protein sulfhydryl groups
IL-1β	Interleukin-1 beta
TNF-α	Tumor necrosis factor-alpha

Introduction

Oral mucositis (OM) is a frequent dose-limiting side effect of radiotherapy (RT) and chemotherapy in patients with cancer [1, 2]. The incidence of OM varies from 10% to 40% of patients receiving conventional chemotherapy for

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solid tumors [3], 97% of head and neck cancer patients receiving conventional fractionated RT (one dose/day, 5 days/week for 5–7 weeks), 100% of patients receiving altered fractionation RT (two or more doses/day) and 89% of patients undergoing high-dose chemotherapy prior to hematopoietic stem cell transplantation [4]. OM is characterized by erythematous, erosive and ulcerative lesions in the oral cavity. The ulcerative lesions are typically painful and affect nutritional intake and quality of life, leading to difficulty in eating and communication and representing a significant risk factor for systemic infection [5]. Patients with mucositis and neutropenia have a relative risk of septicemia that is greater than four times that of individuals without mucositis [6]. In addition, mucositis presence can have other consequences like the interruption of cancer treatment, a limitation in the dose [7], and an increase in therapeutic costs and hospital stays [8]. Various strategies to prevent ulcerative mucositis in radiotherapy and chemotherapy in patients with cancer have been evaluated, but none have demonstrated strong efficacy.

Mechanisms involved in the pathogenesis of mucositis are much more complex than direct damage to the epithelium alone [9]. Sonis et al. [10] demonstrated that in oral mucosa, following exposure to radiation, primary damage to endothelial cells occurred well before any detectable changes were apparent in the epithelium. Based on these data, five continuous overlapping phases have been proposed to describe the pathogenesis of mucositis as follows. First is the initiation phase, which results in direct tissue damage to mucosal components as a result of the production of reactive oxygen species. Second is the upregulation and message generation phase, during which the activation of transcription factors occurs, in particular nuclear factor-kB (NF-kB), and consequently upregulation of genes that have an effect on mucosal integrity by inducing clonogenic cell death, apoptosis and tissue injury, and production of pro-inflammatory cytokines occur. Third is signal amplification, which occurs as a consequence of the pro-inflammatory cytokines acting via positive feedback mechanisms causing further activation of NF-kB and subsequent increased production of cytokines and other pro-inflammatory mediators, such as cyclooxygenase-2 (COX-2). This increased production leads to the activation of matrix metalloproteinases, the production of which elicits further tissue damage. Fourth, the ulcerative phase develops, in which, clinically, there is a breach of the epithelium accompanied by bacterial colonization. Bacterial products can stimulate further amplification of cytokine production leading to further potentiation of tissue injury. And fifth, the healing phase occurs, following cessation of the cancer treatment. This phase results in the restoration of normal mucosal appearance at the clinical level [10, 11].

Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, have emerged as the most important class of lipid-lowering agents. Through inhibition of HMG-CoA reductase, they restrict the rate-limiting step of cholesterol synthesis, resulting in upregulation of low-density lipoprotein (LDL) receptors on the cell membrane and reduction in atherogenic LDL consequences [12]. Several clinical trials have demonstrated that the beneficial effects of statins on cardiovascular disorders go beyond their effects on cholesterol level [13, 14]. Some of the cholesterol-independent or "pleiotropic" beneficial effects of statins include improvement in endothelial function by endothelial nitric oxide synthase (eNOS) upregulation [15], decrease in vascular smooth muscle cell (VSMC) proliferation [16] and macrophage proliferation [17], reduction in platelet activity [18], stabilization of atherosclerotic plaques [19], antioxidant properties [20], and anti-inflammatory and immunomodulatory effects [21, 22]. Many of these pleiotropic effects are mediated by blocking the synthesis of isoprenoid intermediates, via inhibition of the L-mevalonic pathway, with accumulation of inactive small GTP-binding proteins in the cytoplasm (such as Rho, Ras, Rac, Rap and Ral) [23].

The anti-inflammatory effect of statins is well described in atherosclerotic lesions [24]. The anti-inflammatory mechanism of statins may involve inhibition of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM-1) and E-selectin, and cytokines such as interleukin-6 and -8, which are involved in the recruitment of inflammatory cells. In addition, statins can suppress the inflammatory response by binding directly to a novel regulatory site of the β_2 integrin, leukocyte function antigen-1. This regulatory site serves as a major counter receptor for ICAM-1 on leukocytes [25]. Taking into account the anti-inflammatory properties of statins, we investigated the effect of ATV on OM induced by 5-FU. We show that ATV reduced damage and inflammation induced by 5-FU in the oral mucosa of hamsters. We also demonstrate that the association of a higher dose of ATV with 5-FU induced hepatotoxicity and amplified leukopenia.

Methods

Animals

Ninety-six male adult Golden hamsters weighing 140–200 g from the Federal University of Ceará were housed in temperature-controlled rooms and received water and food *ad libitum*. Surgical procedures and animal treatments were conducted in accordance with the Guidelines for Institutional Animal Care and were approved by our local ethics committee for experimental animal use.

Drugs

ATV (Atorvastatina, Pfizer, Inc., Guarulhos, SP, Brasil; prescription formulation) was dissolved in saline and 5% (vol/vol) ethanol (pH 7.6) [26].The 5-FU (Fluoro-Uracil; Roche, Rio de Janeiro, Brazil) was reconstituted in 0.9% NaCl.

Induction of experimental oral mucositis

Oral mucositis was induced by two intraperitoneal (i.p) administrations of 5-FU on the first and second days of the experiment (60 and 40 mg/kg, respectively), according to an experimental oral mucositis model previously described [27]. On day 4, under anesthesia with 2.5% tribromoethanol (250 mg/kg, i.p.), the cheek pouch mucosa was irritated by superficial scratching to potentiate oral mucositis. This was performed by dragging the tip of an 18-gauge needle, twice in a linear manner, across the everted cheek pouch. The animals were killed on the 5th or 10th day after the initial injection of 5-FU, under anesthesia with 2.5% tribromoethanol (250 mg/kg, i.p.). There were at least 6 animals in each experimental group.

Experimental groups

Hamster groups subjected to OM were treated i.p. with ATV 1, 5 or 10 mg/kg (ATV 1/5-FU, ATV 5/5-FU, ATV 10/5-FU), 30 min before the injections of 5-FU and daily until they were killed on the 5th or 10th day after receiving the first injection of 5-FU. Control groups consisted of normal animals (Normal), animals subjected to OM that received saline (5-FU/saline) or saline and 5% (vol/vol) ethanol (5-FU/sal-ethanol), a group that received only mechanical trauma (MT) of cheek pouches on the fourth day and animals subjected to MT that received ATV 5 mg/kg and saline (ATV 5/saline).

ATV effects

Initially, we investigated the effect of ATV on oral mucositis induced by 5-FU on the 10th day. The administration of ATV 5 or 10 mg/kg intraperitoneal significantly increased the mortality rates of hamsters submitted to OM on the 10th day of experiment. Therefore, to evaluate the effect of ATV on 5-FU-induced OM on the 10th day, the dose of 1 mg/kg was selected. ATV (1 mg/Kg), saline or saline and 5% ethanol were administered intraperitoneally (i.p.), 30 min before each injection of 5-FU and daily until they were killed, on day 10. In another set of experiments, ATV 1 or 5 mg/kg, saline or saline and 5% ethanol were administered intraperitoneally (i.p.), 30 min before each injection of 5-FU and daily until they were killed, on day 5. The protocol for induction of oral mucositis was performed as described in the preceding section. On 5th and 10th day, the animals were anesthetized with 2.5% tribromoethanol (250 mg/kg, i.p.) and the cheek pouch was everted and photographed before killing the animal. Samples of cheek pouches were removed from six animals per group for histopathological analysis, determination of cytokines and nitrite and non-protein sulfhydryl groups (NP-SH) levels and myeloperoxidase (MPO) activity. In order to investigate a possible systemic repercussion, blood or tissue samples were collected, and leukogram, analysis of biochemical parameters, investigation of bacteremia in the serum and liver, kidney, heart and lung histopathology were performed on the 5th day.

Macroscopic analysis of cheek pouch

Photographs were used for scoring lesions. For macroscopic analysis, inflammatory aspects such as erythema, erosion, vasodilatation, epithelial ulcerations and abscesses were evaluated in a single-blind fashion and graded as follows. Score 0: completely healthy cheek pouch with erosion or vasodilatation absent. Score 1: presence of erythema, but no evidence of erosion in the cheek pouch. Score 2: severe erythema, vasodilation and surface erosion. Score 3: formation of ulcers in one or more faces of the mucosa, but not affecting more than 25% of the surface area of the cheek pouch; severe erythema and vasodilatation. Score 4: Cumulative formation of ulcers of about 50% of the surface area of the cheek pouch. Score 5: virtually complete ulceration of the cheek pouch mucosa. In this case, the fibrosis makes oral mucosa exposure difficult.

Histopathological analysis of cheek pouch

The specimens were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Sections of 5 μm thickness were obtained for hematoxylin–eosin staining (H&E) and examined by light microscopy (×40). The parameters of inflammatory cell infiltration, vasodilatation, presence of hemorrhagic areas, edema, ulcerations and abscesses were determined in a single-blind fashion and graded as follows. Score 0: normal epithelium and connective tissue without vasodilatation; absence of or discreet cellular infiltration; absence of hemorrhagic areas, ulcerations or abscesses. Score 1: discreet vasodilatation, re-epithelization areas; discreet inflammatory infiltration with mononuclear prevalence; absence of hemorrhagic areas, edema, ulcerations or abscesses. Score 2: moderate vasodilatation, areas of hydropic epithelial degeneration, inflammatory infiltration with neutrophil prevalence, presence of hemorrhagic areas, edema and eventual ulcerations, and absence of abscesses. Score 3: severe vasodilatation, inflammatory infiltration with neutrophil prevalence, presence

of hemorrhagic areas, edema and extensive ulceration, and abscesses.

Myeloperoxidase assay

The extent of neutrophil accumulation in the cheek pouch samples was measured by assaying myeloperoxidase (MPO) activity. In brief, the animals were killed on the 5th or 10th day after the initial injection of 5-FU, and cheek pouch samples were harvested and stored at −70°C until required for assay. After homogenization and centrifugation (2000*g*, 20 min), MPO activity in these samples was determined by a colorimetric method described previously [28]. The results were reported as units of MPO per mg of tissue.

Quantification of TNF-α and IL-1β by ELISA

Cheek pouch samples were also harvested from animals of all groups on the fifth or tenth day after the initial injection of 5-FU for the measurement of TNF-α and IL-1β. The concentrations of these cytokines were measured by enzyme-linked immunosorbent assay (ELISA) as described previously [29]. The results were expressed as pg/ml of TNF-α or IL-1β.

Determination of nitrite levels

The production of NO was determined indirectly by measuring the nitrite levels based on the Griess reaction [30]. Briefly, 100 µl of cheek pouch tissue homogenate was incubated with 100 µl of the Griess reagent (1% sulfanilamide in 1% H_3PO_4/0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride/1% H_3PO_4/distilled water, 1:1:1:1) at room temperature for 10 min. The absorbance was measured at 560 nm in a microplate reader, and nitrite concentration was determined from a standard nitrite curve generated using $NaNO_2$.

Non-protein sulfhydryl groups' (NP-SH) levels

The NP-SH levels were measured following the protocol previously described [31]. One hundred milligrams of cheek pouch tissue was homogenized in 1 ml of 0.02 M EDTA. Aliquots of 400 µl of homogenate were added to 320 µl of distilled water and 80 µl of 50% trichloroacetic acid (TCA) for precipitation of proteins. The tubes were centrifuged (3,000 rpm/15 min/4°C), and 400 µl of supernatant was added to 800 µl of 0.4 M Tris, pH 8.9, and 20 µl of DTNB and agitated for 3 min, immediately before the reading. The absorbance was measured at 412 nm. The results were expressed as mg/g of mucosa.

Immunohistochemical reaction for induced nitric oxide synthase

Immunohistochemistry for iNOS, on day 5 of MO, was performed using the streptavidin–biotin–peroxidase method [32] in formalin-fixed, paraffin-embedded tissue sections (4 µm thick), mounted on poly-L-lysine-coated microscope slides. The sections were deparaffinized and rehydrated through xylene and graded alcohols. After antigen retrieval, endogenous peroxidase was blocked (15 min) with 3% (v/v) hydrogen peroxide and washed in phosphate-buffered saline (PBS). Sections were incubated overnight (4°C) with primary rabbit anti-iNOS antibody diluted 1:100 in PBS plus bovine serum albumin (PBS-BSA). The slides were then incubated with biotinylated goat anti-rabbit, diluted 1:400 in PBS- BSA. After washing, the slides were incubated with avidin–biotin–horseradish peroxidase conjugate (Strep ABC complex by Vectastain® ABC Reagent and peroxidase substrate solution) for 30 min, according to the Vectastain protocol. iNOS was visualized with the chromogen 3,3`diaminobenzidine (DAB). Negative control sections were processed simultaneously as described above but with the first antibody being replaced by PBS-BSA 5%. None of the negative controls showed iNOS immunoreactivity. Slides were counterstained with Harry's hematoxylin, dehydrated in a graded alcohol series, cleared in xylene and coverslipped.

Leukogram

Animals were anesthetized with 2.5% tribromoethanol (250 mg/kg, i.p.), and a sample of blood was collected from heart puncture on day 5. For leukogram determination, 20 µl of blood was added to 380 µl of solution of Turk, and total and differential counts of leukocytes were carried out by standard manual procedures using light microscopy [33]. The results are expressed as the number of cells per milliliter (ml).

Biochemical parameters

Blood of the animals was collected from heart puncture, and biochemical analyses were performed in serum samples obtained after centrifugation of total blood without anticoagulants, at 2,500 rpm for 15 min. Standardized diagnostic kits by LABTEST® spectrophotometer were used in spectrophotometrical determination of the alanine amino transferase (ALT) and aspartate amino transferase (AST), creatinine, urea parameters.

Analysis of liver, lung, heart and kidney

For histopathological analysis, the specimens were fixed in 10% neutral buffered formalin, dehydrated and embedded

in paraffin. Sections of 5 μm thickness were obtained for hematoxylin–eosin staining (H&E) and examined by light microscopy (40× magnification).

Bacteremia

Blood was collected from heart puncture on day 5 under sterile conditions. Ten microliters of blood was diluted tenfold in brain–heart infusion medium. The bacterial growth was analyzed after 24–48 h at 37°C by visual analysis of the turbidity of the culture medium. A turbid medium indicates bacteremia (+), and a non-turbid medium suggests absence of bacterial in the blood (−). Organisms that grew on brain–heart infusion medium were streaked for isolation on 5% sheep blood agar plates, MacConkey agar plates and mannitol-salt-agar plates. Organisms that grew were identified by standard methods, including Gram stain morphology, DNAse, catalase, coagulase, motility, sugar metabolism and other tests as indicated [34].

Statistical analysis

Data were described as either mean ± SEM or median, as appropriate. Analysis of variance (ANOVA) followed by Tukey's test was used to compare means, and Kruskal–Wallis and Mann–Whitney tests to compare medians; $P < 0.05$ was considered as indicating significant differences.

Results

Effect of atorvastatin on oral mucositis

Our group have previously demonstrated that the treatment of hamsters with 5-fluorouracil followed by mechanical trauma of the cheek pouch causes lesion starting by day 5 with peak at day 10, when erythema, hyperemia, hemorrhagic areas, and extensive ulcers and abscesses were detected. These signs of lesion diminished between days 12 and 16. All these aspects were confirmed by histopathological analysis, revealing on day 10 severe vascular ingurgitation and dilatation, accentuated inflammatory infiltration with neutrophil prevalence, hemorrhagic areas, edema and extensive ulceration and abscess [35].

To investigate the effect of atorvastatin (ATV) on lesions induced by 5-FU, groups of animals were killed on the 5th or 10th day after receiving the first injection of 5-FU or saline. The i.p. administration of 5-FU, followed by mechanical trauma (MT) of the cheek pouch of the hamsters, caused significant macroscopic lesions on day 5 and day 10 ($P < 0.05$), represented by accentuated erythema, hemorrhage, vasodilation and ulcers when compared to normal animals (N) or to animals subjected to MT only (Table 1). The lesion peak occurred by day 10, with the presence of abscesses (Fig. 1e). On day 5, the treatment of the animals with ATV 1 or 5 mg/kg (Table 1) significantly prevented the 5-FU-induced oral damage, presenting only a discrete erythema and a small laceration as a result of the mechanical trauma done on the 4th day. On day 10, treatment of the animals with ATV 1 mg/kg significantly reduced the size and severity of the lesions induced by 5-FU ($P < 0.05$) (Table 1). Figure 1g illustrates the protective effect of treatment with ATV 1 mg/kg compared to animals subjected to OM that received saline and 5% ethanol (Fig. 1e).

The histopathology of the cheek pouches of animals subjected to 5-FU-induced OM showed significant alterations ($P < 0.05$) on day 5 and day 10 in relation to that observed in normal animals (Table 1) or in animals that received MT only. On day 5, there was moderate edema, inflammatory cell infiltration with neutrophil prevalence, hemorrhagic

Table 1 Macroscopic and microscopic analysis of hamster cheek pouches subjected to experimental oral mucositis

Analysis	Macroscopic (5 days)	Microscopic (5 days)	Macroscopic (10 days)	Microscopic (10 days)
Normal	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
MT	1 (1–1)	1 (1–1)	1 (1–1)	1 (1–1)
5-FU/saline	3 (2–3)*	2 (1–2)*	4 (3–4)*	3 (2–3)*
5-FU/sal-ethanol	3 (2–3)*	2 (2–2)*	4 (3–5)*	3 (2–3)*
ATV1/5-FU	1 (1–2)**	1 (1–1)**	2 (1–3)**	1 (0–3)**
ATV5/5-FU	1 (1–2)**	1 (1–1)**	–	–
ATV5/saline	1 (1–2)	1 (0–1)	–	–

Oral mucositis was induced in hamsters by intraperitoneal (i.p.) injection of 5-FU followed by mechanical trauma (MT) of the cheek pouch. Animals received i.p. injection of atorvastatin (ATV; 1 or 5 mg/kg), saline or saline/ethanol 30 min before 5-FU and daily for 5 days (5 days) or 10 days (10 days). Control groups consisted of normal animals (*N*), animals subjected to MT only and animals subjected to MT that received ATV 5 mg/kg (ATV 5/saline) and saline. Data represent the median values (and range) of macroscopic or microscopic scores in six animals per group. * $P < 0.05$ compared to normal animals, ** $P < 0.05$ compared to animals submitted to 5-FU-induced oral mucositis, receiving saline or saline/ethanol. Data were analyzed using Kruskal–Wallis and Dunn's tests

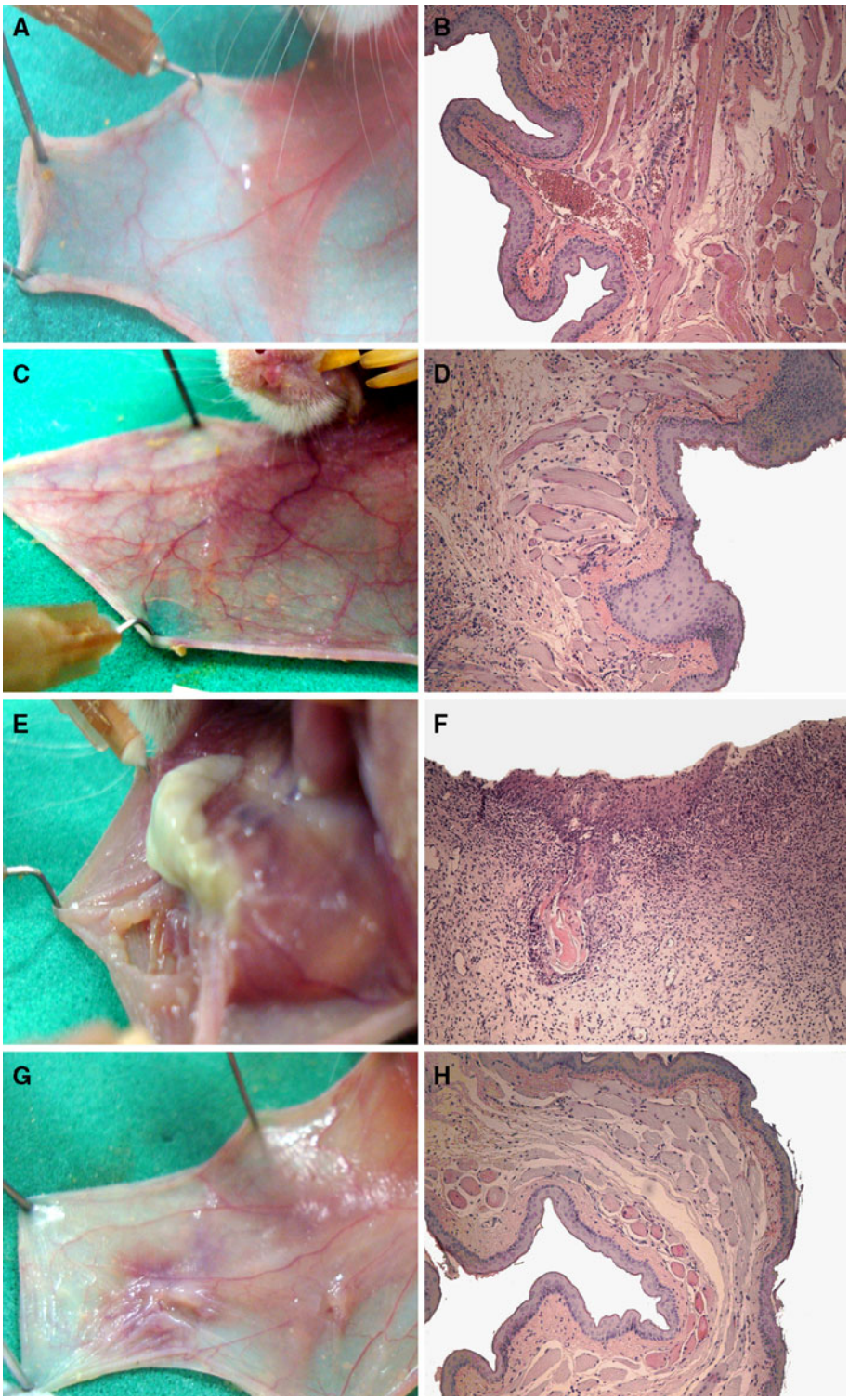

Fig. 1 Macroscopic (*left*) and microscopic (*right*) aspects of normal hamster cheek pouches (**a** and **b**), cheek pouches of hamsters subjected to mechanical trauma (**c** and **d**), cheek pouches of animals subjected to 5-FU-induced oral mucositis that received saline/ethanol (**e** and **f**) or cheek pouches of animals subjected to 5-FU-induced oral mucositis that received atorvastatin (1 mg/kg) (**g** and **h**) (H&E; ×40 magnification). Oral mucositis was induced by intraperitoneal (i.p.) injection of 5-FU followed by mechanical trauma (MT) of the cheek pouch. Animals received i.p. injection of saline/ethanol or ATV 1 mg/kg, 30 min before 5-FU, daily for 10 days

areas, vasodilatation, eventual or discrete ulceration, and the absence of abscesses. On day 10, corresponding to maximal mucositis, the histopathology revealed edema, accentuated inflammatory cell infiltration with neutrophil prevalence, hemorrhagic areas, vasodilatation, extensive ulcers and abscesses (Table 1; Fig. 1f) when compared to normal cheek pouches of hamsters not subjected to OM (Table 1; Fig. 1b) and to the MT group that presented only discrete edema and inflammatory cell infiltration (Fig. 1d; Table 1). On day 5, the treatment with ATV 1 or 5 mg/kg (Table 1) significantly ($P < 0.05$) reduced the 5-FU-induced inflammatory cell infiltration, edema and hemorrhage and prevented the formation of ulceration. Additionally, on day 10, ATV 1 mg/kg significantly reduced the histological

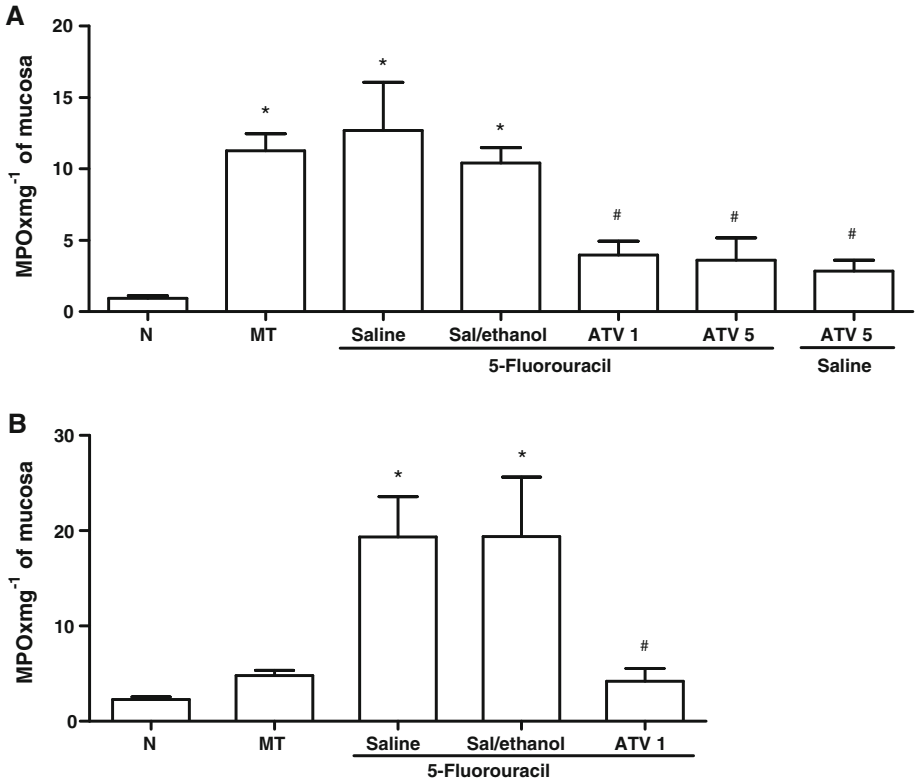

Fig. 2 Atorvastatin (ATV) reduced myeloperoxidase (MPO) activity in the cheek pouches of hamsters subjected to oral mucositis for 5 days (**a**) or 10 days (**b**). Control groups consisted of normal animals (*N*), animals subjected to mechanical trauma (MT) only and animals subjected to MT that received atorvastatin 5 mg/kg (ATV 5) and saline. The experimental groups consisted of animals treated with intraperitoneal (i.p.) injection of 5-FU followed by mechanical trauma and then received i.p. injection of saline, sal/ethanol, atorvastatin 1 mg/kg (ATV 1) or 5 mg/kg (ATV 5) 30 min before 5-FU or saline. Bars represent the mean value ± standard error of the mean (SEM) of MPO units × mg^{-1} of cheek pouch. $^{*}P < 0.05$ represents statistical differences compared to normal hamsters; $^{\#}P < 0.05$ represents statistical differences compared to hamsters subjected to 5-FU-induced oral mucositis and injected with saline/ethanol or saline. The data were analyzed by analysis of variance (ANOVA) and Tukey's multiple comparison test

alterations observed in experimental OM, when compared to saline/ethanol treated animals. This was indicated by the prevention of 5-FU-induced inflammatory cell infiltration, edema hemorrhage and formation of ulcerations or abscesses, resulting in low lesion scores (Table 1). Fig. 1h illustrates the protective effect of treatment with ATV 1 mg/kg, compared to animals subjected to OM that received saline and 5% ethanol (5-FU/sal-ethanol) (Fig. 1f).

Myeloperoxidase activity

The MPO activity was measured in the cheek pouch as an indicator of neutrophil infiltration. The MPO activity of the cheek pouch tissue of animals subjected to MT and of animals subjected to 5-FU-induced experimental OM (5-FU/saline or 5-FU/sal-ethanol) was significantly increased ($P < 0.05$) in comparison with the normal group (N) on the fifth day of OM. ATV at 1 mg/kg or 5 mg/kg significantly ($P < 0.05$) reduced the 5-FU-induced increase in MPO activity. ATV at 5 mg/kg alone did not change the MPO activity compared to normal control (Fig. 2a). On the tenth day of OM, the MPO activity of the cheek pouch tissue of animals subjected to 5-FU-induced experimental OM (5-FU/saline or 5-FU/sal-ethanol) was significantly increased ($P < 0.05$) in comparison with the normal group (N), and ATV 1 mg/kg significantly ($P < 0.05$) reduced the 5-FU-induced increase in MPO activity (Fig. 2b).

Cheek pouch tissue cytokine levels

5-FU significantly ($P < 0.05$) increased the cheek pouch tissue level of TNF-α, but not IL-1β, on day 5. ATV at 1 mg/kg or 5 mg/kg significantly ($P < 0.05$) reduced the 5-FU-induced increase in TNF-α. ATV at 5 mg/kg alone did not change the TNF-α level compared to normal control (Table 2). On the tenth day of OM, 5-FU significantly ($P < 0.05$) increased the cheek pouch tissue level of TNF-α and IL-1β. ATV at 1 mg/kg significantly blocked ($P < 0.05$) this elevation in TNF-α and IL-1β levels in the cheek pouch (Table 2).

Table 2 TNF-α and IL-1β levels (pg/ml) in the cheek pouches of hamsters subjected to 5-FU-induced oral mucositis

Cytokines	TNF-α (5 days)	IL-1β (5 days)	TNF-α (10 days)	IL-1β (10 days)
Normal	1051.0 ± 52.48	288 ± 32.02	96.83 ± 22.44	213.8 ± 30.92
MT	1029.0 ± 96.36	166.3 ± 31.14	172.2 ± 48.85	289.7 ± 11.30
5-FU/saline	2662.0 ± 135.0*	241.3 ± 56.53	417.4 ± 76.74*	435.4 ± 40.50*
5-FU/sal-ethanol	2613.0 ± 115.7*	236.1 ± 38.61	404.0 ± 77.32*	405.0 ± 50.24*
ATV1/FU	1226.0 ± 56.12#	105.0 ± 10.79	177.6 ± 33.13#	206.8 ± 18.39#
ATV5/5- FU	1025.0 ± 72.05#	226.0 ± 46.49	–	–
ATV5/Saline	1087.0 ± 75.93#	183.8 ± 25.61	–	–

Oral mucositis was induced in hamsters by intraperitoneal (i.p.) injection of 5-FU followed by mechanical trauma (MT) of the cheek pouch. Animals received i.p. injection of atorvastatin (ATV; 1 or 5 mg/kg), saline or saline/ethanol, 30 min before 5-FU and daily for 5 days (5 days) or 10 days (10 days). Control groups consisted of normal animals (*N*), animals subjected to MT only and animals subjected to MT that received ATV 5 mg/kg (ATV 5/saline) and saline. The data represent the median values ± standard error of the mean (SEM) of TNF-α and IL-1β level in six animals per group. * $P < 0.05$ represents statistical differences compared to normal hamsters, # $P < 0.05$ represents statistical differences compared to hamsters submitted to 5-FU-induced oral mucositis and injected with saline or saline/ethanol. Data were analyzed using analysis of variance (ANOVA) and Tukey's multiple comparison test

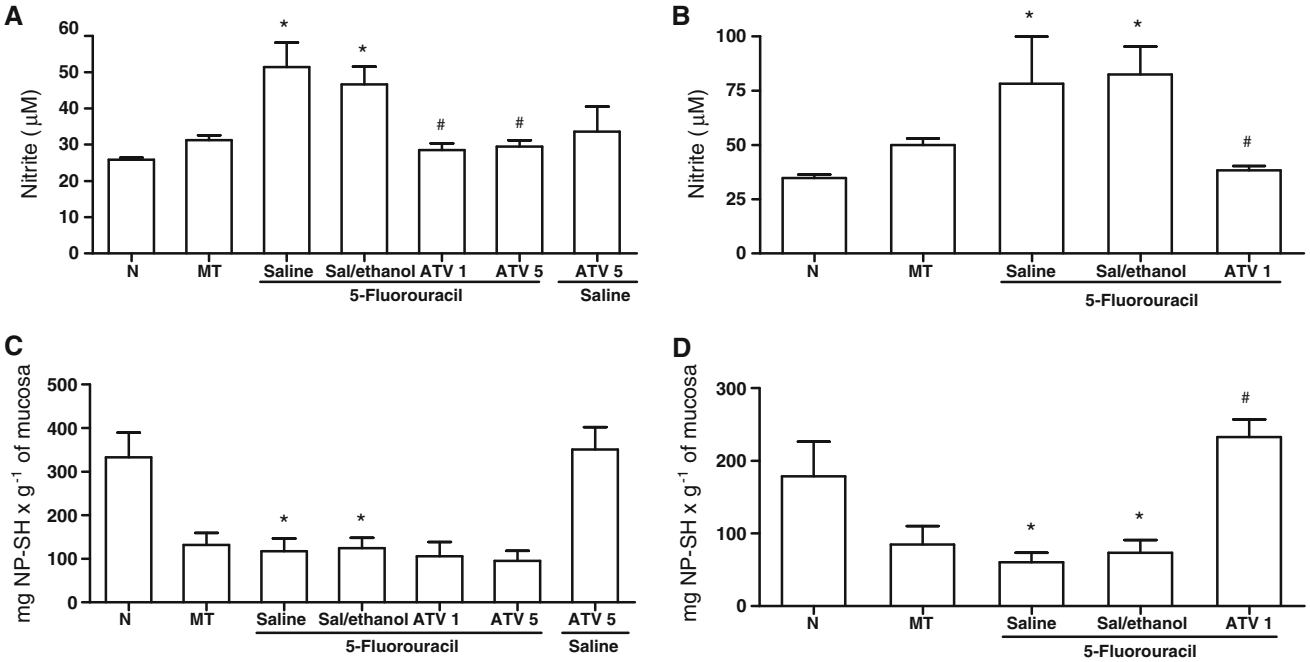

Fig. 3 Quantification of nitrite levels in cheek pouches of hamsters submitted to oral mucositis for 5 days (**a**) or 10 days (**b**) and quantification of non-protein sulfhydryl groups (NP-SH) in cheek pouches of hamsters submitted to oral mucositis for 5 days (**c**) or 10 days (**d**). Oral mucositis was induced in hamsters by the intraperitoneal (i.p.) injection of 5-fluorouracil (5-FU) followed by mechanical trauma (MT). Animals received i.p. injection of atorvastatin (1 or 5 mg/kg), saline or saline–ethanol, 30 min before 5-FU and daily for 5 days (5 days) or 10 days (10 days). Control groups consisted of normal animals (*N*), animals subjected to mechanical trauma (MT) only and animals subjected to MT that received atorvastatin 5 mg/kg and saline (ATV 5/saline). Data represent the median values ± standard error of the mean (SEM) of nitrite levels or non-protein sulfhydryl groups (NP-SH) in six animals per group. * $P < 0.05$ represents statistical differences compared to normal hamsters; # $P < 0.05$ represents statistical differences compared to hamsters submitted to 5-FU-induced oral mucositis and injected with saline or saline–ethanol. Data were analyzed using analysis of variance (ANOVA) and Tukey's multiple comparison test

Cheek pouch tissue nitrite and non-protein sulfhydryl groups (NP-SH) levels

The nitrite levels in the cheek pouch tissue of animals subjected to 5 or 10 days of 5-FU-induced experimental OM were significantly increased ($P < 0.05$) in comparison with the normal group (N) or with the group of animals subjected to MT (MT) (Fig. 3a, b, respectively). The administration of ATV (1 mg/kg or 5 mg/kg) significantly ($P < 0.05$) reduced the 5-FU-induced increase in nitrite levels (Fig. 3a). ATV at 1 m/kg also reduced the 5-FU-induced increase in nitrite levels on the tenth day of OM, restoring this parameter to

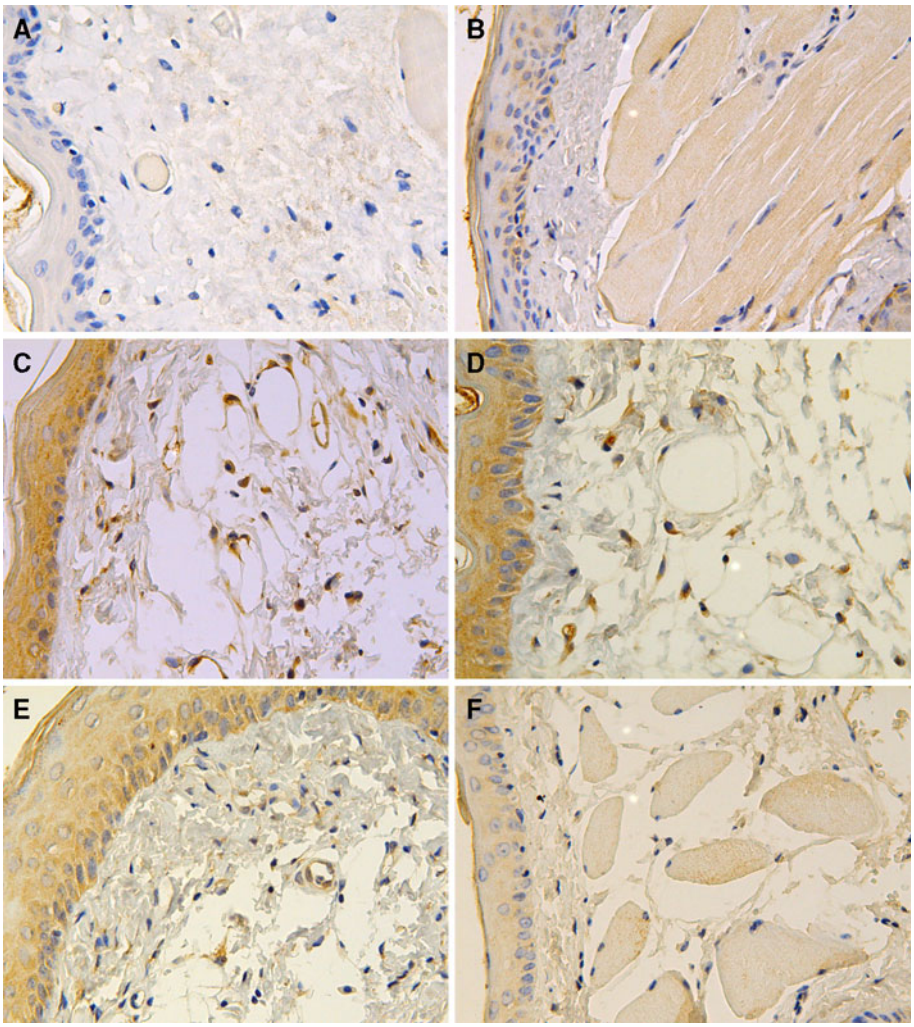

Fig. 4 Representative photomicrographies of iNOS immunohistochemistry in the cheek pouches of hamsters subjected to 5-fluorouracil (5-FU)-induced oral mucositis for 5 days. The cheek pouch tissue of hamster submitted to 5-FU-induced oral mucositis, receiving saline (**c**) or saline/ethanol (**d**) presented intense immunostaining for iNOS in the inflammatory cells when compared to the cheek pouch of normal hamster (**b**). The treatment with atorvastatin 1 mg/kg (**e**) or 5 mg/kg (**f**) considerably reduced the immunostaining in the conjunctive tissue. Negative control represents a sample of the cheek pouch where the first antibody was replaced by PBS-BSA 5% and no immunostaining was detected (**a**). Magnification ×400

normal levels (Fig. 3b). The levels of non-protein sulfhydryl groups (NP-SH) in the cheek pouch tissue of animals subjected to 5-FU-induced OM and MT were significantly decreased ($P < 0.05$) at 5 and 10 days when compared to the normal control group (N) (Fig. 3c, d). The administration of ATV 1 mg/kg significantly ($P < 0.05$) reversed the effects of experimental OM on stores of NP-SH levels in the mucosa on the 10th day (Fig. 3d).

Immunohistochemical reaction for induced nitric oxide synthase

The cheek pouches of hamsters submitted to oral mucositis for 5 days and treated with saline or saline/ethanol showed marked immunostaining for iNOS on inflamed conjunctive tissue (Fig. 4c, d) compared with the cheek pouches of the normal control group (Fig. 4b). Atorvastatin 1 and 5 mg/Kg caused considerable reduction in the iNOS immunostaining in the check pouches tissue when compared with the group of animals subjected to experimental mucositis that received saline or saline/ethanol (Fig. 4e, f).

Effect of ATV on the survival rates of hamsters subjected to OM

The control group, consisting of animals not subjected to OM (normal) and animals that received only mechanical trauma (MT), showed 100% survival during 10 days of investigation. The groups subjected to OM and treated with saline or saline and 5% (vol/vol) ethanol (5-FU/saline and 5-FU/sal-ethanol) showed 40% mortality. The animals with OM and treated i.p. with ATV 1 mg/kg (ATV 1/5-FU) showed 60 % mortality over 10 days. The groups with OM and treated i.p. with ATV 5 mg/kg or 10 mg/kg (ATV 5/5-FU or ATV 10/5-FU) exhibited 100% mortality on the 10th day. However, all animals not subjected to OM that received MT of cheek pouches or those treated with ATV 5 mg/kg (ATV 5/saline) survived throughout the 10 days of the experiment (Fig. 5). These findings indicate that high doses (5 mg/kg or 10 mg/kg) of ATV in addition to 5-FU significantly decreased ($P < 0.05$) the survival rate of hamsters when compared to animals undergoing experimental OM treated with saline or saline/ethanol.

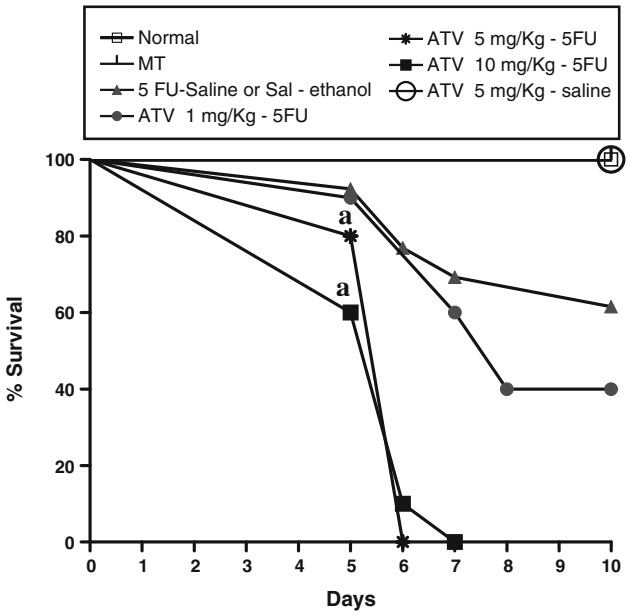

Fig. 5 Pretreatment with atorvastatin (ATV) 5 mg/kg reduced survival rates of the hamsters submitted to 5-FU-induced oral mucositis. Oral mucositis was induced in hamsters by intraperitoneal (i.p.) injection of 5-FU followed by mechanical trauma (MT) of the cheek pouch. The survival rates of hamsters were determined daily up to 10 days. The hamsters were pretreated for 10 consecutive days with ATV (1, 5 or 10 mg/kg i.p.), saline or saline/ethanol 5% once a day. The doses of ATV were administered 30 min before i.p. injection of 5-FU or saline. The results are expressed as % survival. (*a*, $P < 0.05$; Kaplan–Meier log rank test)

Effects of ATV on liver, kidney, heart and lung histopathology

Liver histology of hamsters subjected to 5 days of 5-FU-induced experimental OM and treated with ATV 5 mg/kg (ATV5/5-FU) presented vacuolization (Fig. 6d), focal necrosis and inflammatory cell infiltration (Fig. 6e, f) in comparison with normal (N) (Fig. 6a), 5-FU/sal-ethanol (Fig. 6b) or ATV 5/saline groups (Fig. 6c). The kidney, heart and lung presented normal histology.

Effects of ATV on systemic changes: biochemical analyses and leukogram

To explain the high mortality of animals with OM that were treated with ATV (5 and 10 mg/kg) for 10 days, biochemical analyses and a leukogram were performed on the 5th day of the experiment. Figure 7 shows that the levels of hepatic enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), of animals subjected to 5 days of 5-FU-induced experimental OM and treated with ATV 5 mg/kg (ATV5/5-FU), were significantly increased ($P < 0.05$) in comparison with the 5-FU/saline or 5-FU/sal-ethanol groups. However, the animals treated with ATV 5 mg/kg that did not receive 5-FU (ATV 5/saline) had no increase in serum transaminases. Other biochemical values, specifically creatinine and urea, did not differ between groups (Table 3). Animals subjected to 5 days of 5-FU-induced experimental OM and treated with ATV 5 mg/kg presented significant intensification of leukopenia, neutropenia and lymphopenia ($P < 0.05$) in comparison with the 5-FU/saline or 5-FU/sal-ethanol groups. ATV 5 mg/kg alone did not change the leukocyte number compared to normal control (N) (Fig. 8).

Effects of ATV on systemic changes: Bacteremia

Table 4 shows the bacterial infection in the blood of animals in the various experimental groups on the 5th day of the experiment. The normal animals did not exhibit bacteremia (−). In the MT and ATV 5/saline groups, there was bacteremia (+) in a few animals, 40% (2/5) and 50% (3/6), respectively, caused by Gram-positive bacteria (*Staphylococcus* coagulase negative). The 5-FU/saline, 5-FU/sal-ethanol, ATV 1/5-FU and ATV 5/5-FU hamsters exhibited bacteremia (+) in 80 % (4/5), 83.3% (5/6), 85.7% (6/7) and 100% (5/5) of animals, respectively. The infection of 5-FU/saline, 5-FU/sal-ethanol and ATV1/5-FU groups was caused primarily by Gram-positive bacteria (*Staphylococcus* coagulase negative) in 80% (4/5), 67% (4/6) and 57% (4/7) of animals, respectively. Curiously, the animals with OM and treated with ATV 5 mg/kg were not infected by Gram-positive bacteria but showed 100 % (5/5) infection by Gram-negative bacilli (*Escherichia coli, Klebsiella* sp., *Citrobacter* sp. or *Arizona* sp.) compared with 28.5% (2/7) of ATV 1 mg/kg and 16.6% (1/6) of 5-FU-saline group (Table 4).

Discussion and conclusions

In the present study, we demonstrated that atorvastatin (ATV) significantly reduced the macroscopic and microscopic lesions induced by 5-FU in the oral mucosa of hamsters. The macroscopic protective effects were associated with reduced production of TNF-α and IL-1β and decreased neutrophil infiltration evidenced by histopathological analysis and MPO activity. In addition, ATV also reduced oxidative stress and induced an increase in non-protein sulfhydryl groups. This effect of ATV is consistent with previous reports showing that ATV has anti-inflammatory and immunomodulatory action that is distinct from its cholesterol-reducing action [25, 36–38]. OM is characterized by an intense inflammatory reaction caused by effects of chemotherapeutic agents on the mucosa lamina propria cells, which results in activation of the transcription factor NF-kB [39, 40]. The activation of NF-kB leads to transcription

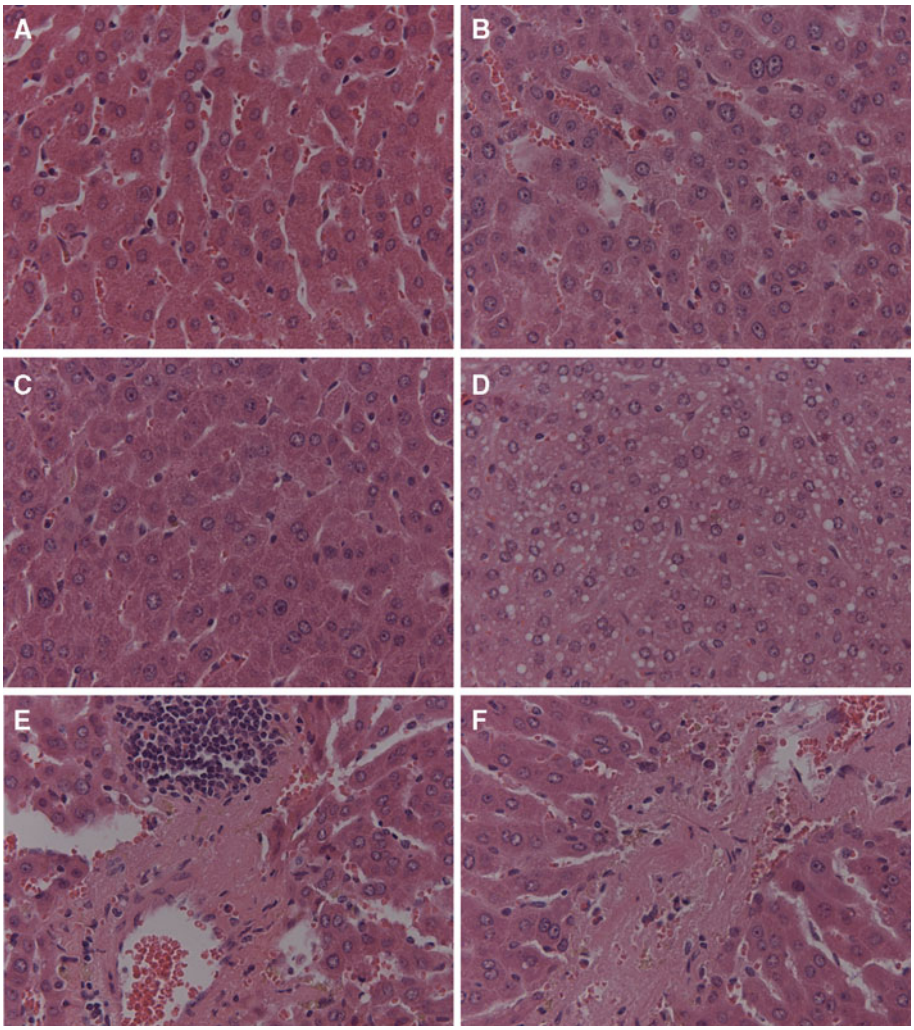

Fig. 6 Effect of atorvastatin (ATV; 5 mg/kg) on liver histology of hamsters subjected to 5-FU-induced oral mucositis for 5 days. Aspects of normal hamster liver (**a**), liver of animals subjected to oral mucositis that received saline/ethanol (**b**), liver of hamsters subjected to MT that received ATV 5 mg/kg (ATV 5/saline) (**c**) and liver of animals subjected to 5-FU-induced oral mucositis that received ATV 5 mg/kg (ATV 5/5-FU) (**d**), (**e**), (**f**). Samples of liver were processed for hematoxylin and eosin staining (×40 magnification)

of genes involved in the synthesis of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α [39, 40], and agents known to attenuate the expression of cytokines have demonstrated efficacy in the prevention of experimental mucositis [10, 35].

In this investigation, we demonstrated that ATV (1 or 5 mg/kg) significantly ($P < 0.05$) reduced the 5-FU-induced increase in TNF-α and IL-1β levels. In agreement with our data, various articles have shown that ATV decreases TNF-α and IL-1β production [41, 42]. These data are supported by the finding that the release of pro-inflammatory cytokines is largely dependent on the NF-kB transcription pathway [43], and other authors suggest that statins diminish the activity of NF-kB [44, 45]. Thus, the protective effect of ATV found in OM could be related to its capacity to inhibit cytokine production. Cytokines have been shown to stimulate the expression of the inducible NOS synthase isoform (iNOS) with consequent production of nitric oxide (NO). In this study, we demonstrated that 5-FU-induced OM presented marked expression of iNOS and increased nitrite level in the cheek pouch, suggesting increased synthesis of NO in the tissue. This gas appears to play a beneficial and detrimental role. Beneficial effects may include antimicrobial activity and immune modulation [46, 47]. On the other hand, detrimental effects may include a cytotoxic action toward the adjacent host tissues, resulting in tissue lesions [48–52]. It has been extensively demonstrated that iNOS-derived NO mediates pathogenesis of experimental and human inflammatory diseases, including OM [27]. Accordingly, the present study demonstrated that ATV clearly decreased the nitrite level and reduced the iNOS immunostaining in the cheek pouch, and this could result in tissue protection.

According to the literature, statins downregulate the expression of ICAM-1 and LFA-1, and through binding to LFA-1, they interfere with ICAM-1–LFA-1 interaction resulting in statin-induced inhibition of neutrophil activity [25]. Treatment of post-infarction patients with ATV also reduced soluble ICAM and E-selectin, adhesion molecules involved in neutrophil migration [53]. In addition, ATV significantly reduced neutrophil transendothelial migration toward the chemoattractant formyl-methionyl-leucyl-phe-

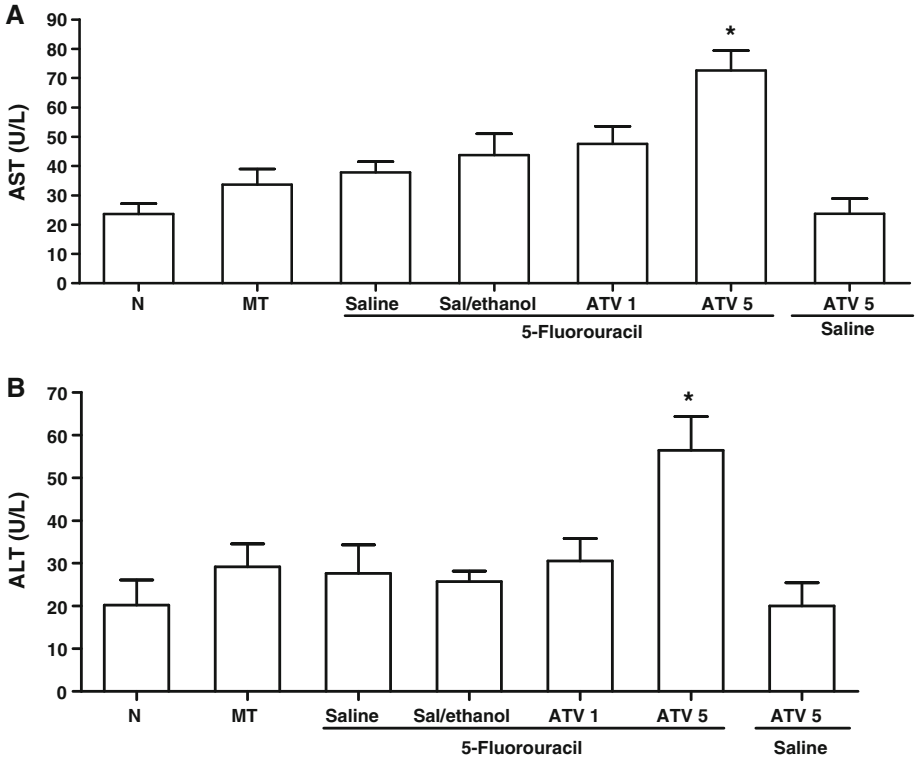

Fig. 7 Atorvastatin 5 mg/kg (ATV 5) increased aspartate aminotransferase (AST) and alanine aminotransferase (ALT) serum levels in animals with oral mucositis after 5 days. Oral mucositis was induced in hamsters by intraperitoneal (i.p.) injection of 5-FU followed by mechanical trauma (MT) of the cheek pouch. Animals received saline, saline–ethanol 5% or ATV (1 or 5 mg/kg i.p.) 30 min before 5-FU injection daily for 5 days. Control groups consisted of normal animals (*N*), animals subjected to mechanical trauma (MT) only and animals subjected to MT that received atorvastatin 5 mg/kg and saline (ATV 5/saline). Blood was collected by heart puncture when the hamsters were killed on day 5. *Bars* represent the mean value ± standard error of the mean (SEM) of AST (**a**), and ALT (**b**). $^*P < 0.05$ represents statistical differences compared to hamsters submitted to 5-FU-induced oral mucositis and injected with saline or saline/ethanol. The data were analyzed using analysis of variance (ANOVA) and Tukey's multiple comparison test

nylanine (fMLP), by reducing fMLP-induced Rho activity in neutrophils. In a rat model of adjuvant-induced arthritis, ATV (1–10 mg) decreased neutrophil influx and concentrations of the cytokines IL-1β, IL-6 and TNF-α and the chemokines CCL5 and CCL2 [41]. These data point to an important inhibitory effect of statins on neutrophil migration. Although neutrophils are essential components of host defense, they are also associated with tissue destruction. In 5-FU-induced OM, MPO activity is increased [27, 54], indicating a crucial role for neutrophils in this condition. Therefore, the inhibitory effect of ATV on neutrophil migration is probably related to the protective effect of ATV on 5-FU-induced oral mucosal damage.

In addition, we also demonstrated that the cheek pouch tissue glutathione stores of the animals subjected to 5-FU-induced experimental OM were significantly decreased when compared to normal animals. These data are consistent with a previous study [54]. In the present study, ATV 1 mg/kg restored glutathione levels on the 10th day, which might be related to a decrease in oxidative stress. Glutathione, the major intracellular antioxidant, is involved in several fundamental biological functions, including free radical scavenging, detoxification of xenobiotics and carcinogens, redox reactions, and biosynthesis of DNA and proteins [55] and is essential to normal cell function and replication [56]. In addition, glutathione has an inhibitory effect on a number of cytokines [57, 58]. Thus, our results suggest that ATV administration restored glutathione levels, thereby enhancing cell protection and regulating cell proliferation after exposure to 5-FU that generates toxic quantities of free radicals.

Although ATV has considerably improved local 5-FU-induced OM, the higher doses of ATV (5 mg/kg and 10 mg/kg), proportionally higher than those used in clinical practice, reduced the survival rate of animals with OM after the fifth day, suggesting a negative drug interaction between 5-FU and ATV. To clarify this hypothesis, we performed systemic evaluation of main organ histology, leukocytes and biochemical parameters.

Our results revealed that the animals treated with ATV 5 mg/kg alone showed no changes in serum levels of transaminases. This result is consistent with studies that show a low incidence of increased transaminases and exceedingly

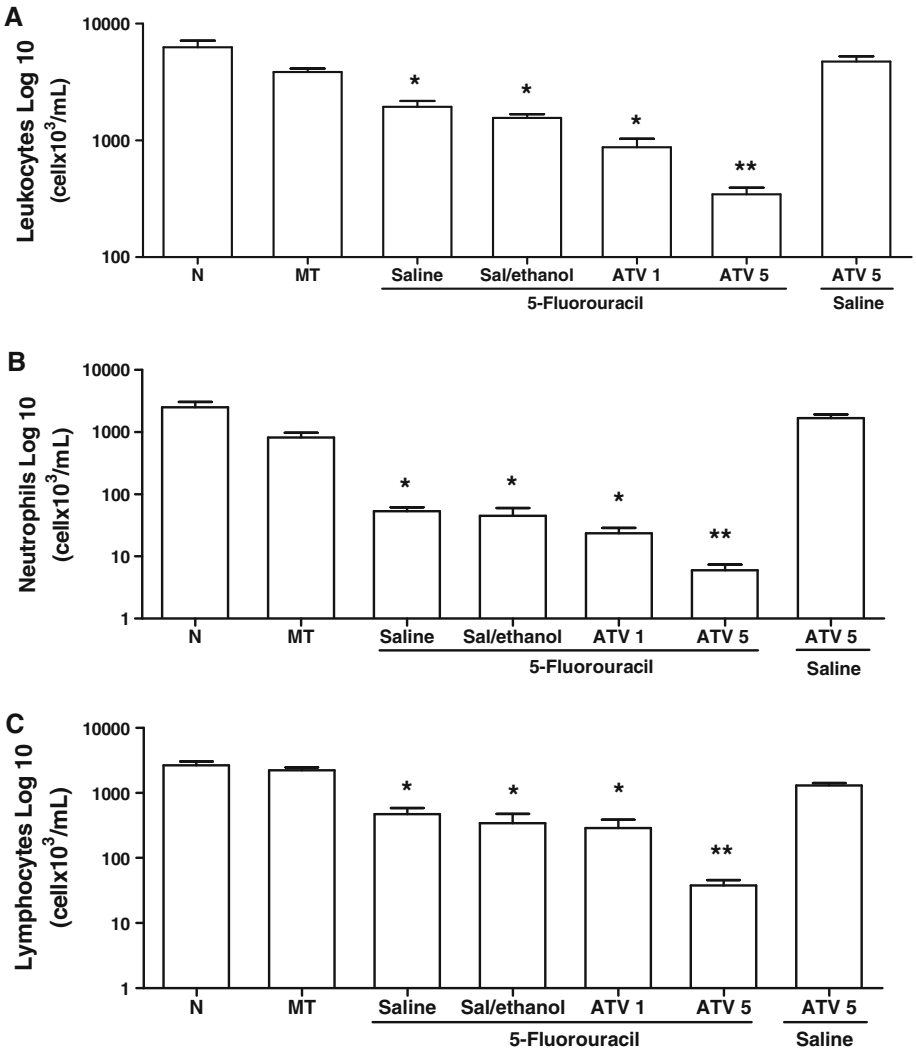

Fig. 8 Atorvastatin 5 mg/kg (ATV 5) amplified the leukopenia, neutropenia and lymphopenia in oral mucositis for 5 days. Oral mucositis was induced in hamsters by intraperitoneal (i.p.) injection of 5-FU followed by mechanical trauma (MT) of the cheek pouch. Animals received saline, saline–ethanol 5% or ATV (1 or 5 mg/Kg i.p.) 30 min before the injections of 5-FU and daily until they were killed. Control groups consisted of normal animals (*N*), animals subjected to mechanical trauma (MT) only and animals not subjected to MT that received atorvastatin 5 mg/kg (ATV 5/saline) and saline. Blood was collected by heart puncture when the hamsters were killed on day 5. *Bars* represent the mean value ± standard error of the mean (SEM) of total leukocytes (**a**), neutrophils (**b**) and lymphocytes (**c**) $\times 10^3$/ml of at least 5 animals. $^*P < 0.05$ represents statistical differences compared to normal hamsters; $^{**}P < 0.05$ represents statistical differences compared to hamsters subjected to 5-FU-induced oral mucositis and injected with saline or saline/ethanol. The data were analyzed using analysis of variance (ANOVA) and Tukey's multiple comparison test

Table 3 Urea and creatinine levels (mg/dl) in the cheek pouches of hamsters subjected to 5-FU-induced oral mucositis for 5 days

Experimental groups	Urea	Creatinine
Normal	39.20 ± 4.044	0.03833 ± 0.02386
MT	38.27 ± 2.283	0.0175 ± 0.01436
5-FU/saline	61.54 ± 11.38	0.04667 ± 0.02963
5-FU/sal-ethanol	41.17 ± 5.356	0.0475 ± 0.02323
ATV1/5-FU	49.16 ± 7.351	0.0860 ± 0.03043
ATV 5/5-FU	39.55 ± 6.775	0.0750 ± 0.03122
ATV 5/saline	50.91 ± 7.341	0.0400 ± 0.03082

Data are presented as the mean ± SEM of six animals. $P > 0.05$ ANOVA followed by Tukey's multiple comparison test

rare progression to liver failure during statin therapy [59–61]. The combination of the highest dose (5 mg/kg) of ATV with 5-FU resulted in increased levels of AST and ALT on the fifth day of the experiment associated with vacuolization, focal necrosis and inflammatory cell infiltration seen in liver histopathology. However, the intensity of the liver lesion is not likely to be responsible for increased mortality.

The leukocyte count in the fifth day revealed amplification of leukopenia, neutropenia and lymphopenia in animals treated with ATV 5 mg/kg and subjected to 5-FU-induced OM. Leukopenia is a well-known event in patients treated with most chemotherapeutic agents and is consistent with our previous study that shows leukopenia in animals subjected to experimental OM [35]. Various statins have been previously shown to exhibit antiproliferative effects on tumor cells via regulatory effects on cell cycle [62–64], but leukopenia is not a usual side effect of statins alone. Together with liver lesion, we speculate if -the amplification of 5-FU-induced leukopenia by ATV 5 mg/kg is causing high mortality of the animal group subjected to OM and treated with ATV 5 mg/kg. Reports from the literature show that infection is one of the major causes of morbidity in patients with OM and treated with a chemotherapeutic agent. The risk of infection increases with the severity and duration of neutropenia [65], and patients with mucositis and neutropenia have a relative

Table 4 Bacteria in the serum of hamsters subjected to 5-FU-induced oral mucositis for 5 days and identification of microorganisms

Experimental groups	No. with bacteremia/total	Microorganism				
		E. coli	*Klebsiella* sp.	*Citrobacter* sp.	*Arizona* sp.	*Staphylococcus* Coagulase negative
Normal	0/6 (−)	−	−	−	−	−
MT	2/5 (+)	−	−	−	−	+ (2)
5-FU/saline	4/5 (+)	−	−	−	−	+ (4)
5-FU/sal-ethanol	5/6 (+)	+ (1)	−	−	−	+ (4)
ATV1/5-FU	6/7 (+)	−	+ (2)	−	−	+ (4)
ATV5/5-FU	5/5 (+)	+ (1)	+ (2)	+ (1)	+ (1)	−
ATV5/saline	3/6 (+)	−	−	−	−	+ (3)

Bacteremia was determined, varying from none (−) to positive (+). The identification of microorganisms is expressed as negative (−) and positive (+) for *Escherichia coli*, *Klebsiella* sp., *Citrobacter* sp., *Arizona* sp. or *Staphylococcus* coagulase negative. The numbers in the microorganism column indicate the number of animals with positive results/number of animals in the tested group

risk of septicemia that is greater than four times that of individuals without mucositis [6].

To investigate whether the animals had bacteremia, microbiological cultures of the blood of animals were performed on the fifth day of experiment. The bacteremia was detected in groups MT and ATV 5/saline in a few animals and was not associated with deaths; all hamsters survived throughout the 10 days of the experiment. We speculated that the stress of anesthesia and mechanical trauma may result in the translocation of microflora of the jugal mucosa to systemic circulation of animals and result in bacteremia by Gram-positive bacteria. The hamsters treated with 5-FU-saline or saline/ethanol and ATV 1-FU exhibited bacteremia but with a predominance of Gram-positive bacteria (*staphylococcus* coagulase negative), and although leukopenic, were able to survive. However, the ATV 5-FU animals developed a systemic infection by Gram-negative bacteria. The bacteremia may have contributed to the high mortality rate in this group We speculate if the inability of animals to resolve the infection may be due to severe leukopenia and more specifically neutropenia, which was amplified after the association treatment of 5-FU with ATV 5. This fact is an apparent contradiction with the demonstration that treatment with ATV reduces the mortality of patients with severe sepsis [66, 67] and improves survival of mice subjected to experimental sepsis [68]. A possible explanation is that ATV controls inflammation during sepsis in patients that are initially immunocompetent [69] or in experimental models of sepsis in which ATV does not potentiate the leukopenia [68].

In conclusion, treatment with ATV showed an important protective action in OM. However, the association of 5-FU with high doses of ATV deserves attention regarding hepatotoxicity and leucopenia. The possibility of using ATV in the treatment of human oral mucositis merits further investigation.

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Conflict of interest The authors state no conflict of interest.

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