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ORIGINAL ARTICLE

Role of nitric oxide on pathogenesis of 5-fluorouracil induced experimental oral mucositis in hamster

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Abstract
Introduction Mucositis induced by antineoplastic drugs is an important, dose-limiting, and costly side effect of cancer therapy. *Aim*: To investigate the role of nitric oxide (NO) on the pathogenesis of 5-fluorouracil (5-FU)-induced oral mucositis.
Materials and Methods 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) in male hamsters. Animals were treated subcutaneously with saline (0.4 ml), 1,400 W (1 mg/kg), aminoguanidine (5 or 10 mg/kg) or Nφ-Nitro-L-Arginine Methyl Ester (L-NAME) (5, 10, or 20 mg/kg) 1 h before the injections of 5-FU and daily until sacrifice, on the tenth day. Macroscopic and histopathological analyses were evaluated and graded. Tissues from the cheek pouches were harvested for measurement of myeloperoxidase (MPO) activity, nitrite level, and immunohistochemistry for induced nitric oxide synthase (iNOS).
Results Treatment with 1,400 W or aminoguanidine reduced macroscopic and histological parameters of oral mucositis, and reduced the inflammatory cell infiltration as detected by histopathology and by MPO activity. In contrast, the administration of L-NAME did not significantly reverse the inflammatory alterations induced by experimental mucositis. Increased NOS activity, nitrite level and immunostaining for iNOS were detected on the check pouch tissue of animals submitted to 5-FU-induced oral mucositis on the tenth day.
Conclusion These results suggest an important role of NO produced by iNOS in the pathogenesis of oral mucositis induced by 5-FU.

Keywords Nitric oxide · Nitric oxide synthase · 5-fluorouracil · Oral mucositis · 1,400 W · Aminoguanidine · Nφ-Nitro-L-Arginine Methyl Ester

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Introduction

Ulcerative oral mucositis is one of the most common, bothersome, and dose-limiting complications of cancer chemotherapy [14, 43, 44, 58]. An overall frequency of ~40% of oral mucositis has been reported to be associated with the use of a variety of agents including 5-fluorouracil (5-FU) [6]. In addition to severe pain, as a consequence of the disruption of the mucosal barrier, local and systemic infections may develop, representing a clinically significant risk factor for sepsis [15]. Furthermore, the intensification of oral mucositis interferes with patient ability to maintain oral nutrition [30].

It has been suggested that oral mucositis results from a sequential interaction of drug-related direct

epithelial injury, local factors such as saliva and oral microbiota with oral mucosa cells, resulting in release of pro-inflammatory cytokines and free radicals [55]. The literature indicates an important role for cytokines, particularly IL-1 and TNF-α in the pathophysiology of mucositis [32, 33, 41].

Cytokines have been shown to stimulate the expression of the inducible NOS synthase isoform (iNOS) with consequent production of nitric oxide (NO). This gas appears to play beneficial as well as detrimental role. Beneficial effects may include antimicrobial activity and immune modulation [2, 34]. On the other hand, detrimental effects may include a cytotoxic action toward the adjacent host tissues, resulting in pain, tissues lesions, apoptosis, and bone resorption [17, 29, 37, 47, 53]. In fact, a biphasic effect of iNOS in inflammation has also been reported. Depending on the setting, the role or iNOS has ranged from enhancing inflammation to retarding it [50]. For example, an exacerbation of antigen-induced arthritis in inducible NO synthase-deficient mice [64] was observed which suggests that NO production by iNOS has antiinflammatory effects in experimental arthritis. On the contrary, it has been extensively demonstrated that iNOS-derived NO mediates pathogenesis of experimental and human inflammatory diseases, including periodontitis [36, 39, 40, 46], joint diseases such as rheumatoid arthritis [12, 61], and hemorrhagic cystitis [60]. Thus, the aim of this study was to investigate the role of NO on the pathogenesis of 5-FU-induced oral mucositis.

Materials and methods

Animals

Seventy-two male adult golden hamsters weighing 140–160 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 Use of Federal University of Ceará, Brazil.

Materials

Nφ-Nitro-L-Arginine Methyl Ester (L-NAME), aminoguanidine, and *N*-(3-(Aminomethyl)benzyl)acetamidine (1,400 W) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 5-FU used is a product of Roche, Rio de Janeiro, Brazil. Rabbit anti-NOS-2 and Biotinylated goat anti-rabbit were purchased from Santa Cruz Biotechnology. Vectastatin® ABC detection system, and the VIP substrate kit used in immunohistochemistry were from Vector Laboratories (Burlingame, CA, USA).

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 [53]. In order to mimic the friction to which the oral mucosa is normally subjected, the animal cheek pouch mucosa was irritated by superficial scratching with the tip of an 18-gauge needle on the fourth day, under anesthesia with chloral hydrate (250 mg/kg, i.p.). The needle was dragged twice in linear fashion across the everted cheek pouch until erythematous changes were noted. The animals were sacrificed on the tenth day after the initial injection of 5-FU, under anesthesia with chloral hydrate (250 mg/kg, i.p.), the cheek pouches were everted and photographed, and then the hamster was sacrificed. Samples of cheek pouches were removed from six animals per group for histopathological analysis, immunohistochemistry for iNOS, and measurement of NOS activity, determination of nitrite level and for myeloperoxidase (MPO) assay.

Experimental groups

Hamster groups with oral mucositis were treated subcutaneously with aminoguanidine (5 or 10 mg/kg), 1,400 W (1 mg/kg), selective inhibitors of iNOS [20, 22], or L-NAME (5, 10, or 20mg/kg), a non-selective inhibitor of NOS [48] 1 h before the injections of 5-FU and daily until sacrifice, on the tenth day. Control groups consisted of animals not subjected to oral mucositis (control), a non-treated group which was subjected to the experimental mucositis by 5-FU administration and mechanical irritation and received saline (5-FU) and a group which received only mechanical trauma (MT) of cheek pouches on the fourth day.

Macroscopic analysis of cheek pouch

Photographs were used for scoring lesions. For macroscopic analysis, inflammatory aspects such as erythema, hyperemia, hemorrhagic areas, epithelial ulcerations, and abscesses were evaluated in a single-blind fashion and graded as follows. Score 0: normal cheek pouch with erythema and hyperemia absent or discreet; absence of hemorrhagic areas, ulcerations or abscess.

Score 1: moderate erythema and hyperemia; absence of hemorrhagic areas, ulcerations, or abscess. Score 2: severe erythema and hyperemia; presence of hemorrhagic areas, small ulcerations, or scarred tissue, absence of abscess. Score 3: severe erythema and hyperemia; presence of hemorrhagic areas, extensive ulcerations, and abscesses.

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). 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, reepithelization 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, absence of abscesses. Score 3: severe vasodilatation, inflammatory infiltration with neutrophil prevalence, presence of hemorrhagic areas, edema and extensive ulceration, and abscesses.

Myeloperoxidase assay

Cheek pouch samples were harvested and stored at −70°C until required for assay. After homogenization and centrifugation (4,500*g*, 20 min), MPO activity, an enzyme found in azurophil neutrophil granules, used as a marker for the presence of neutrophils in inflamed tissue, was determined by a colorimetric method described previously [59] and expressed as units of MPO per 5 mg of tissue.

Determination of nitric oxide synthase activity

After the incubation the cheek pouch fragments were homogenized with 0.2 ml 20 mM HEPES (pH 7.4) containing 1.25 mM $CaCl_2$, 1 mM dithiothreitol (DTT), and 100 mM tetrahydrobiopterin (BH4). After homogenization, 1 mM NADPH and 200,000 cpm of [^{14}C] arginine (270 μCi/mmol) were added and incubated for 15 min at 37°C, then the homogenates were centrifuged at 10,000*g* for 10 min at 4°C. The resulting supernatants were applied to 2 ml columns of Dowex AG WX-8 (Na+ form) and these were eluted with 3 ml of double-distilled water [^{14}C]. Citrulline was then determined in a beta counter. This method is based on the equimolar production of citrulline and NO from arginine, mediated by NOS activity. The results are expressed as citrulline production per mg of tissue [21].

Determination of nitrite levels

The production of NO was determined indirectly by measuring the nitrite levels based on the Griess reaction [7]. Briefly, 100 μl of cheek pouch tissue homogenate were incubated with 100 μl of the Griess reagent (1% sulfanilamide in 1% H_3PO_4/0.1%N-(1-naphthyl)ethylenediamine dihydrochoride/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, nitrite concentration was determined from a standard nitrite curve generated by using $NaNO_2$.

Immunohistochemical reaction for induced nitric oxide synthase

Immunohistochemistry for iNOS was performed using the streptavidin-biotin-peroxidase method [27] 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.

Statistical analysis

Data were described as either mean ± SEM or median, as appropriate. Analysis of Variance (ANOVA) followed by Bonferroni's test was used to compare means and Kruskal–Wallis- and Mann–Whitney-tests to compare medians; $p < 0.05$ was defined as statistically significant.

Results

Effect of Nφ-nitro-L-arginine methyl ester, aminoguanidine and 1,400 W in oral mucositis

The i.p. administration of 5-FU, followed by MT of the cheek pouch of the animals caused significant lesions ($p < 0.05$), represented by accentuated erythema, hemorrhage, extensive ulcers, and abscesses (Fig. 1c; Table 1), when compared with the group of animals not subjected to oral mucositis (Control; Fig. 1a; Table 1) or with animals submitted to MT only (MT; Table 1). The treatment of the animals with specific (1,400 W; 1 mg/kg) or selective (aminoguanidine; 10 mg/kg) inhibitor of iNOS significantly prevented the 5-FU induced oral damage presenting reduced erythema and absence of ulcerations and abscesses on the tenth day ($p < 0.05$; Fig. 1e,g, respectively; Table 1). Aminoguanidine at dose of 5 mg/kg was ineffective. In contrast, treatment of the animals with L-NAME (5, 10, or 20 mg/kg), which is a non-selective inhibitor of NOS [23], did not significantly prevent the lesions induced by experimental oral mucositis (Table 2).

The histopathology of the cheek pouch of animals subjected to 5-FU-induced oral mucositis revealed accentuated vasodilatation, intense cellular infiltration with neutrophil prevalence, hemorrhagic areas, edema, abscesses, and extensive ulcers (Fig. 1d; Table 1), when compared with normal cheek pouches of hamsters not subjected to oral mucositis (Fig.1b, Table 1) and of the MT group (Table 1). The treatment with 1,400 W (Fig. 1f; Table 1) and aminoguanidine (Fig. 1h; Table 1)

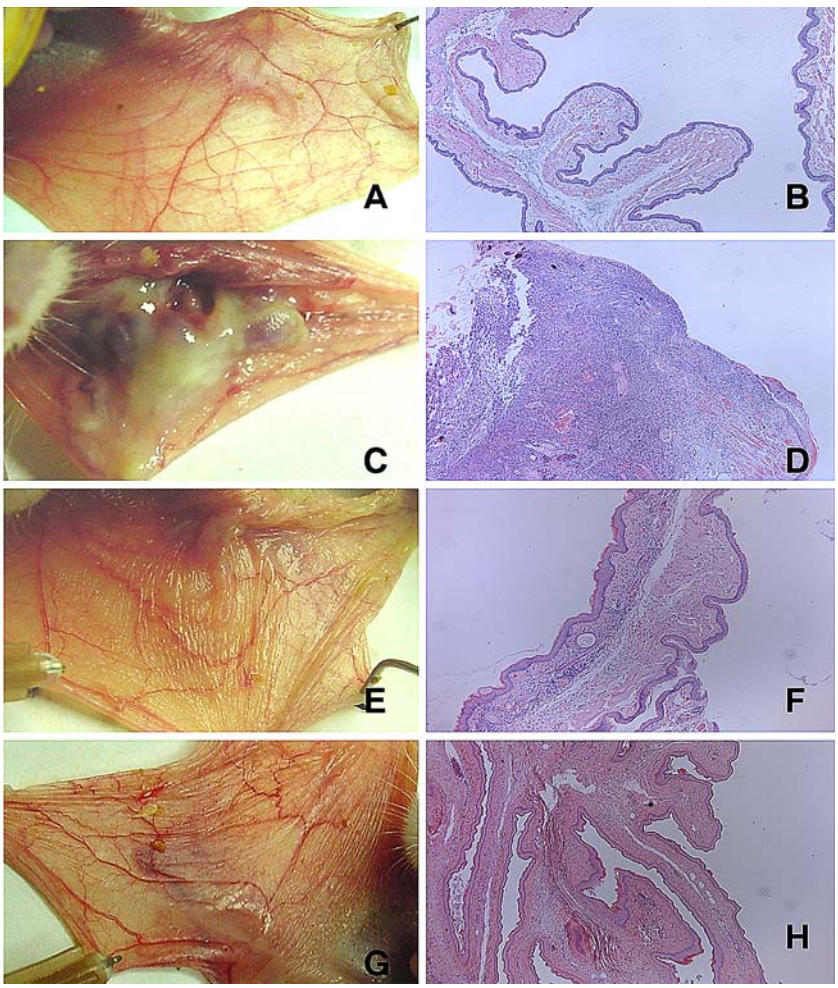

Fig. 1 Macroscopic and microscopic aspects of normal hamster cheek pouches (**a** and **b**) or cheek pouches of animals submitted to 5-FU-induced oral mucositis, receiving saline (**c** and **d**), aminoguanidine (**e** and **f**) or 1,400 W (**g** and **h**). Oral mucositis was induced by i.p injection of 5-FU followed by MT of the cheek pouch. Animals received subcutaneous injection of aminoguanidine (10 mg/kg), 1,400 W (1 mg/kg) or 0.5 ml of saline, 1 h before 5-FU and daily for 10 days. Each cheek pouch was everted and photographed, and samples were removed and processed for hematoxylin and eosin staining (×40 magnification) after the animal was killed

Table 1 Macroscopic and microscopic analysis of hamster cheek pouch submitted to experimental oral mucositis and treated with aminoguanidine or 1,400 W

Experimental groups	Normal	MT	5-FU		
			Saline	AG	1,400 W
Macroscopic analysis	0 (0–0)	0.5 (0–1)	3 (2–3)*	2 (1–3)*,**	1 (1–3)*,**
Microscopic analysis	0 (0–0)	0.5 (0–1)	3 (1–3)*	1.5 (1–3)*,**	1 (1–3)*,**

Oral mucositis was induced in hamsters by intraperitoneal (*i.p.*) injection of 5-FU and by mechanical trauma (*MT*) of the cheek pouch. Animals received subcutaneous injection of aminoguanidine (10 mg/kg), 1,400 W (1 mg/kg) or 0.4 ml of saline, 1 h before 5-FU and daily for 10 days. Data represent the median values (and range) of macroscopic or microscopic scores in six animals per group. Data were analyzed by using Kruskal–Wallis- and Mann–Whitney-tests

* $p < 0.05$ compared to normal animals

** $p < 0.05$ compared to animals that received irritation of cheek pouch only (MT)

Table 2 Macroscopic and microscopic analysis of hamster cheek pouch submitted to experimental oral mucositis and treated with L-NAME

Experimental groups	Normal	MT	Saline	5-FU		
				L-NAME (mg/Kg)		
				5	10	20
Macroscopic analysis	0 (0–0)	0.5 (0–1)	3 (2–3)*	3 (2–3)*	3 (2–3)*	3(2–3)*
Microscopic analysis	0 (0–0)	0.5 (0–1)	3 (1–3)*	3 (1–3)*	3 (2–3)*	2, 5(2–3)*

Oral mucositis was induced in hamsters by intraperitoneal (*i.p.*) injection of 5-FU and by mechanical trauma (*MT*) of the cheek pouch. Animals received subcutaneous injection of L-NAME (5, 10, or 20 mg/kg), or 0.4 ml of saline, 1 h before 5-FU and daily for 10 days. Data represent the median values (and range) of macroscopic or microscopic scores in six animals per group. Data were analyzed by using Kruskal–Wallis- and Mann–Whitney-tests

* $p < 0.05$ compared to normal animals

significantly ($p < 0.05$) reduced the 5-FU-induced inflammatory cell infiltration, edema, and hemorrhage, and prevented the formation of ulceration and abscess. Re-epithelization areas were observed.

Myeloperoxidase activity

The MPO activity on the cheek pouch tissue of animals subject to 10 days of 5-FU-induced experimental oral mucositis was significantly increased ($p < 0.05$) in comparison with the normal group or to the group of animals submitted to MT. Both 1,400 W (1 mg/kg) and aminoguanidine (10 mg/kg) significantly ($p < 0.05$) reduced the 5-FU-induced increase of MPO activity, restoring this parameter to the level of normal and MT controls. In contrast, a significant ($p < 0.05$) increase of MPO activity in the cheek pouch of animals subjected to 5-FU-induced oral mucositis and treated with L-NAME was observed, when compared with the group subjected to 5-FU-induced experimental mucositis treated with saline (Fig. 2).

Nitric oxide synthase activity

The 5-FU-induced experimental oral mucositis resulted in a significant ($p < 0.05$) increase in NOS activity in the cheek pouch tissue of animals on the tenth day, when compared with normal group (Fig. 3).

Nitrite levels

The nitrite levels in the cheek pouch tissue of animals subject to 10 days of 5-FU-induced experimental oral mucositis was significantly increased ($p < 0.05$) in comparison with the normal group or with the group of animals submitted to MT. Systemic administration of 1,400 W (1 mg/kg) significantly ($p < 0.05$) reduced the 5-FU-induced increase of nitrite levels, restoring this parameter to the level of normal and MT controls (Fig. 4).

Immunohistochemical reaction for induced nitric oxide synthase

The cheek pouches of hamsters submitted to oral mucositis by 5-FU showed marked immuno-staining of iNOS on inflamed conjunctive tissue (Fig. 5c) compared with the cheek pouches of the normal control group (Fig. 5b). Both aminoguanidine and 1,400 W caused a significant decrease of iNOS immuno-staining in the check pouches tissue when compared with the group of animals subjected to experimental mucositis that did not receive treatment (Fig. 5d, e).

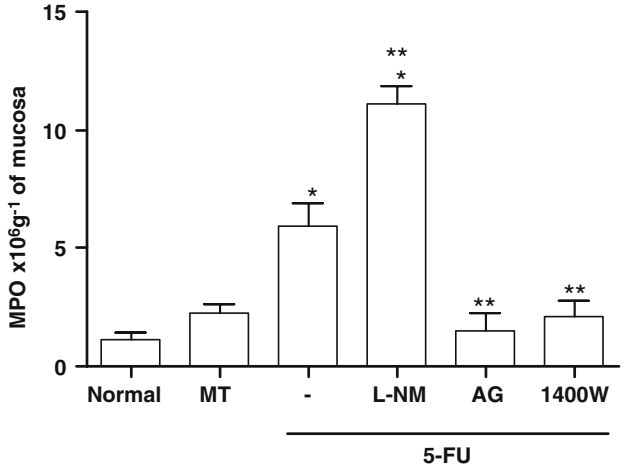

Fig. 2 About 1,400 W and aminoguanidine (*AG*) inhibit myeloperoxidase (*MPO*) activity in the cheek pouch of hamsters submitted to oral mucositis. Oral mucositis was induced in hamsters by the intraperitoneal (*i.p.*) injection of 5-FU followed by mechanical trauma (*MT*). Animals received subcutaneous administration of Nφ-Nitro-L-Arginine Methyl Ester (*L-NM*; 20 mg/kg), aminoguanidine (*AG*; 10 mg/kg), 1,400 W (1 mg/kg) or 0.4 ml of saline, 1 h before each 5-FU injection and daily for 10 days. After sacrifice, a sample of the cheek pouch was removed for MPO activity. Bars represent the mean value ± standard error of the mean (*SEM*) of the concentration of MPO×10^6/g of cheek pouch. *$p < 0.05$ represents statistical differences compared to 5-FU-induced oral mucositis group. The number animals in each group was at least three. Data were analyzed by using analysis of variance (*ANOVA*) and Bonferroni tests

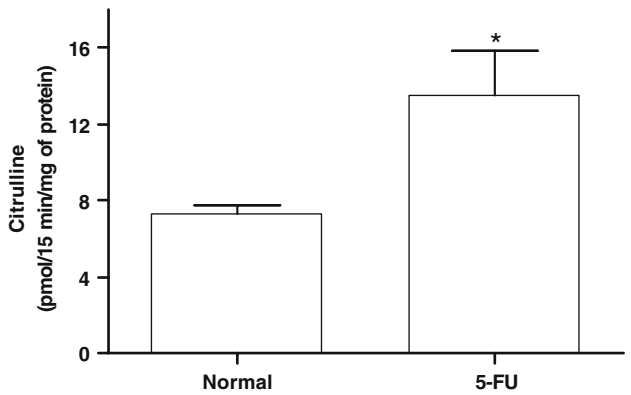

Fig. 3 Effect of 5-fluorouracil (5-*FU*) followed by MT on NOS activity from cheek pouches of hamsters. Data are reported as mean ± SEM. *$p < 0.05$ compared to normal animals. The number animals in each group was at least five [data were analyzed by using analysis of variance (*ANOVA*) and Bonferroni tests]

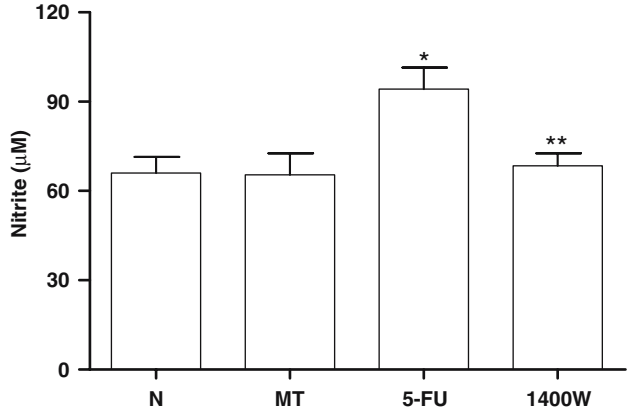

Fig. 4 Systemic administration of 1,400 W (1 mg/kg) significantly ($p < 0.05$) reduced the 5-FU-induced increase of nitrite levels in the cheek pouch of hamsters submitted to oral mucositis. Oral mucositis was induced in hamsters by the intraperitoneal (*i.p.*) injection of 5-fluorouracil (5-*FU*) followed by mechanical trauma (*MT*). Animals received subcutaneous administration 1,400 W (1 mg/kg) or 0.4 ml of saline, 1 h before each 5-FU injection and daily for 10 days. After sacrifice, a sample of the cheek pouch was removed for determination of NO production by the Griess reaction. Results are expressed as the mean ± SEM of values for each group of six animals. *$p < 0.05$ represents statistical differences compared to normal and MT controls **$p < 0.05$ represents statistical differences compared to 5-FU-induced oral mucositis group. Data were analyzed by using analysis of variance (*ANOVA*) and Bonferroni tests

Discussion

This study has clearly demonstrated that treatment of hamsters with aminoguanidine and 1,400 W, which are iNOS inhibitors, but not with L-NAME, a non-selective NOS inhibitor, significantly reduced the lesions found in 5-FU-induced experimental oral mucositis. The treatments decreased the inflammatory cell infiltration, edema, hemorrhage, and the formation of ulcerations and abscess. The protective effects of these inhibitors suggest a key role for endogenous iNOS-derived NO in the pathogenesis of 5-FU-induced mucosistis.

Mucositis is a complex process that is initiated by injury of cells of the basal epithelium and underlying tissue. One of the primary effects of chemotherapy agents is the generation of reactive oxygen species (ROS). ROS directly damage cells, tissues, blood vessels and stimulate transcription factors, such as nuclear transcription factor-κB (NF-κB). Once activated NF-κB leads to the up-regulation of many genes, including those that result in the production of pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis alpha (TNF-α) [23, 32, 33, 56]. Agents known to attenuate the expression of cytokines have demonstrated efficacy in the prevention of both experimental [41, 56] and clinical [16] mucositis. Thus, the participation of NO in 5-FU-induced oral mucositis is consistent with previous reports showing that pro-inflammatory cytokines stimulates iNOS-derived NO production, and that NO mediates cytokines cytotoxicity

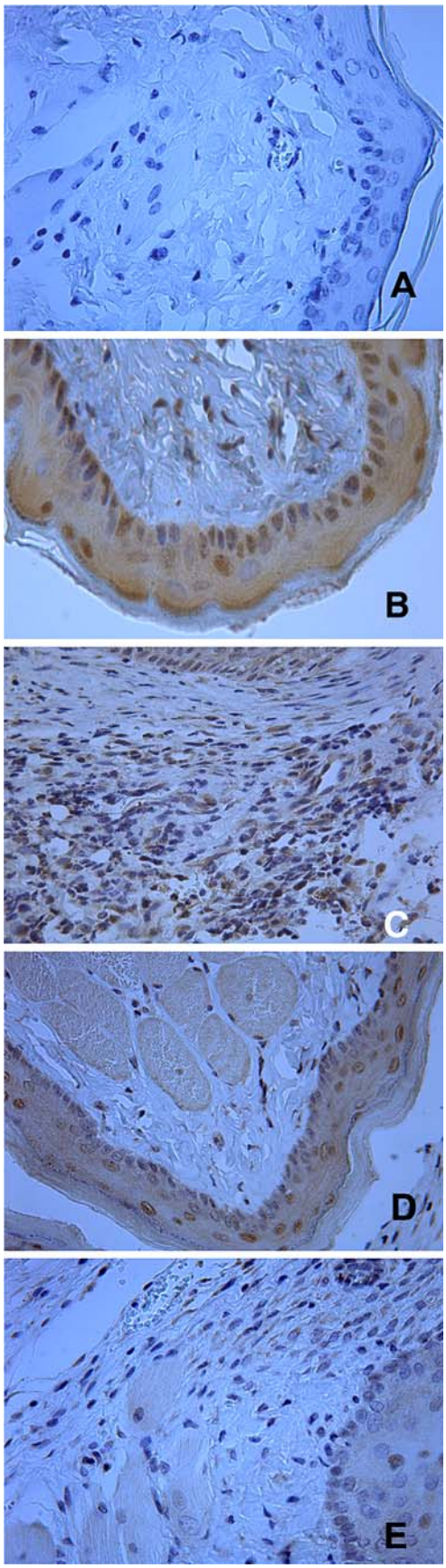

Fig. 5 Representative examples of iNOS immunohistochemistry in the cheek pouches of hamsters subjected to 5-fluorouracil (5-*FU*)-induced oral mucositis. The cheek pouch tissue of hamster which received (5-FU) and subcutaneous saline (**c**) presented intense immuno-staining of iNOS in the inflammatory cells when compared to the cheek pouch of hamster which received only saline (**b**). The treatment with aminoguanidine (10 mg/kg) (**d**) or 1,400 W (1 mg/kg) (**e**) considerably reduced the immuno-staining 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 immuno-staining was detected (**a**)

and inflammatory events [17, 42, 53]. Moreover, the role of NO on pathogenesis of other inflammatory pathophysiological conditions has been extensively demonstrated, including periodontitis [36, 39, 40, 46], joint diseases such as rheumatoid arthritis [12, 61], and hemorrhagic cystitis [60].

In the present study, we observed that the protective effect of 1,400 W and aminoguanidine were associated with reduced neutrophil infiltration detected by histopathology and myeloperoxidase activity. In accordance with our data, it has been demonstrated that NOS inhibitors or iNOS-deficient mice generated by gene knockout have a reduced neutrophil migration induced by staphylococcal enterotoxin B, streptococcal cell wall, carragenin, or zymosan [1, 10, 18, 19]. In contrast, it has been also shown that NO down-regulates the expression of adhesion molecules in the vascular endothelium, thereby decreasing neutrophil trafficking into inflamed tissues [13, 35, 51]. We do not have an accurate explanation for these differences. However, other authors suggest a biphasic pattern of the regulation of neutrophil accumulation by different NO concentrations in inflamed tissue, i.e., low level of NO production (by constitutive or inducible NOS) is known to suppress neutrophil adhesion to endothelial cells [24, 25], while high levels of NO increase neutrophil infiltration [1]. The mechanism by which iNOS derived NO can enhance neutrophil migration and tissue damage are still unclear. One possible explanation for these findings could be the fact that during severe inflammation, as observed after 5-FU treatment, peroxynitrite are formed due the reaction between iNOS-derived NO and, ROS [9, 62, 63], which are produced concomitantly in response to inflammatory cytokines [11]. This very toxic compound has the ability to initiate lipid peroxidation [54], oxidation of protein sulfhydryls [52], and nitration of tyrosine residues on a variety of proteins, including inactivation of enzymes and/or receptors [3, 4, 28, 31, 62], resulting in tissue injury which may lead to an excessive local amplification of the inflammatory response, and leading to migration of inflammatory cells. Furthermore, it has been demonstrated that

iNOS-derived NO can up-regulate the production of a number of pro-inflammatory mediators, such as TNF-α, IL-6, and CXC chemokines [1, 5, 26], and that the inhibition of NOS can increase IL-10 production, an antiinflammatory cytokine [49]. Thus, the decrease in the neutrophil migration observed when aminoguanidine and 1,400 W were administered in animals subjected to 5-FU induced mucositis is probably due to suppression of pro-inflammatory effects of cytokines mediated high level of NO via iNOS activation.

On the other hand, it was observed that L-NAME, a non-selective NOS inhibitor [48], did not significantly prevent the lesions induced by experimental oral mucositis nor the neutrophil infiltration observed in histopathology analysis and in myeloperoxidase activity. On the contrary, the L-NAME treatment promoted a significant increase in the myeloperoxidase activity on the cheek pouch tissue of animals subject to 5-FU-induced mucositis. In accordance with our data, it has been shown that endothelial dysfunction which results in loss in basal level of NO, leads to up-regulation of cell adhesion molecules (CAMs), particularly P-selectin, leading to increased neutrophil infiltration and tissue injury [38]. Furthermore, it has been demonstrated that NO synthase inhibitors, including L-NAME, increase leukocyte adhesion to cat mesenteric endothelium, possibly by up-regulating the expression of CD11/CD18 [62], since this effect was attenuated by monoclonal antibodies against CD18 [35]. Thus, the inhibition of both inducible and constitutive NOS by L-NAME could be an explanation for the lack of effect of L-NAME in preventing oral mucositis or even for the enhancement of the neutrophil infiltration. Together these results lead to the conclusion that basal levels of NO, appear to be essential to maintain homeostasis, whereas, increased and sustained NO levels, produced by iNOS, during inflammatory reactions may contribute to local tissue damage [1, 5, 26, 45].

The detection of a significant increase of the NOS activity, nitrite levels and more specifically iNOS immuno-staining in the cheek pouch tissue of hamsters on the 10th day of 5-FU-induced oral mucositis, reinforce the role of NO upon pathogenesis of this condition. According to our data, low level of iNOS gene expression was demonstrated in a model of radiation-induced oral mucositis [57]. However, the role of NO on chemotherapy-induced oral mucositis was not yet demonstrated.

Interestingly, a reasonable amount of iNOS staining was noted even in the cheek pouch epithelium of hamsters not subjected to oral mucositis. We believe that iNOS can be constitutively expressed in this normal epithelium in response to the highly contaminated oral environment, but additional studies are necessary in order to confirm this hypothesis. It was also observed that the treatment with both aminoguanidine and 1,400 W caused a significant decrease of iNOS immuno-staining in the cheek pouches of hamsters when compared with the group of animals subjected to experimental mucositis by 5-FU that did not receive treatment. This finding may be a consequence of the reduction of inflammatory cell infiltration in the connective tissue induced by these NOS inhibitors, leading to decreased iNOS expression. According to our data, it has been demonstrated that N-Iminoethyl-L-lysine attenuated the inducible NO synthase immunoreactivity in adjuvant-treated rats [8].

Thus, this study has demonstrated for the first time that aminoguanidine and 1,400 W display substantial protective effects against experimental mucositis induced by 5-FU. These results associated with the detection of NOS activity, increased nitrite levels and iNOS immuno-staining in the cheek pouches of hamster subjected to oral mucositis suggest that endogenous NO, via iNOS, is involved in mucosal damage and in the inflammatory events leading to 5-FU-induced oral mucositis. Elucidation of the mechanism involved on 5-FU-induced mucositis should lead to improved control of this dose-limiting and costly side effect of cancer therapy.

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