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ORIGINAL ARTICLE

Involvement of nitric oxide on the pathogenesis of irinotecan-induced intestinal mucositis: role of cytokines on inducible nitric oxide synthase activation

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Abstract
Purpose Intestinal mucositis and the closely associated diarrhea are common costly side effects of irinotecan. Cytokine modulators, such as thalidomide and pentoxifylline, are found capable of attenuating intestinal mucositis progression. Nitric oxide (NO) seems to be a key mediator of the antineoplastic drug toxicity. The aim of this study was to investigate the role of NO on the pathogenesis of intestinal mucositis, as well as the participation of cytokines upon inducible nitric oxide synthase (iNOS) expression in irinotecan-induced intestinal mucositis.
Methods iNOS-knockout ($iNOS^{-/-}$) and C57BL/6 (WT, wild type) animals ($n = 5$–6) were given either saline or irinotecan (60 mg/kg i.p for 4 days), with or without pretreatment with aminoguanidine (50 mg/kg s.c.), thalidomide (60 mg/kg s.c), infliximab (5 mg/kg i.v.), or pentoxifylline (1.7 mg/kg s.c). On day 5, diarrhea was assessed, and following euthanasia, proximal intestinal samples were obtained for myeloperoxidase (MPO) and iNOS activity, morphometric analysis, western blot and immunohistochemistry to iNOS, cytokine dosage, and for in vitro evaluation of gut contractility.
Results Irinotecan induced severe diarrhea and intestinal smooth muscle over-contractility, accompanied with histopathological changes. Additionally, increased MPO and iNOS activity and iNOS immunoexpression were found in WT animals treated with irinotecan. The rise in MPO, smooth muscle over-contractility, and diarrhea were abrogated in aminoguanidine-treated and $iNOS^{-/-}$ mice. Moreover, through western blot, we verified that infliximab and pentoxifylline significantly inhibited irinotecan-induced iNOS expression. In addition, cytokine concentration was found only partially decreased in irinotecan-treated $iNOS^{-/-}$ mice when compared with wild-type animals that were given irinotecan.
Conclusions This study suggests a role of nitric oxide in the pathogenesis of irinotecan-induced intestinal mucositis and also provides evidence for the participation of cytokines on iNOS induction.

Keywords Irinotecan · Intestinal mucositis · Nitric oxide · Cytokines

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Introduction

Intestinal mucositis is a common, dose-limiting, and costly side effect of cancer treatment. Oral and gastrointestinal mucositis can affect up to 100% of patients undergoing high-dose chemotherapy and hematopoietic stem cell transplantation, 80% of patients with malignancies of the head and neck receiving radiotherapy, and a wide range of patients receiving

chemotherapy [1]. About 15–40% of patients undergoing standard-dose chemotherapy in clinical trials present oral and intestinal mucositis, which may compromise the patient's quality of life and the efficacy of chemotherapy regimens [1, 2]. Diarrhea, abdominal pain, nausea, and vomiting are frequent clinical manifestations of intestinal mucositis, although severe cases may be associated with febrile neutropenia, sepsis, and even death [3]. The incidence, severity, and localization of mucositis are strictly dependent on the chemotherapeutic protocol and may depend on one or multiple drugs as well as the dose regimen adopted. Patients with advanced colorectal cancers who receive common chemotherapy regimens have a low risk of severe oral mucositis, but a high risk (16%) of overall diarrhea, which approaches 25% (grade 3 and 4) when both irinotecan and oxaliplatin are used (IROX regimen) [2]. One of the critical problems in understanding mucositis, and its closely associated diarrhea, is that the mechanisms behind the development of the pathology are not completely known.

Irinotecan, a topoisomerase I inhibitor, is an antiproliferative drug with activity against several types of cancers. Clinically, irinotecan is mainly used to treat colorectal cancer [4, 5]. Irinotecan induces mild and moderate diarrhea, which is often and effectively controlled by loperamide. On the other hand, more severe and life-threatening events are poorly managed, and several supplemental strategies have been proposed, such as the use of octreotide, acetorphan, antibiotics, glutamine, budesonide, cyclosporin, and alkalization of intestinal lumen [6]. In an attempt to determine the mechanism by which irinotecan induces diarrhea and mucositis, our group evaluated the effect of thalidomide, a tumor necrosis factor (TNF)-α inhibitor, and pentoxifylline, a methylxanthine derivative that blocks both TNF-α and interleukin (IL)-1β synthesis, along with other cytokines [7]. We found that irinotecan-induced intestinal damage may be negatively modulated by the administration of pentoxifylline and thalidomide through the inhibition of cytokine production. However, only pentoxifylline reduced the severity of diarrheal events in this model [7].

Cytokines, such as IL-1β and TNF-α, have been shown to stimulate the expression of the inducible isoform of nitric oxide synthase (iNOS) with consequent production of nitric oxide (NO) [8]. Interestingly, iNOS appears to have a biphasic role during the onset of the inflammatory process. Depending on the setting, the role of iNOS ranges from enhancing inflammation to retarding it [9]. A number of studies report that iNOS-born NO exhibits harmful properties in several inflammatory conditions, such as sepsis [10], pancreatitis [11], and also in antineoplastic drug-induced tissue injury, such as hemorrhagic cystitis [12] and oral mucositis [13].

Thus, this work addressed the involvement of nitric oxide on the pathogenesis of irinotecan-induced intestinal mucositis, and cytokine contribution to inducible nitric oxide synthase activation.

Materials and methods

Animals

Inducible nitric oxide synthase–knockout mice (iNOS$^{-/-}$) and counterpart wild-type animals, C57BL/6 (WT), weighing 20–22 g, were maintained in microisolator cages in the animal facility of the School of Medicine of Ribeirão Preto, University of São Paulo. The animals were kept in a temperature-controlled room under dark–light cycles, with free access to food and water until use. The Local Ethics Committee for Animal Experiments approved the entire protocol (number 02/04), complying to laboratory animal care and use principles (NIH publication no. 85–23, revised 1985). The original breeding pairs of mice with a targeted disruption of the iNOS gene were obtained from The Jackson Laboratories (Bar Harbor, Maine, USA). The genotype of iNOS$^{-/-}$ mice was confirmed by DNA PCR.

Drugs

The following drugs were used: irinotecan hydrochloride (irinotecan, Camptosar®, Pharmacia and Upjohn Co, Kalamazoo, MI, USA, 100 mg ampoule); aminoguanidine (Sigma, St. Louis, MO, USA); loperamide chloride (Hexal do Brasil Ltda., São Paulo, Brazil), pentoxifylline (Trental®, Hoechst, São Paulo, Brazil, 100 mg ampoule); thalidomide (Talidomida®, CEME, Minas Gerais, Brazil, 100 mg tablet); and infliximab (Remicade®, Schering Plough, Rio de Janeiro, Brazil, 100 mg ampoule).

Induction of experimental intestinal mucositis

Induction of experimental intestinal mucositis in mice was based on a model previously described [14], and modified for our experimental conditions. Briefly, WT animals were given either saline (3.5 ml/kg s.c.), aminoguanidine (50 mg/kg s.c., twice a day/4 days), or loperamide chloride (10 mg/kg s.c., twice a day on the first two days and 30 mg/kg on the third and fourth days) alone or in combination with irinotecan (a topoisomerase I inhibitor, 60 mg/kg i.p., once a day/4 days), 1 h before and 12 h following the administration of irinotecan. iNOS$^{-/-}$ mice were treated with saline or irinotecan (same protocol used for WT mice). Animals were killed on the fifth day (measured from the first dose of irinotecan), and the animals had portions of the proximal intestine collected for the measurement of myeloperoxidase and iNOS activities, morphometric and immunohistochemistry analysis, and functional studies (in vitro

contractility of isolated duodenum). In another experimental setting, WT animals were given saline (3.5 ml/kg s.c.) or irinotecan (60 mg/kg, i.p.), alone or in combination with infliximab (5 mg/kg, i.v, single injection), thalidomide (60 mg/kg, s.c.), or pentoxifylline (1.7 mg/kg, s.c.) given daily 1 h before the administration of irinotecan. These pretreatment regimens began 1 day before the initiation of irinotecan treatment. On the fifth day after the first dose of irinotecan, the animals were killed and a further intestinal segment was collected and halved for iNOS western blot studies.

Morphometric 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 subsequent examination by light microscopy (×100). For the morphometric analysis, length of the intestinal villus and depth of the Lieberkühn crypts were calibrated and converted to µm (Software ImageJ 1.4, NIH, USA), and the villus/crypt ratio was determined [49]. Between 5 and 10, villi and crypts were measured per slice. A range of 5–8 slices were analyzed per group.

Myeloperoxidase assay (MPO)

MPO is an enzyme most abundantly found in azurophilic granules in neutrophils; it can be used as a neutrophil marker in inflamed tissue, following a previously described colorimetric method [15]. Briefly, a duodenum sample was homogenized in hexadecyltrimethylammonium bromide (HTAB) buffer (50 mg of tissue/ml). The homogenate was then centrifuged at 2,000*g* for 15 min at 4°C. MPO activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm using a reading solution (5 mg *O*-dianisidine; 15 µL of 1% H_2O_2; 3 ml phosphate buffer; 27 ml H_2O). The change in absorbance was recorded and plotted in a standard curve of neutrophil density, with the obtained data expressed as myeloperoxidase activity (neutrophils/mg of tissue).

Determination of nitric oxide synthase activity

After incubation, intestinal samples were homogenized with 0.2 ml of 20 mM HEPES (pH 7.4) containing 1.25 mM CaCl2, 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, and then the homogenates were centrifuged at 10,000*g* for 10 min at 4°C. The resulting supernatants were loaded 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. Results were expressed as citrulline production per mg of tissue [16].

Immunohistochemical reaction to inducible nitric oxide synthase

Immunohistochemistry for iNOS was performed using the streptavidin–biotin-peroxidase method [17] in formalin-fixed, paraffin-embedded tissue sections (5 µm thick) mounted on poly-L-lysine-coated microscope slides. Duodenal cross-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 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in PBS plus bovine serum albumin (PBS-BSA). The slides were then incubated with biotinylated goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) 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). Slides were counterstained with Harry's hematoxylin, dehydrated in a graded alcohol series, cleared in xylene, and coverslipped. Qualitative immunohistochemistry was performed as described by Yeoh et al. [18]. Staining was observed using light microscopy, and iNOS expression was blinded scored based on the intensity of the staining as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, moderate–intense staining; and 4, intense staining.

Western blot for iNOS

The animals had a sample of their duodenum removed on day 5 to determine the amount of iNOS. Specimens were immediately frozen in liquid nitrogen and stored at −70°C until required for the assay. Briefly, the tissues were homogenized in 0.5 ml of lysis buffer (50 mM Tris–HCl [pH 8.5]; 50 mM NaCl; 0.1 mM EDTA; 1% Tween 20; 1 mM [each] dithiothreitol, leupeptin, aprotinin, and phenylmethylsulfonyl fluoride). Total protein (50 µg protein/well) was resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, Amersham, UK). The membranes were blocked with 5% skimmed milk/Tris-buffered saline with 0.1%

Tween 20 for 14 h at 4°C followed by an incubation period of 1 h at room temperature with the primary antibodies (rabbit polyclonal anti-iNOS, 1:800 or anti-β-Actin, 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). The blots were washed with and incubated with horseradish peroxidase–conjugated secondary antibody (Monkey anti-rabbit immunoglobulin G, 1:1,000; Amersham Pharmacia BioTech UK) for 1 h at room temperature. The membranes were washed, incubated with electrogenerated chemiluminescence (ECL; Amersham Pharmacia Biotech), and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) to develop the western blot. Densitometry analyses were performed by ImageJ 1.4 software (National Institute of Health, USA). Data were expressed as the relative density of iNOS/β-Actin bands.

Detection of cytokines (TNF-α, IL-1β, and KC)

iNOS$^{-/-}$ mice had a sample of intestine removed on day 5 for analysis of cytokines. The specimen was stored at −70°C until required for assay. TNF-α, IL-1β, and KC (murine IL-8 homologue) concentrations were determined by ELISA, as described previously [7]. Briefly, microtiter plates were coated overnight at 4°C with antibody against mice TNF-α, IL-1β, and KC (2 µg/ml). After blocking the plates, the samples and standard at various dilutions were added in duplicate and incubated at 4°C for 24 h. The plates were washed three times with buffer. After washing the plates, biotinylated sheep polyclonal anti-TNF-α or anti-IL-1β or anti-KC (diluted 1:1,000 with assay buffer 1% BSA) was added to the wells. After further incubation at room temperature for 1 h, the plates were washed and 50 µl of avidin-HRP diluted 1:5,000 was added. The color reagent *o*-phenylenediamine (OPD; 50 µl) was added 15 min later, and the plates were incubated in the dark at 37°C for 15–20 min. The enzyme reaction was stopped with H2SO4, and the absorbance was measured at 490 nm. The results are expressed as pg/mg protein and reported as mean ± SD.

Diarrhea assessment

Diarrhea observed after the final administration of irinotecan was considered to be delayed-onset diarrhea. The severity of the diarrhea was scored as described by Kurita et al. [19] as follows: 0—normal, normal stool or absent; 1—slight, slightly wet and soft stool; 2—moderate, wet and unformed stool with moderate perianal staining of the coat; and 3—severe, watery stool with severe perianal staining of the coat.

In vitro contractility of duodenum

Intestinal segments (0.6 cm long) from WT and iNOS$^{-/-}$ mice were removed and processed as described by Araújo et al. [20]. Briefly, the segments were placed into a Petri dish containing Tyrode's solution (Composition in mM: NaCl 136.0; KCl 5.0; $MgCl_2$ 0.98; $CaCl_2$ 2.0; NaH_2PO_4 0.36; $NaHCO_3$ 11.9; and glucose 5.5). After dissection, the luminal contents were washed away with physiological solution. Each segment was then placed in a glass organ bath (filled with 20 ml Tyrode's solution, 37°C, pH 7.4, bubbled continuously with carbogen mixture 95% O_2/5% CO_2) and connected to a force transducer (AD Instruments, model MLT0201, USA) set at 1-g resting tension. Longitudinal muscle tension was recorded on a computer-coupled data-acquisition system (Chart Pro, USA). Half an hour was allowed for equilibration. Spontaneous contractility was obtained, following two standard contractions recorded using 60 mM KCl. A dose–response curve to the cholinergic agonist acetylcholine (concentrations varying from 1×10^{-10} to 1×10^{-4} M) was also constructed. The data obtained from the cholinergic curve were analyzed as the percentage of response in comparison with the mean of the two contractions observed with the KCl standard.

Statistical analysis

Data were submitted to one-way or two-way ANOVA followed by Bonferroni's test; Kruskal–Wallis followed by Dunn's test, as appropriate. Values are expressed as mean ± SD (parametric data) or Median (minimum–maximum) for non-parametric data. The level of significance was set at $P < 0.05$.

Results

In irinotecan-treated mice, microscopic damage was observed in duodenal structures (Fig. 1c, d). It presents shortened recovered villi with flattened and vacuolated cells, loss of crypt architecture, epithelial cell line disarrangement, and marked infiltration of inflammatory cells (Fig. 1c, d) in comparison with intact structures seen for saline-treated WT mice (Fig. 1a, b). Aminoguanidine-treated (Fig. 1e, f) and iNOS-knockout (Fig. 1g, h) mice showed preservation of the villi and crypts and maintenance of epithelial cell surface. Figure 2 shows irinotecan-induced morphometric alterations in intestinal structures in WT mice, with shortened villi and deepening of crypts (Fig. 2a, b, respectively). A more pronounced view of these alterations can be seen in Fig. 2c, depicting changes in villus/crypt ratio. In all of these cases, irinotecan-treated WT mice showed statistically significant differences when compared to saline-treated mice ($P < 0.05$). Despite the administration of irinotecan, genetically iNOS-deficient mice (iNOS$^{-/-}$) and aminoguanidine-treated WT animals do not develop significant villus blunting (Fig. 2a) when compared

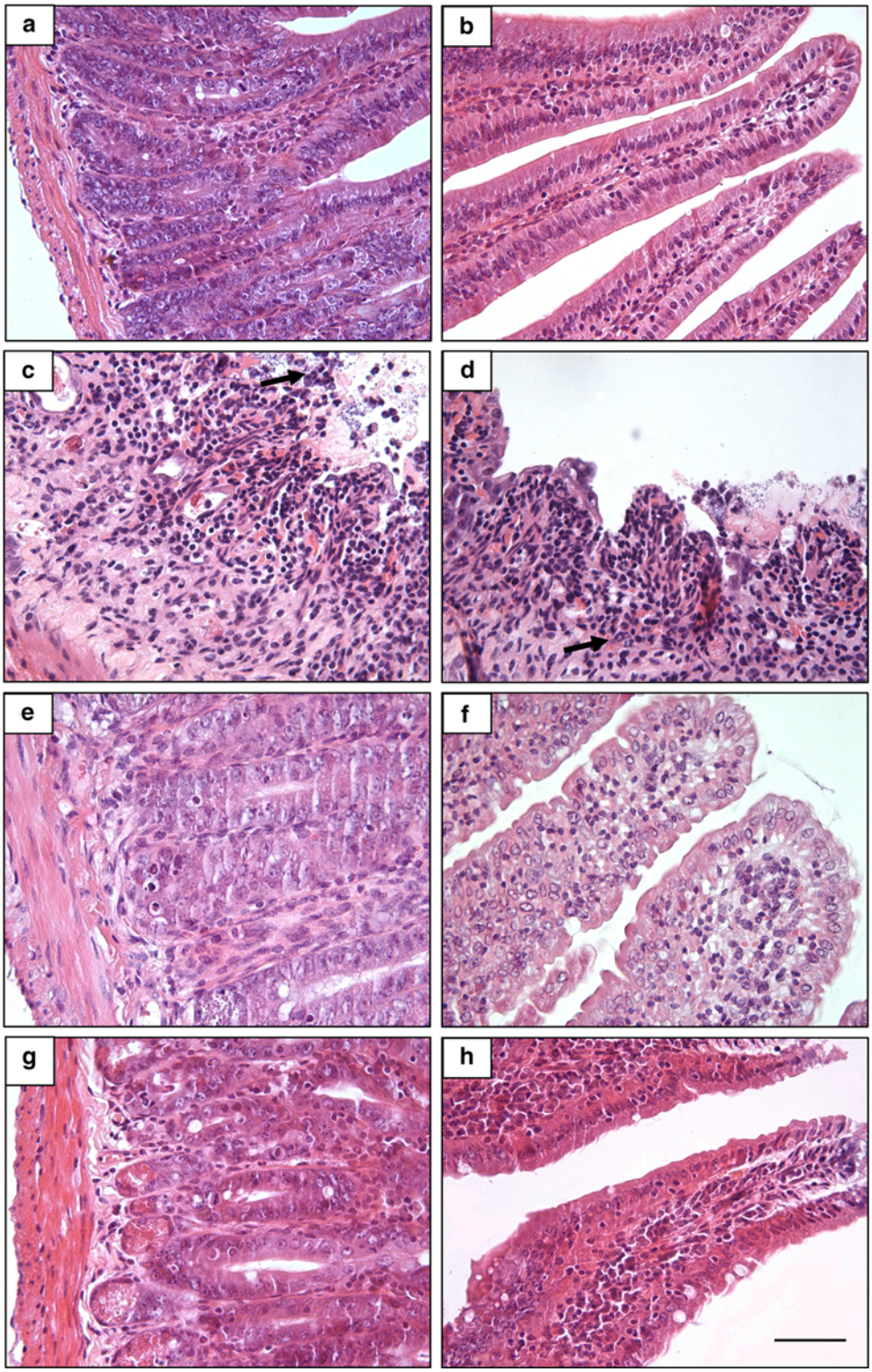

Fig. 1 Photomicrographs of duodenum. Crypts and villi of normal mice (**a** and **b**, respectively) presented intact. Mice submitted to intestinal mucositis by irinotecan, showing shortened villi recovered with flattened cells (**c**), and loss of crypt architecture (**d**), hemorrhagic areas and marked infiltration of inflammatory cells (*black arrows*). Mice submitted to intestinal mucositis by irinotecan and treated with aminoguanidine showing preservation of the crypts (**e**) and villi (**f**) and of the epithelial cell surface. The same is observable in iNOS-knockout mice (**g** crypt; **h** villi). H&E staining (magnification ×400). *Scale bar* represents 50 μm

with their respective controls ($P > 0.05$), denoting protective effects on intestinal mucosa. When the crypt depth parameter is taken into consideration, statistically significant differences between all irinotecan-treated groups were found only among aminoguanidine-treated animals ($P < 0.05$), but not for iNOS$^{-/-}$ mice (Fig. 2b). However, the villus/crypt ratio reveals that both aminoguanidine treatment and iNOS deficiency are significant protective factors ($P < 0.05$ in comparison with WT mice that were given irinotecan, Fig. 2c).

A significant increase in intestinal MPO activity is observed in mice injected with irinotecan ($P < 0.05$ in

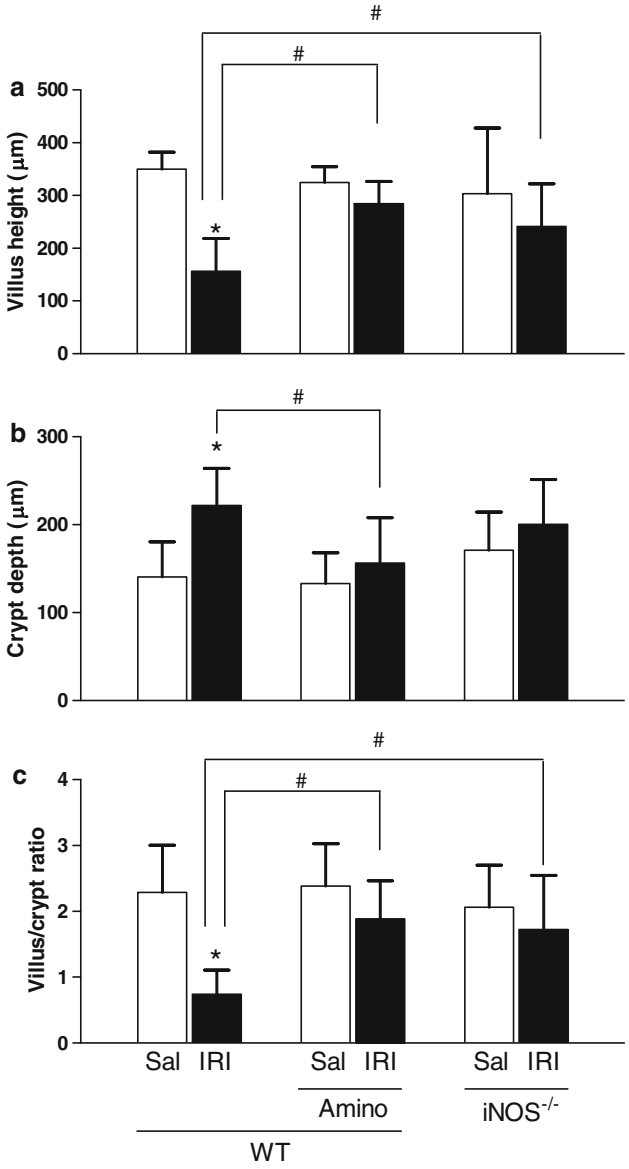

Fig. 2 Irinotecan-induced morphometric alterations in duodenum are prevented in iNOS$^{-/-}$ and aminoguanidine-treated mice. Mice were treated for 4 days with saline (vehicle of irinotecan, 3 ml/kg, *open* and *striped bars*) or irinotecan (60 mg/kg i.p, *filled bars*) and were killed on the fifth day after the first dose. A portion of the duodenum was collected and processed for histological studies. Morphometric analysis shows that animals treated solely with irinotecan exhibit a decreased villus/crypt ratio when compared with normal control animals treated only with saline. Additionally, iNOS-deficient mice (iNOS$^{-/-}$) and aminoguanidine-treated animals, to which irinotecan was given, do not develop alterations in this parameter in comparison with irinotecan-treated animals pretreated with saline only. Values are expressed as mean ± SD (n = 5–8). *$P < 0.05$ versus group treated only with saline, $^{\#}P < 0.05$ versus irinotecan-treated group that was given saline

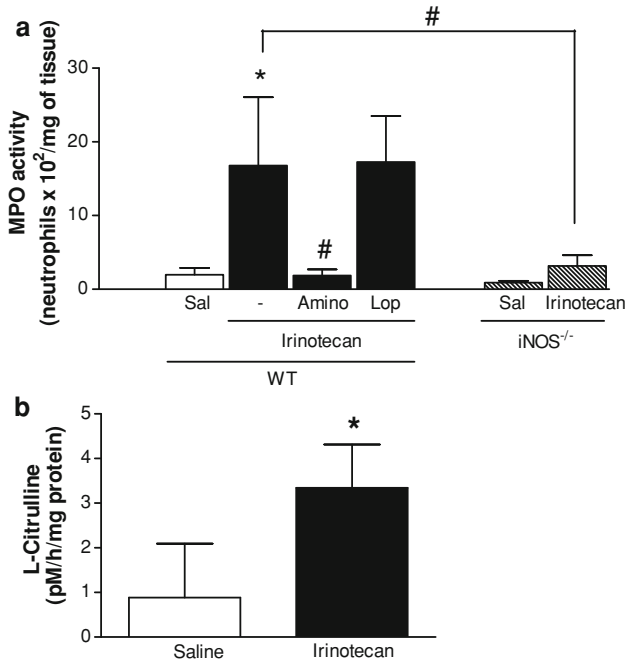

Fig. 3 Irinotecan-induced increases in duodenal iNOS and MPO activity are abolished in iNOS$^{-/-}$ and aminoguanidine-treated mice. Mice were treated for 4 days with saline (vehicle of irinotecan, 3 ml/kg, *open* and *striped bars*) or irinotecan (60 mg/kg i.p, *filled bars*) and were killed on the fifth day after the first dose. A section of the duodenum was collected and kept frozen at −70°C until the performance of the myeloperoxidase and iNOS activity assay. The proinflammatory effect of irinotecan upon the duodenum was detected as an increase in the number of tissue neutrophils. This observation was not reflected in the iNOS$^{-/-}$ and aminoguanidine-treated groups, demonstrating a protective effect against the administration of irinotecan (**a**). Additionally, irinotecan injection leads to a significant increase in the activity of iNOS in intestinal samples (**b**). Values are expressed as mean ± SD (n = 5–8). *$P < 0.05$ versus group treated only with saline, $^{\#}P < 0.05$ versus irinotecan-treated group that was given saline

comparison with WT, saline-treated animals; Fig. 3a). Pharmacological (aminoguanidine, 50 mg/kg) or genetic (iNOS$^{-/-}$) inhibition of iNOS completely prevented this response ($P < 0.05$). On the other hand, loperamide-treated mice showed the levels of intestinal MPO activity similar to irinotecan-treated animals ($P > 0.05$). Figure 3 also shows that irinotecan treatment leads to a significant increase in iNOS activity ($P < 0.05$) in comparison with saline-treated mice (Fig. 3b).

Irinotecan caused prolonged and severe diarrhea in WT mice when compared with saline-treated animals ($P < 0.05$). Aminoguanidine treatment and iNOS knockout were protective factors, since these animals exhibit normal stool excretion to slight diarrhea upon irinotecan treatment ($P < 0.05$ compared with irinotecan-treated WT animals). The positive control treatment adopted for this experiment, loperamide, also significantly inhibited the development of severe diarrhea ($P < 0.05$, Table 1). Our in vitro contractility studies (Fig. 4) showed that duodenal segments collected from WT mice treated with irinotecan presented a higher responsiveness to the cholinergic agent, acetylcholine, in comparison with the baseline curve found for animals treated only with saline ($P < 0.05$). Intestinal samples collected from iNOS$^{-/-}$ or aminoguanidine-treated mice

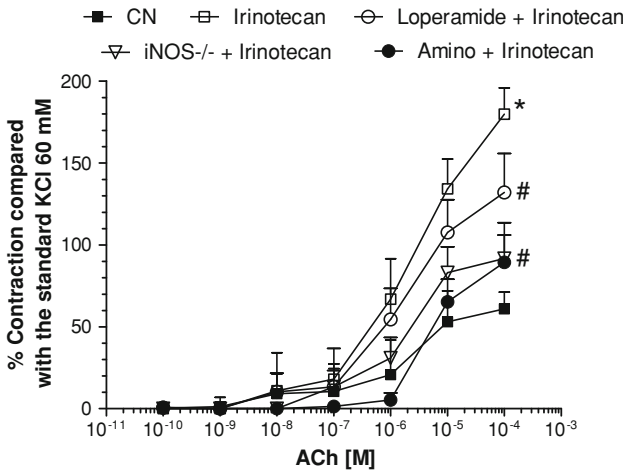

Fig. 4 Irinotecan induces functional alterations in isolated duodenum contractility, which is prevented in iNOS$^{-/-}$, aminoguanidine-treated, or loperamide-treated mice. Animals were treated for 4 days with saline (vehicle of irinotecan, 3 ml/kg, *filled squares*) or irinotecan (60 mg/kg i.p.) and were killed on the fifth day after the first dose. Samples of duodenum were then collected for an in vitro contractility study. This functional study reveals an increased sensitivity of tissues collected from animals treated with irinotecan to acetylcholine when compared with mice treated only with saline. This effect was not replicated when irinotecan was administered to iNOS$^{-/-}$ and aminoguanidine-treated mice. Furthermore, this protective effect was more pronounced in comparison with that exerted by the standard drug, loperamide. Values are expressed as mean ± SD (n = 5–8). *$P < 0.05$ versus group treated only with saline, $^{\#}P < 0.05$ versus irinotecan-treated group that was given saline

Table 1 The severity of irinotecan-induced diarrhea is reduced in iNOS$^{-/-}$, aminoguanidine-treated, and loperamide-treated mice

Diarrhea assessment	
Group	Scores
WT + saline	0 (0–0)
WT + irinotecan	3 (2–3)[a]
WT + aminoguanidine + irinotecan	1 (1–1)[b]
iNOS$^{-/-}$ + irinotecan	1 (0–2)[b]
Loperamide + irinotecan	1.5 (1–2)[b]

Data were analyzed through Kruskal–Wallis and Dunn's test. Values are expressed as medians (minimum–maximum)

[a] $P < 0.05$ versus group treated only with saline, [b] $P < 0.05$ versus irinotecan-treated group that was given saline

expressed a significantly lower response to the cholinergic stimulus in comparison with irinotecan-treated WT animals ($P < 0.05$). Loperamide also appeared to be effective, but less so.

iNOS immunohistochemistry in intestinal tissues (Fig. 5) from irinotecan-treated mice (60 mg/kg) showed intense epithelial cell staining (panel c) scored as 4(3–4). This was also seen at the lamina propria level (panel d, black arrows). Saline-treated, normal control animals (panel b) showed a milder staining ($P < 0.05$) to iNOS in comparison with animals treated with irinotecan, scored 1(1–2). Intestinal samples that did not receive a primary antibody for immunohistochemistry are shown in panel a (background). In addition to that, the western blot analysis (Fig. 6a–d) showed that irinotecan increased iNOS intestinal expression, which was inhibited in a significant manner with pentoxifylline (Fig. 6a, b) and infliximab (Fig. 6c, d) pretreatment. However, thalidomide failed to significantly diminish the expression of iNOS.

Cytokine detection in irinotecan-treated WT and iNOS$^{-/-}$ mice was also performed. Table 2 shows that TNF-α, IL-1β, and KC concentration rose due to irinotecan injection ($P \leq 0.05$). In addition, tissue concentration of IL-1β and KC was only partially attenuated in iNOS$^{-/-}$ animals that were given irinotecan ($P \leq 0.05$) when compared with irinotecan-treated iNOS ±/± mice, but TNF-α remained increased ($P \geq 0.05$).

Discussion

This study shows, for the first time, the participation of the inducible isoform of NOS (iNOS) on the pathogenesis of irinotecan-induced intestinal mucositis in mice.

A study published by our group [7] established that doses of irinotecan ranging between 60 mg/kg to 75 mg/kg induce significant intestinal mucositis with mucosal damage. Moreover, it was observed that cytokines, mainly IL-1β and TNF-α, are involved in the trigger of this pathology, since the treatment of the irinotecan-injected mice with pentoxifylline or thalidomide prevented the inflammatory events and tissue damage [7]. Taking into account that these cytokines are involved in the induction of iNOS in several pathologies, including sepsis [21] and cystitis [12], we aimed to determine whether NO derived from iNOS accounts for irinotecan-induced IM. The treatment of the mice with aminoguanidine, a selective inhibitor of iNOS, reduced the villus/crypt ratio. It suggests that the inhibition of iNOS has a protective role, since less villus flattening and alterations in crypt size induced by irinotecan injection were observed. Confirming these results, similar beneficial outcome was obtained with iNOS-knockout mice. Along to this, the detection of an increased activity and expression of iNOS in the intestinal tissue of irinotecan-treated mice reinforces the role of this NOS isoform on the pathogenesis of this condition. Similarly, we have demonstrated the role of NO in a hamster model of 5-fluorouracil-induced oral mucositis [13]. It is important to mention that the inhibition of iNOS seems not to interfere with irinotecan-induced cytotoxic effect. Neither aminoguanidine nor the genetic ablation of iNOS

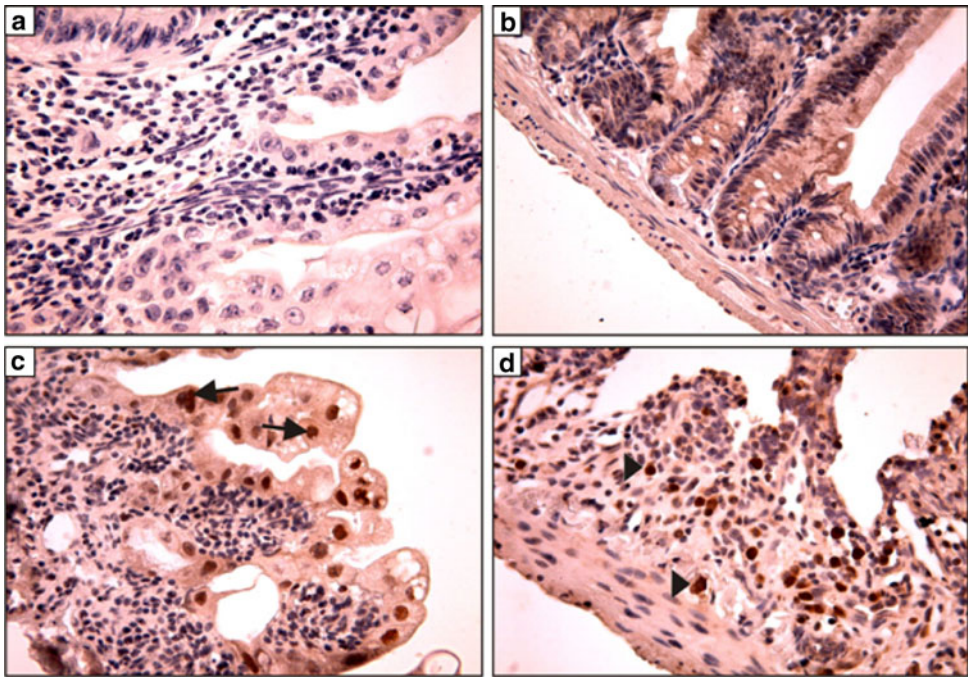

Fig. 5 Irinotecan induces increased iNOS immunostaining in the gut. Mice were treated for 4 days with saline (vehicle of irinotecan, 3 ml/kg) or irinotecan (60 mg/kg i.p.) and were killed on the fifth day after the first dose. A portion of the proximal intestine was collected and processed for immunohistochemical staining. Analysis shows that treatment with irinotecan markedly increased the staining of inflammatory cells (**c, d**) when compared with the normal control group (**b**) treated only with saline. The negative control represents a sample of intestine where the primary antibody was replaced by PBS/BSA 5%; no staining was detected (**a**). Magnification (400×)

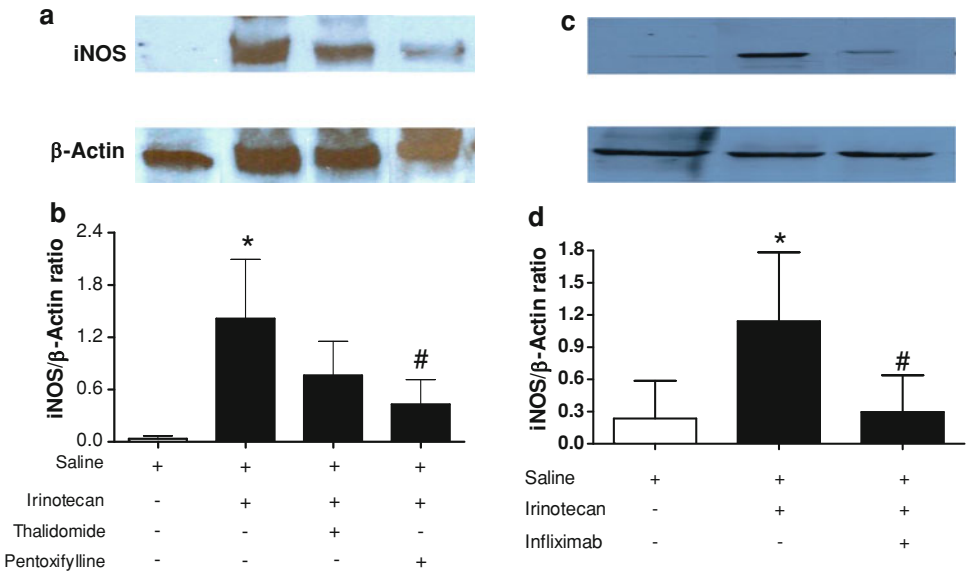

Fig. 6 Cytokine inhibition leads to a reduced expression of iNOS in irinotecan-treated mice. Mice treated with saline, thalidomide (TLD), infliximab (Inflix), or pentoxifylline (Pentox) were given irinotecan (60 mg/kg, 4 injections i.p.). A control group saline was adopted. The animals were killed on the fifth day after the first dose of irinotecan. A section of the intestine was collected and kept frozen at −70°C until the assay. Irinotecan was able to induce an increase in iNOS expression when compared with control group (**a**–**d**). On the other hand, infliximab (**c**, **d**) and pentoxifylline (**a**, **b**) prevented iNOS expression in comparison with mice to which irinotecan was given. **a** and **c** are representative blottings of 5 experiments. **b** and **d** represent the relative analysis of the iNOS and β-actin expression. Values are expressed as mean ± SD ($n = 4$–6). $^{*}P < 0.05$ versus saline-treated group that was not given irinotecan; $^{\#}P < 0.05$ versus irinotecan-treated group

prevented the leukopenia induced by irinotecan treatment (data not shown).

The intestinal injury following irinotecan administration is associated with inflammatory infiltrate within the gut wall [7, 19]. In the present study, neutrophil infiltration into intestinal lesion was indirectly evaluated by the myeloperoxidase activity assay. Interestingly, we found a marked reduction in MPO activity in intestinal segments obtained from iNOS$^{-/-}$ or aminoguanidine-treated mice, but not in those treated with loperamide, suggesting that there is a possible correlation between the lesion induced by NO and the recruitment of neutrophils in intestinal mucositis.

Table 2 Intestinal levels of proinflammatory cytokines in irinotecan-treated WT and iNOS$^{-/-}$ mice

Intestinal level of cytokines (pg/mg protein)			
Group	TNF-α	IL-1β	KC
iNOS$^{+/+}$ + saline	82.31 ± 27.70	8.15 ± 1.82	3.52 ± 0.27
iNOS$^{+/+}$ + irinotecan	341.70 ± 127.60*	537.50 ± 106.30*	452.30 ± 42.94*
iNOS$^{-/-}$ + saline	29.70 ± 9.99	4.18 ± 0.93	9.00 ± 0.68
iNOS$^{-/-}$ + irinotecan	248.90 ± 54.90	277.50 ± 46.66$^{\#,\S}$	116.80 ± 68.58$^{\#,\S}$

Data were analyzed through one-way ANOVA and Bonferroni's test. Values are expressed as mean ± SD

* $P < 0.05$ versus group treated only with saline, $^{\#}$ $P < 0.05$ versus irinotecan-treated iNOS$^{+/+}$ group, § $P < 0.05$ versus saline-treated iNOS$^{-/-}$ group

Additionally, some evidence shows that the deleterious effects ascribed to NO are mediated by peroxynitrite ($ONOO^-$), produced when superoxide anion (O_2^-) and NO react at an almost diffusion-limited rate (6.7×10^9 M^{-1} s^{-1}) [22]. $ONOO^-$ is an oxidizing and nitrating agent that promotes protein nitration, lipid peroxidation, inhibition of cellular metabolic pathways and signal transduction processes, and DNA strand breakages [23].

The effects of nitric oxide on intestinal function and injury range from beneficial to detrimental. iNOS activity has an association with increase of apoptosis [24], and cytotoxicity [23], which seems to be dose-dependent. At high concentration, nitric oxide promotes mutagenesis, DNA damage [25], alters the function of iron-sulfur–containing enzymes [26], and disrupts mitochondrial respiration [27]. Moreover, high levels of nitric oxide are associated with intestinal injury and loss of intestinal barrier function. Xu et al. [28] demonstrated that nitric oxide can directly increase ileal mucosal membrane and enterocyte monolayer permeability, as well as bacterial translocation, and that increased NO production and iNOS mRNA expression are associated with endotoxin- and/or cytokine-induced loss of enterocyte monolayer barrier function. The level of NO production, the isoform involved in this process, the redox status of the epithelial cells, the degree of microvascular dilatation, and other factors concerning the cellular environment may be of decisive importance in the effect of nitric oxide on mucosal permeability and protection [29].

Several studies show that TNF-α regulates inflammatory cascades and represents a therapeutic target in inflammatory diseases such as Crohn's disease, rheumatoid arthritis, and renal cell carcinoma (reviewed by Franks et al. [30]), some of which have been associated with raised concentrations of this cytokine in patients' tissues. Furthermore, other studies also demonstrated that IL-1β is also involved on these, and other inflammatory diseases [31]. Recently, we demonstrated that both pentoxifylline and thalidomide are protective agents in animal models of oral and intestinal mucositis induced by 5-fluorouracil [32] and irinotecan [7], respectively. In this context, the authors proposed that TNF-α and IL-1β are involved in the development of these pathological conditions. In addition, Govindarajan et al. [33] demonstrated in a human pilot study that thalidomide largely eliminated the dose-limiting gastrointestinal toxic effects of irinotecan. However, other studies did not confirm this antidiarrheal activity [7, 34] in spite of protecting mice from inflammatory events associated with mucositis [7]. In our point of view, this observation might evidence that the pathogenesis of mucositis and diarrhea seems to be different. The study by Melo et al. [7] did not assess specific mediators activated by TNF-α and IL-1β that could account for the damage caused by the administration of irinotecan. We employed a western blot assay to verify whether the cytokines TNF-α and IL-1β are essential mediators for iNOS induction in irinotecan-induced intestinal mucositis. For that purpose, we used a specific anti-TNF-α antibody, infliximab, and thalidomide, which has been reported to inhibit the production of TNF-α [35–39] and to inhibit NO effects in immunological diseases [40, 41]. We also used pentoxifylline, which exerts multiple beneficial immunomodulatory effects through the downregulation of TNF-α synthesis, decreasing in IFN-γ, granulocyte–macrophage colony-stimulating factor, and IL-6 secretion, and by attenuating cell surface expression of the IL-2 receptor (reviewed by Kreth et al. [42]). In addition to that, pentoxifylline also suppress iNOS at the mRNA level [43, 44]. In our present study, despite the fact that thalidomide failed to significantly reduce iNOS expression, most likely because of a less potent action regarding TNF-α inhibition, a significant reduced intensity of iNOS expression was found in tissues extracted from mice treated with pentoxifylline and infliximab. This observation reinforces the role of iNOS in driving intestinal mucositis pathogenesis, and that TNF-α and other cytokines, such as IL-1β, seem to be important in nitric oxide overproduction.

In mice, IFN-γ, IL-1β, and TNF-α stimulate NO production by macrophages and therefore serve as the key signals that promote the activation of these cells [45]. Despite the fact that cytokine activation leads to increased iNOS expression and tissue injury, such damage could further

enhance cytokine release by inflammatory cells in a feedback loop [46], as observed for other pathologies [47, 48]. Then, we tested the TNF-α, IL-1β, and KC production in irinotecan-treated iNOS$^{-/-}$ mice. Accordingly, we detected a partial significant inhibition on the expression of IL-1β and KC in iNOS$^{-/-}$ mice. Such result corroborates the milder inflammatory profile found in iNOS-knockout mice. However, TNF-α concentration remained significantly increased. Such observation suggests that TNF-α, IL-1β, and KC are upstream of the iNOS expression. Thence, in spite of iNOS gene deletion, the signaling operated by IL-1β and KC and mainly by TNF-α is still activated.

Irinotecan-induced mucosal injury has been examined in several animal studies [7, 14]. In this study, we showed that NO accounts for the diarrhea development, since controlling of NO production resulted in mild diarrheic events in a comparable manner to the standard drug loperamide. Considering that diarrhea can be associated with gut dysmotility, the direct study of intestinal smooth muscle function is of prime importance. The changes observed in intestinal contractility, along with associated villus/crypt ratio disruption, may partially jeopardize the intestinal absorptive function and could contribute to the development of diarrhea. We are aware of only one in vitro functional report assessing intestinal contractility patterns secondary to anticancer drug injections in animals [49].

Several studies addressed the role of NO in the gastrointestinal tract motility in different animal species [50–53]. It is well known that the NO is an inhibitory neurotransmitter in the gastrointestinal tract, and abnormal NO production or release has been implicated in functional gastrointestinal disorders. However, in our experimental protocol, where an anticancer agent is administered, we found an increased intestinal contractility verified in vitro and that the modulation of the underlying inflammation with genetic depletion of iNOS or treatment with aminoguanidine ameliorated the over-contractility due to irinotecan injection in a more effective manner than did loperamide, probably because the chronic exposure to the latter is involved with tolerance [54]. Opposingly, Lundberg et al. [55] showed that the induced overproduction of NO is likely to be responsible for the decreased motility in colitis where NO is suggested to exert a suppressive tone on colonic contractility, which is reversed by blockade of the enzyme. Until now, it is not clear whether in our model, the higher responsiveness of intestinal samples to the acetylcholine could be due to increased cholinergic receptor expression or a result of local inflammation mediated by cytokines and NO, which merits further investigation.

Some information with potential clinical applicability provided by this study suggests that the use of loperamide prevents functional disorders but is ineffective in modifying the course of the remaining inflammatory process which continues leading to tissue damage. Therefore, the modulation of inflammatory mediators could be the best approach in mucositis treatment with a focus on both inflammatory and functional components.

In summary, our data show that irinotecan-induced intestinal mucositis can be negatively modulated by the inhibition of inducible nitric oxide synthase, suggesting for the first time a harmful role of nitric oxide in the pathogenesis of this condition and the participation of cytokines in the inducible nitric oxide synthase cascade induction. This study provides perspectives on the effective management of anticancer agent-induced gastrointestinal toxic actions with a focus on the modulation of nitric oxide.

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Conflict of interest The authors indicated no potential conflict of interest.

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