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ORIGINAL ARTICLE

Gastrointestinal dysmotility in 5-fluorouracil-induced intestinal mucositis outlasts inflammatory process resolution

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Abstract
Aim To evaluate gastrointestinal motility during 5-fluorouracil (5-FU)-induced intestinal mucositis.
Materials and methods Wistar rats received 5-FU (150 mg kg^{-1}, i.p.) or saline. After the 1st, 3rd, 5th, 15th and 30th day, sections of duodenum, jejunum and ileum were removed for assessment of epithelial damage, apoptotic and mitotic indexes, MPO activity and GSH concentration. In order to study gastrointestinal motility, on the 3rd or 15th day after 5-FU treatment, gastric emptying in vivo was measured by scintilographic method, and stomach or duodenal smooth muscle contractions induced by CCh were evaluated in vitro.
Results On the third day of treatment, 5-FU induced a significant villi shortening, an increase in crypt depth and intestinal MPO activity and a decrease in villus/crypt ratio and GSH concentration. On the first day after 5-FU there was an increase in the apoptosis index and a decrease in the mitosis index in all intestinal segments. After the 15th day of 5-FU treatment, a complete reversion of all these parameters was observed. There was a delay in gastric emptying in vivo and a significant increase in gastric fundus and duodenum smooth muscle contraction, after both the 3rd and 15th day.
Conclusion 5-FU-induced gastrointestinal dysmotility outlasts intestinal mucositis.

Keywords 5-Fluorouracil · Gastric emptying · Mucositis · Smooth muscle contractility

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Introduction

Cancer chemotherapy-associated dyspepsia syndrome (CADS) is one of the most important causes of chemotherapy-related cancer morbidity [10, 20] but its pathophysiological mechanisms are still not fully established [17]. Gastrointestinal dysmotility-related symptoms such as early satiety, anorexia, nausea and vomiting have been associated with delayed gastric emptying [17].

The antimetabolite agent 5-fluorouracil (5-FU) has been used in the treatment of a range of cancers, including colorectal and breast cancers [18, 34] and can induce intestinal damage, referred as intestinal mucositis [4]. Duncan and Grant [13] proposed that intestinal mucositis is developed through three interlinked stages of increasing epithelial dysfunction: the initial inflammatory phase, the epithelial degradation phase, and the ulceration/bacterial phase. Subsequently, there is a re-establishment of functional epithelia [29, 40].

Intestinal inflammation is associated with gastrointestinal dysmotility, not only at the site of inflammation, but also at distant non-inflamed sites [1, 3, 21, 22]. A number of inflammatory mediators have been implicated both in acute and long-lasting alterations of smooth muscle contractility induced by intestinal inflammation [9]. Recently,

Riezzo et al. [31] demonstrated that gastrointestinal symptoms induced by cancer chemotherapy are associated with electrogastrography alterations. However, the relation between cancer chemotherapy-induced intestinal mucositis and changes in gastrointestinal motility has not been elucidated.

Our present hypothesis is that 5-FU-induced intestinal inflammation leads to persistent gastrointestinal dysmotility in rats.

Materials and methods

Animals

Male Wistar rats (180–220 g), provided by our Institution's Central Station, were kept in a temperature-controlled room with water supplied ad libitum, and fasted for 24 h before all experiments. Animal treatment and surgical procedures were performed in accordance to the Brazilian College of Animal Experimentation (COBEA) and were approved by the ethics committee.

Model of intestinal mucositis induced by 5-FU (adapted by Carneiro-Filho et al. [6])

Rats were randomly assigned to six groups of at least six animals and treated i.p. with a single dose of 5-FU (150 mg/kg^{-}1, ICN Farmacêutica) or vehicle (saline). After the 1st, 3rd, 5th, 15th or 30th day, rats were weighed and killed by cervical dislocation. Intestinal tissue samples were harvested and prepared for histological analysis. Additional samples were excised from the same region and immediately frozen at −70°C for further measurement of myeloperoxidase (MPO) activity and glutathione dosage (GSH). Blood was collected for total leukocyte evaluation by heart puncture.

Leukocyte count [30]

Rats were anesthetized with an ether overdose and blood samples were collected by heart puncture. The total number of white cells was determined after dilution in Turk's solution using a Neubauer chamber. Results were expressed as number of leukocytes per ml of sample.

Intestinal morphometry and histopathology

Segments of duodenum, jejunum (3-cm immediately distal to the ligament of Treitz), and ileum (6-cm section of distal ileum adjacent to the ileocecal valve) were taken. Specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (5 μm) and stained with hematoxylin and eosin. Microscopic slides were reviewed by histologist (GCB) blinded to the experimental groups and damage extent. Mucosa and muscular inflammation were assessed using a modification of the Macpherson and Pfeiffer histopathological grading system [19] (Table 1).

Villus heights measurements (from villus tip to villus–crypt junction) and crypt depths (defined as invagination depth between adjacent villi) were performed by light microscopy using a calibrated micrometer (10X). Ten intact and well-oriented villi and crypts were measured and averaged for each sample in all experimental days. Crypts of duodenum, jejunum and ileum were also assessed to establish apoptosis and mitotic indexes (1st, 3rd, 5th and 15th experimental days) in animals treated or not treated with 5-FU. Ratio of number of crypt cells with morphological apoptosis features (nuclear condensation and segmentation, cell shrinkage, and apoptotic bodies) and total number of cells per crypt were determined for at least 20 crypts for each sample. To evaluate recovery of injured small intestinal mucosa, the mitotic index was assessed by blindly counting well-defined mitotic figures at crypt bases. Mitotic figures per crypt were scored in 20 longitudinal crypt sections. Measurements were done under light microscopy at high magnification (400×). Absolute values were averaged to produce mitotic and apoptotic indexes for each group.

Intestinal MPO activity

The MPO enzyme is found primarily in azurophilic neutrophil granules and has been extensively used as a biochemical marker for granulocyte infiltration into various tissues, including the gastrointestinal tract. The extent of neutrophil accumulation in the intestinal mucosa was measured by an MPO activity assay [5]. Briefly, 50 mg/ml of intestinal tissues were homogenized in HTAB buffer (Sigma). Homogenates were centrifuged at 4,500 rpm for 7 min at 4°C. MPO

Table 1 Histopathological grading scores

Scores	Microscopic findings
0	Normal histological findings
1	Mucosa: villus blunting, loss of crypt architecture, sparse inflammatory cell infiltration, vacuolization and edema
	Normal muscular: layer
2	Mucosa: villus blunting with fattened and vacuolated cells, crypt necrosis, intense inflammatory cell infiltration, vacuolization and edema
	Normal muscular layer
3	Mucosa: villus blunting with fattened and vacuolated cells, crypt necrosis, intense inflammatory cell infiltration, vacuolization and edema
	Muscular: edema, vacuolization, sparse neutrophil infiltration

activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm using *o*-dianisidine dihydrochloride (Sigma) and 1% hydrogen peroxide (Merck). Results were reported as MPO units/mg of tissue. The unit of MPO activity was defined as the one converting 1 μmol/min of hydrogen peroxide into water at 22°C.

GSH assay

GSH concentration in intestinal tissue was assessed using a non-protein sulfhydryl group (NP-SH) assay [32]. Briefly, 100 mg/ml of frozen intestinal tissue were homogenized in 0.02 M EDTA. Aliquots of 400 μl of homogenate were mixed with 320 μl distilled H_2O and 80 μl of 50% trichloroacetic acid to precipitate proteins. Material was centrifuged for 15 min at 3,000*g* at 4°C. Aliquots of 400 μl of supernatant were mixed with 800 μl of 0.4 M Tris buffer, pH 8.9 plus 20 μl 5,5-dithiobis-2-nitro-benzoic acid (DTNB, Fluka) and mixture was shaken for 3 min. Absorbance was read within 5 min after addition of DTNB at 412 nm against a reagent blank without homogenate. The GSH concentration was reported as μg/mg of tissue.

Gastric emptying and intestinal transit

Two groups of rats ($N = 10$) were treated with 5-FU or vehicle (saline). After the 3rd or 15th day, animals previously fasted for 18 h received intragastric instillation of 5% glucose (1.0 ml/100 g) labeled with 500 μCi ^{99m}technetium (Institute of Nuclear and Energy Research—IPEN, São Paulo, SP, Brazil) coupled to phytate ("Phytosid", Sydma Medical Reagents and Equipment, Ribeirão Preto, SP, Brazil) as an unabsorbable carrier. Thirty minutes later, animals were killed by decapitation and the stomach and the small and large bowels were isolated by consecutive ligatures at the esophagogastric, gastroduodenal, ileocecal and retosigmoidal junctions. Stomachs and small bowels (separated into five consecutive segments P1–P5 in similar lengths) were inserted and kept into rubber glove fingers until counting, in order to avoid content spillage. Radioactivity from isolated tissues was counted using a gamma camera (Orbiter Stand; Siemens Gamasonics, Hoffman Estates, IL, USA). Results are reported as number of counts per minute, after subtracting background activity.

Gastric radioactivity retention was calculated through the following formula: gastric retention = amount of radioactivity recovered in stomach/total amount of radioactivity recovered from all six segments (stomach, P1–P5, small intestine) and expressed in percentages [36].

Intestinal transit was calculated through the following formula: intestinal retention in each small intestine segment = amount of radioactivity recovered in each small intestine segment/total amount of radioactivity recovered from all five small intestine segments (P1–P5) and expressed in percentages [36].

Gastric and duodenum smooth muscle contractility

Two groups of rats were treated with 5-FU or vehicle (saline). After the 3rd or 15th day, gastric fundus or duodenum were rapidly cut into segments of 1.0–2.0 cm in length. Tissues were mounted vertically in an organ bath containing Tyrode's solution bubbled with 95% O_2/5% CO_2 and maintained at 37°C, pH 7.4. Preparation was stabilized under an initial resting tension of 2 g (stomach) and 1 g (duodenum) for 1 h before experimental protocols. Active tension was developed isometrically using a force transducer connected to a computerized data acquisition system (Chart 4.1; PowerLab, ADInstruments).

Experimental protocols were initialized with a contraction control KCl (60 mM). Following tissue washing with Tyrode´s solution, a cumulative concentration–response curve with carbachol (CCh 10^{-10} to 10^{-4} M) was performed. Data were expressed as contraction control percentage.

Statistical analysis

Results are reported as means ± standard error of the mean (SEM) for each group. Statistical analysis was performed using analysis of variance (ANOVA) followed by Bonferroni's test, and for non-parametric data, Kruskal–Wallis and Dunn's test were used. Differences between groups were considered significant at $P < 0.05$.

Results

I.p. treatment of rats with 5-FU (150 mg/kg^{-1}) induced severe animal body mass reduction on the 3rd, 5th and 15th day after treatment, showing maximal reduction on the third experimental day (17.24 ± 2.16%). Significant leucopenia was also observed after 5-FU treatment on experimental days 1 (5,733 ± 1,013 cells/ml), 3 (2,917 ± 904 cells/ml) and 5 (5,308 ± 1,202 cells/ml), when compared to controls (1,0730 ± 518.2 cells/ml). This effect disappeared after 15 days of treatment (12,230 ± 886.4 cells/ml).

Figure 1 shows that on the third day after 5-FU treatment, there was a significant villi shortening with fattened and vacuolated cells, inflammatory cell infiltration in the lamina propria, loss of normal crypt architecture and presence of vacuolization and neutrophil infiltration in the duodenum muscular layer. After the 15th day, we could observe preservation of the villi and crypts, and presence of sparse neutrophil infiltration in the duodenum muscular layer.

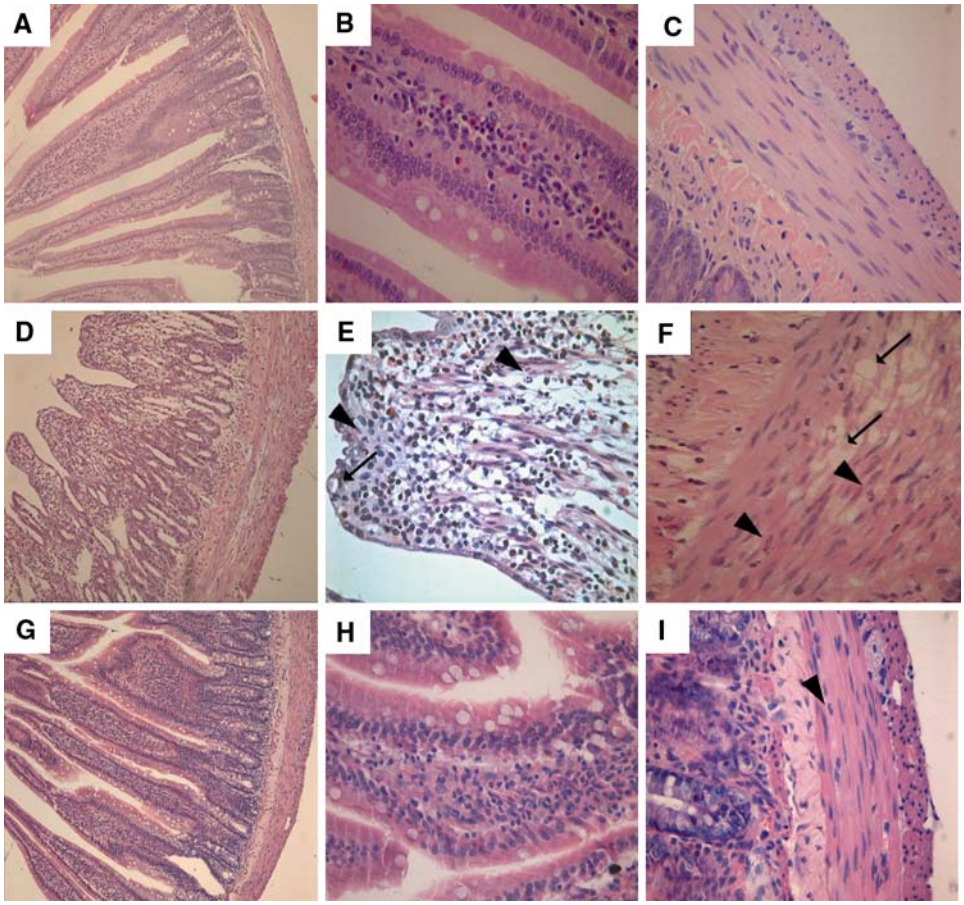

Fig. 1 Photomicrographs of duodenum. Control rat, showing normal villi, crypts and muscular (**a**, **b**, **c**). Rat submitted to 5-FU-induced intestinal mucositis (third experimental day) (**d**, **e**, **f**), showing shortened villi with fattened and vacuolated cells (**e**; *arrow)*, and inflammatory cell infiltration in the lamina propria (**e**; *arrowhead*), loss of normal crypt architecture (**d**), and presence of vacuolization *(arrow)* and neutrophil infiltration *(arrowhead)* in the muscular layer (**f**). Rat submitted to intestinal mucositis by 5-FU (15th experimental day) (**g**, **h**, **i**) showing preservation of the villi (**g**, **h**) and crypts (**g**), but with presence of sparse neutrophil infiltration in muscular layer (**i**, *arrowhead*). H&E staining (**a**, **d**, **g**, ×100 and **b**, **c**, **e**, **f**, **h**, and **i** ×400 magnification)

On the thirrd day after 5-FU treatment, there was significant microscopic mucosa and muscular duodenum inflammation (third day: histological score 3; control: histological score 0). After the 15th day of 5-FU treatment, there was no microscopic mucosa inflammation in duodenum. However, sparse neutrophil infiltration and edema in muscular layer were detected (histological score: 1).

Figure 2 shows that on the third day of 5-FU treatment, there was significant villi shortening (Fig. 2a), increase in crypt depth (Fig. 2b), and decrease in the villus/crypt ratio (Fig. 2c) in all analyzed tissues (duodenum, jejunum and ileum). After the fifth day of treatment, we noticed an increase in crypt depth in duodenum and ileum (Fig. 2b) and on the 15th experimental day, we could observe a complete reversion of the intestinal morphometry alteration in duodenum, jejunum and ileum (Fig. 2a, b, c). In Fig. 2b, it can also be detected that after the 30th day of treatment there was a significant increase in ileum crypt depth (Fig. 2b) in comparison to controls (Fig. 2c, saline).

In Fig. 3, we can observe that only on the first day after 5-FU treatment there was an increase in the apoptosis index (Fig. 3a) and a decrease in the mitosis index (Fig. 3b) in duodenum, jejunum and ileum crypts if compared to controls (Fig. 3c, saline). In Fig. 3b, a significant decrease in mitosis index was also seen on the third day after treatment. On the 5th and 15th day after treatment, no alterations in apoptosis and mitosis indexes were observed in all small bowel segments (Fig. 3a, b).

On the first day after 5-FU treatment there was an increase in MPO activity in all small bowel segments (Fig. 4a) and a decrease in GSH concentration was only found in the ileum (Fig. 4b). On the third day, we noticed an increase in MPO activity and a decrease in GSH concentration in the duodenum, jejunum and ileum (Fig. 4a, b). On the 5th and 15th day, a complete alteration reversion of MPO activity and GSH concentration was observed in duodenum, jejunum and ileum (Fig. 4a, b).

Figure 5a shows that there was an increase in gastric retention, both on the 3rd and 15th day after 5-FU treatment, when compared to control. In regards to intestinal retention, Fig. 5b presents a growth in P1 and P5 segments and a reduction in P4 on the third day. On the 15th day, no changes could be observed in any of the intestinal segments analyzed.

In the experiments carried out to verify alterations in the mechanical activity of gastrointestinal smooth muscle, 5-FU treatment induced a significant hypercontractility in gastric fundus contracted by carbachol at cumulative

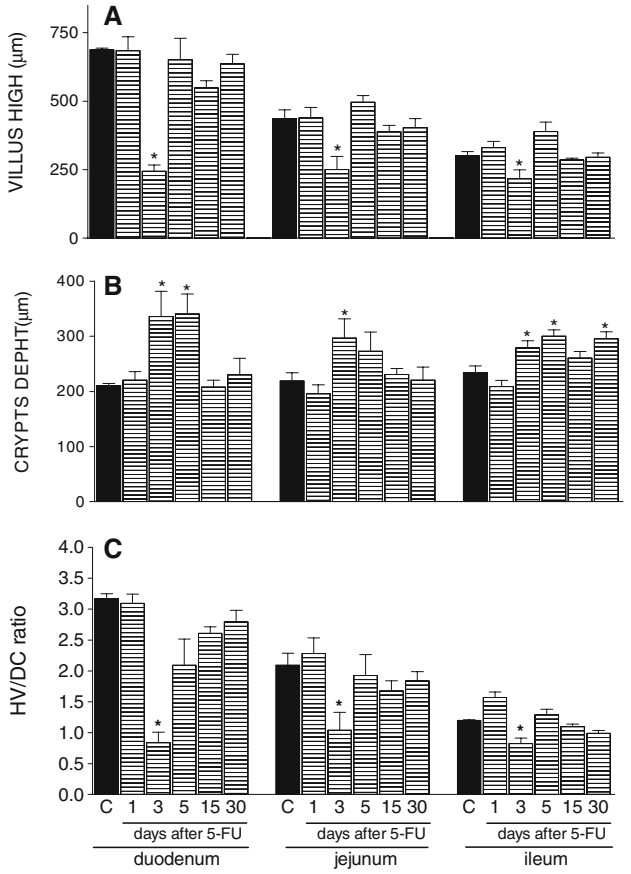

Fig. 2 Morphometric analyses of intestinal tissues in rats ($N = 6$) treated with 5-FU or saline. Animals were pretreated with 5-FU (150 mg kg^{-1}) or saline (**c**). After the 1st, 3rd, 5th, 15th or 30th day of treatment, they were killed and segments of duodenum, jejunum and ileum were taken for villus height measurement (**a**), crypt depth (**b**) and villus/crypt ratio (**c**). Values were reported as mean ± SEM. * $P < 0.05$ compared to control (**c**), ANOVA and Bonferroni's test

concentrations (10^{-10}–10^{-4} M). The maximal carbachol contraction occurred at 10^{-5} M after the third day of treatment (CCh 10^{-5} M, control = 171.90 ± 26.22%, 5-FU = 290.78 ± 36.17%) and at 10^{-4} M after the 15th day (CCh 10^{-4}M, control = 174.78 ± 28.24%, 5-FU = 282.55 ± 23.83%), in comparison to control contractions with KCl (Fig. 6a). The same improvement in duodenum contractility was observed in the tissue contracted with carbachol at 10^{-5} M, after the third day (control = 35.41 ± 15.63%, 5-FU = 105.80 ± 16.87%), and on the 15th day (control = 35.41 ± 15.63%, 5-FU = 224.38 ± 21.61%) (Fig. 6b).

Discussion

The most frequent side effects of antineoplasic chemotherapy are gastrointestinal symptoms, mainly nausea and vomiting. Patients with cancer may suffer from other gastrointestinal symptoms, such as dyspepsia, dysphagia

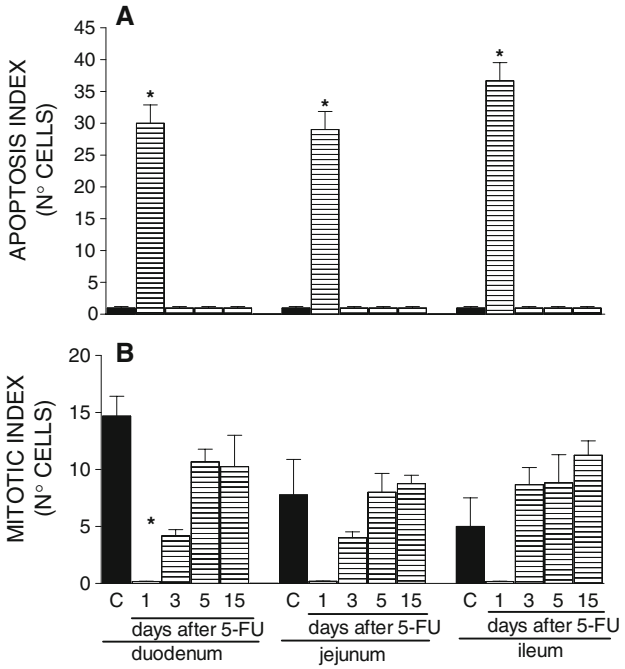

Fig. 3 Mitotic (**a**) and apoptotic (**b**) indexes of rat intestinal tissues from ($N = 6$) treated with 5-FU or saline. Animals were pretreated with 5-FU (150 mg kg^{-1}) or saline (**c**) on the 1st, 3rd, 5th, 15th or 30th day before killing and segments of duodenum, jejunum and ileum were taken for evaluation of mitotic figures (**a**) and apoptotic bodies (**b**) per crypt, in 20 longitudinal crypt sections. Values were reported as mean ± SEM. * $P < 0.05$ compared to control (**c**), ANOVA and Bonferroni's test

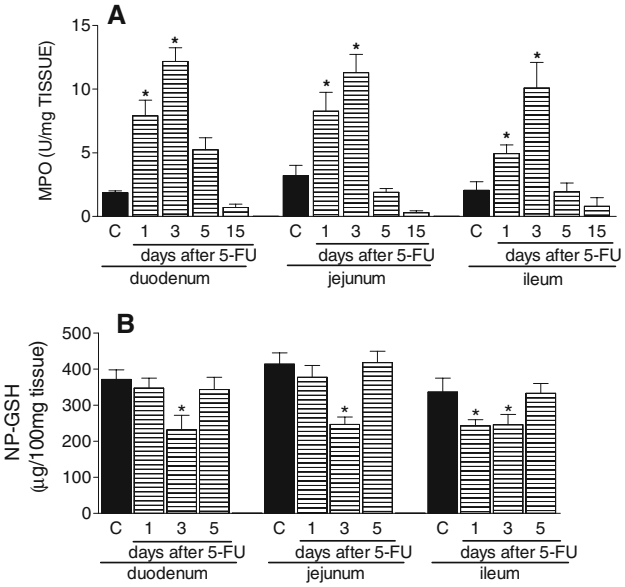

Fig. 4 MPO activity and GSH concentration in duodenum, jejunum and ileum in rats treated with 5-FU or saline. Animals were pretreated with 5-FU (150 mg kg^{-1}) or saline (**c**). After the 1st, 3rd, 5th, 15th or 30th day of treatment, they were killed and samples of duodenum, jejunum and ileum were taken for measurement of MPO activity (**a**) and GSH concentration (**b**). Results are presented as mean ± SEM. * $P < 0.05$ compared to control (**c**), ANOVA and Bonferroni's test

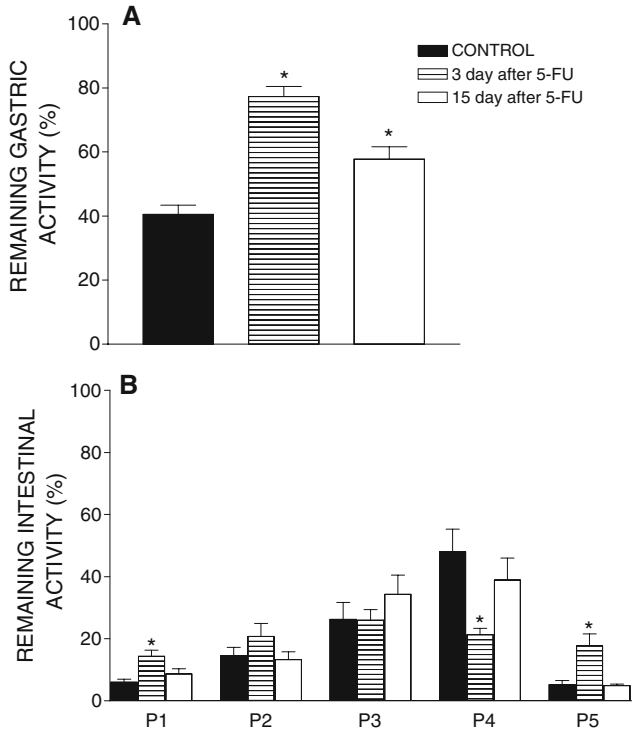

Fig. 5 Gastric emptying and intestinal transit of liquids in 5-FU or saline (**c**) treated rats. Remaining radioactivity in stomach (*S*, **a**) and intestinal segments (P1–P5, **b**), 30 min after intragastric instillation of 5% glucose (1.0 ml/kg body weight) labeled with ^{99m}technetium is expressed as percentage of total activity in gastrointestinal tract. Results are presented as mean ± SEM. $^*P < 0.05$ vs. control, ANOVA and Bonferroni's test

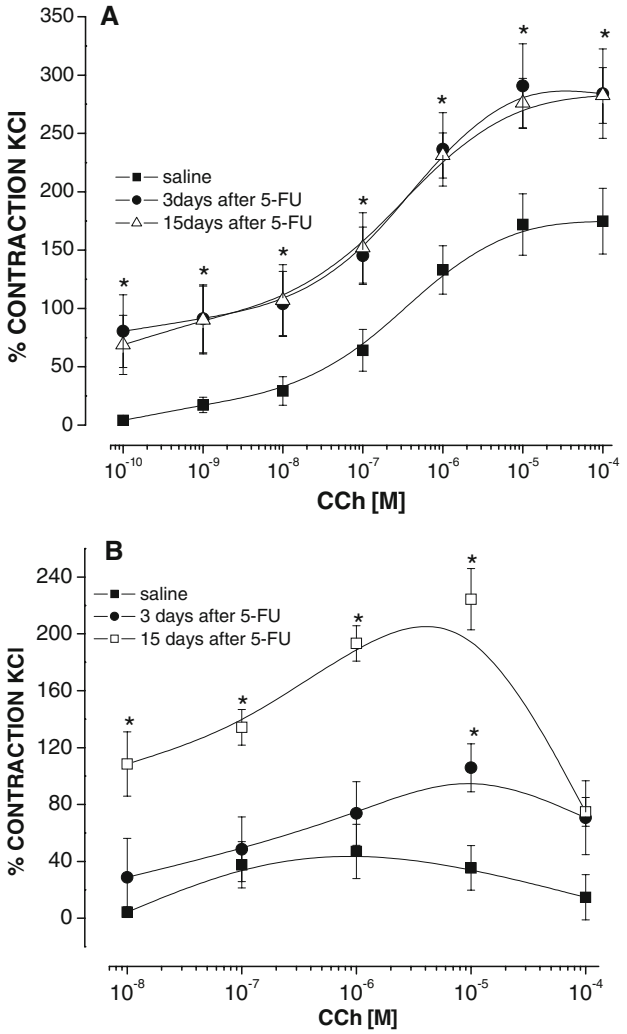

Fig. 6 Contractility of the stomach fundus and duodenum smooth muscles in 5-FU and saline treated rats. **a** Mean stomach fundus data showing effects of CCh + saline (*filled square*, $n = 8$), CCh + 5-FU after the third day (*filled circle*, $n = 6$), and CCh + 5-FU after 15 days (Δ, $n = 5$). **b** Mean duodenum data showing effects of CCh + saline (*filled square*, $n = 7$), CCh + 5-FU after the third day (*filled circle*, $n = 4$), and CCh + 5-FU after the 15th day ((*open square*, $n = 4$). Data is shown as mean ± SEM. $^*P < 0.05$ compared to saline

and diarrhea [31]; this cluster of symptoms has been referred as chemotherapy-associated cancer dyspepsia syndrome (CADS). Recent researches suggest that abnormal gastrointestinal motility may be one of the causes of CADS [24, 31]. 5-FU is one of the most important antineoplasic drugs, and it can induce intestinal mucositis, which is associated to diarrhea and dyspepsia syndrome in clinical practices [4]. We are unaware of any previous studies examining the effects of 5-FU-induced intestinal mucositis on gastrointestinal motility.

This manuscript demonstrated that intestinal mucositis induced by 5-FU is associated to a delay in gastric emptying/intestinal transit of liquid, and related to hypercontractility in gastric fundus and duodenum muscles, in both inflammatory (third day) and post-inflammatory phases (15th day), after 5-FU treatment.

Administration of 5-FU in rats resulted in severe body mass loss, leucopenia, important decrease in villus/crypt ratio, neutrophil infiltration and decrease in intestinal GSH concentration, which could be secondary to the consumption of reaction oxygen species (ROS). Theses events were observed on the third day after 5-FU administration with complete reversion after the 15th day. Our results are in agreement with literature, which showed that oral mucositis is a complex process that is initiated by basal epithelium cell injury and underlying tissue. Non-DNA injury is initiated by a variety of mechanisms, including ROS generation [35]. Intestinal injuries can include alterations in brush-border hydrolase activity [26], blunted villus heights, crypt depths, and increased apoptosis of crypt cells with decreased proliferation [6, 12, 14, 25].

Our data also showed alterations in cellular crypt cycles, with intense apoptosis on the first experimental day, which is linked to mitosis reduction. These evidences were not observed after the fifth experimental day. As demonstrated by Pritchard et al. [27, 28] 5-FU induces maximal p53

expression in intestinal crypts after 24 h of treatment and declines after the third day. This could be the mechanism involved in the 5-FU-induced apoptosis observed in our study.

Delay in gastric emptying may be due to an increase in gastric compliance and/or an increase in antro-duodenal resistance [15]. This explanation could be used in our results, showing that in the 5-FU-induced intestinal mucositis, we could observe a delay in gastric emptying, both in the inflammatory (after the third day) or post-inflammatory (after the 15th day) phases. At present, to the best of our knowledge, there are no studies available on the effect of 5-FU-induced intestinal mucositis in gastric compliance or in gastrointestinal muscle contractility. At this point, two hypotheses can be formulated: (1) 5-FU-induced decrease in gastric motility can be resultant of gastric inflammation; (2) 5-FU-induced intestinal inflammation leads to changes in gastric motility. The former hypothesis seems to be less probable, since in our model, 5-FU treatment neither induced macroscopical damage nor growth in MPO activity in the stomach (data not shown). On the other hand, the second hypothesis seems to be more reliable, since the intestinal inflammatory parameters studied (morphometric, MPO activity and GSH concentration) were present on the third day after 5-FU treatment. Accordingly, it was demonstrated that intestinal inflammation is associated to gastrointestinal motility control abnormalities, not only at the site of inflammation, but also at distant non-inflamed sites [1, 3, 21, 23]. More recently, Demedts et al. [9] showed that intestinal inflammation induces acute and long-lasting alterations in smooth muscle contractility, involving a number of inflammatory mediators. In addition, there are several other experimental results that clearly show an interaction between inflammation and motility. For instance, sepsis inhibits gastrointestinal motility, which may influence nitric oxide production [8] and intestinal hypomotility. Delayed gastric emptying has also been shown to be characteristic in postoperative ileum mouse model [7]. In Fig. 5b, we also demonstrated that an acceleration in intestinal transit only occurred on the third day (inflammatory phase), which could be associated to the diarrhea observed in patients under 5-FU treatment.

Our results showed that contractility induced by the muscarinic agonist carbachol in gastric fundus and also in duodenum, was increased both in the inflammatory (after the third day) and in the post-inflammatory (after the 15th day) phases of the 5-FU-induced intestinal mucositis. As also described in functional dyspeptic patients [33, 39], the growth in gastric fundus contractility could induce an impaired gastric accommodation and then, dyspeptic symptoms.

Furthermore, other studies have demonstrated a significant enhancement in gastrointestinal muscle contractility in response to carbachol at inflammatory/infectious situations, as in *T. Spiralis* [41] infection in rats or during the chronic inflammation phase induced by *Schistosoma* in murine model [22]. Moreover, in inflammatory bowel disease models, changes in colonic smooth muscle contractility were demonstrated. [11]. These alterations, associated with inflammation/infection, could be secondary to the production of contracturant mediators [11]. Following this pattern, an increased response of muscarinic receptor to IL-4, IL-13, TGF-β cytokines [1, 2] in the gastrointestinal smooth muscle was reported, as well as a reduction in inhibitory non-adrenergic and non-cholinergic (NANC) neurotransmission [23].

These disturbances in gastrointestinal motility could contribute to the generation of dyspepsia symptoms related to chemotherapy-induced cancer. Thus, it is possible to infer that the persistence of gastrointestinal dysmotility could be related to chemotherapy-associated cancer dyspepsia syndrome (CADS) as previously suggested [16, 24, 31]. Besides, several researchers demonstrated that the persistence of motility alterations could be linked to functional diseases, such as irritable bowel syndrome and functional dyspepsia [37, 38].

In conclusion, our results support the hypothesis that gastrointestinal dysmotility in 5-fluorouracil-induced intestinal mucositis outlasts the resolution of inflammatory processes. It could explain, at least in part, the persistence of CADS symptoms in patients under 5-FU treatment.

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