Biomedicine & Pharmacotherapy 133 (2021) 111012

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

# Prophylactic and therapeutic supplementation using fructo-oligosaccharide improves the intestinal homeostasis after mucositis induced by 5- fluorouracil

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ARTICLE INFO

*Keywords:*
Mucositis
Intestinal damage
Tight junction
Short-chain fatty acids
5-Fluorouracil
Fructo-oligosaccharide
Prebiotics

ABSTRACT

The beneficial effects of prebiotic, such as fructo-oligosaccharides (FOS), in intestinal inflammation have been demonstrated in several studies. Herein, we evaluate whether joint treatment with FOS, both before and during mucositis, had additional beneficial effects and investigated the mechanisms underlying in the action of FOS on the intestinal barrier. BALB/c mice were randomly divided into five groups: CTR (without mucositis + saline solution), FOS (without mucositis + 6 % FOS), MUC (mucositis + saline solution), PT (mucositis + 6 % FOS supplementation before disease induction), and TT (mucositis + 6 % FOS supplementation before and during disease induction). Mucositis was induced by intraperitoneal injection (300 mg/kg) of 5-fluorouracil (5-FU). After 72 h, the animals were euthanized and intestinal permeability (IP), tight junction, bacterial translocation (BT), histology and morphometry, and immunoglobulin A secretory (sIgA), inflammatory infiltrate, and production of short-chain fatty acids (acetate, butyrate and propionate) were evaluated. The MUC group showed an increase in the IP, BT, and inflammatory infiltrate but a decrease in the tight junction expression and butyrate and propionate levels ($P < 0.05$). In the PT and TT groups, FOS supplementation maintained the IP, tight junction expression, and propionate concentration within physiologic levels, increased butyrate levels, and reduced BT and inflammatory infiltrate ($P < 0.05$). Total treatment with FOS (TT group) was more effective in maintaining histological score, morphometric parameters, and sIgA production. Thus, total treatment (prophylactic and therapeutic supplementation) with FOS was more effective than pretreatment alone, in reducing 5-FU-induced damage to the intestinal barrier.

*Abbreviations:* 5-FU, 5-fluorouracil; $^{99m}$Tc- DTPA, diethylenetriaminepenta acetic acid labeled with technetium-99m; $^{99m}$Tc-*E. coli*, *Escherichia coli* labeled with technetium-99m; BT, bacterial translocation; EPO, eosinophil peroxidase; FOS, fructo-oligosaccharides; IP, intestinal permeability; MLN, mesenteric lymph nodes; MPO, myeloperoxidase; SCFA, short-chain fatty acids; sIgA, immunoglobulin A secretory; TJs, tight junctions.

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https://doi.org/10.1016/j.biopha.2020.111012
Received 6 August 2020; Received in revised form 3 November 2020; Accepted 11 November 2020
Available online 27 November 2020

## 1. Introduction

Mucositis is a common side effect of cancer treatment, especially when using antimetabolite drugs, such as 5-fluorouracil (5-FU). The 5-FU is metabolized to intermediates that cause DNA damage and, act on both tumor and healthy cells, particularly those replicate rapidly [1,2].

It is condition is characterized by inflammation of the mucosa of the entire gastrointestinal tract with impaired intestinal integrity leading to increased intestinal permeability (IP) and bacterial translocation (BT), thereby enhancing the risk of sepsis among these patients [3–5].

The pathogenesis of mucositis is multifactorial, and involves the release of free radicals and activation of the inflammatory immune response [6]. In recent years, studies have also reported changes in the intestinal microbiota after anticancer treatments [7–9]. Fijlstra et al. [8] showed that the number and diversity of microbiota decreased after four days of mucositis in a model of methotrexate-induced gastrointestinal mucositis.

Therefore, it has been hypothesized that the modulation of intestinal microbiota with prebiotics, probiotics or synbiotics could had a prophylactic and therapeutic benefit for the development of mucositis. However, studies that have evaluated the effect of these compounds against mucositis have reported controversial results, with few studies showing partial improvement in the intestinal barrier [7,9,10].

Prebiotics are defined as substrates that are selectively used by the host microorganisms and confer health benefits [11]. Prebiotics such as fructo-oligosaccharides (FOS) have demonstrated good therapeutic action against intestinal infections. It exerts maximum effects on the colonic epithelium by increasing the concentration of short chain fatty acids (SCFA) such as acetate, propionate and butyrate, stimulating the proliferation of crypts, and modulating both the microbiota and the immune function [12].

However, the benefits of FOS on the intestinal barrier during mucositis are still controversial, as well the dose and timing of supplementation of FOS are not known [9,10]. A study carried out by our research group [9] evaluated the effects of supplementation with 6% FOS in the pre-mucositis and after the mucositis induction in distinct groups. The results showed that both period of FOS supplementation had protective effects on the intestinal barrier function. In addition, only pretreatment with FOS increased the production of SCFA, such as acetate and butyrate, suggesting that modulation of intestinal microbiota is time-dependent. In contrast, other study evaluated supplementation with 5 % FOS just before mucositis. The results showed that prebiotic supplementation was unable to improve intestinal integrity and immunity [10].

Finally, the last guideline published by the MASCC/ISOO Mucositis Study Group concluded [13] that supplementation with prebiotics, such as FOS, in order to prevent intestinal mucositis had conflicting results in terms of effectiveness, and thus there is no specific guideline. Therefore, more studies evaluating the mechanisms of action are needed.

The purpose of this work was to investigate possible FOS additional beneficial effects and also the mechanism underlying of this action on the intestinal barrier, employing a new treatment modality: total treatment with FOS (prophylactic and therapeutic supplementation).

## 2. Materials and methods

### 2.1. Animals and experimental design

Male BALB/c mice, weighing 18−25 g, were provided by the Animal Care Center at Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais (UFMG). The animals were housed in cages, subjected to 12-h light–dark cycles and controlled temperature, and allowed free access to commercial chow and water. This study was approved by the Ethics Committee in Animal Experimentation of UFMG (CEUA/UFMG; number 66/2018) and complied with the Institutional and National guidelines for the Care and Use of Laboratory Animals.

The animals were randomized into five groups: CTR (without mucositis + saline), FOS (without mucositis + supplementation with FOS), MUC (mucositis + saline), PT (mucositis + supplementation with FOS before the induction of the mucositis i.e. 1st to 6th day), and TT (mucositis + supplementation with FOS during the entire experimental duration i.e. 1st to 10th day). The animals in the FOS, PT, and TT groups received 240 mg of FOS (6% of total kilocalories), diluted in 0.2 ml of saline, by gavage (once a day) at the same time. The FOS used was NutraFlora®, GTC Nutrition LLC, Golden, CO, USA.

Each day, the amount of food consumption was measured based on the difference between the amount of offered chow and residual chow. The weight of the mice was measured with a semi-analytical balance (Filizola-MF, São Paulo, Brazil). The weight loss was calculated by subtracting the weight on day 10 from that on day 7.

### 2.2. Mucositis induction

Intestinal mucositis was induced by the method described by Maioli [14]. On the 7th day, animals in the MUC, PT, and TT groups received an intraperitoneal injection of 300 mg/kg 5-FU (Eurofarma®) to induce mucositis. The CTR and FOS groups received intraperitoneal injections of the same volume of sterile saline. After 72 h (10th experimental day), all animals were euthanized under anesthesia and blood, organs, and the small intestine were removed for further analysis.

### 2.3. Intestinal permeability

After 72 h of mucositis induction, all mice received 0.1 ml of diethylenetriaminepentaacetic acid labeled with technetium-99 m solution ($^{99m}$Tc-DTPA) with 18.5 MBq of activity, by gavage. After 4 h, all animals were anesthetized, and blood was collected and placed in appropriate tubes for the determination of radioactivity. Blood radioactivity levels were determined using an automatic gamma counter (PerkinElmer Wallac Wizard 1470−020 Gamma Counter; PerkinElmer, Waltham, MA). The data were expressed as dose % using the following equation:

$$\%dose/g = (cpm\ in\ g\ ofblood/cpmof\ standard) \times 100$$

Where, cpm represents the counts of radioactivity per minute [15,16].

### 2.4. Bacterial translocation

The procedure for radiolabeling bacterial cells (*Escherichia coli*) was based on the method described by Diniz et al. [17]. The percentage of $^{99m}$Tc incorporated into the bacterial cells was determined using the following equation: $\%\ \text{labeling} = \frac{(\text{cpm precipitate})}{\text{cpm precipitate} + \text{cpm supernatant}} \times 100.$

After 72 h, 0.1 ml of the suspension of *E. coli* labeled with technetium-99 m ($^{99m}$Tc-*E.coli*), containing 1.8 MBq of activity, was administered by gavage to all animals, corresponding to $10^8$ colony forming units/mL. After 4 h, the animals were anesthetized and euthanized, and their blood, mesenteric lymph nodes (MLNs), liver, spleen, and lungs were collected, weighed, and placed in tubes to measure the radioactivity present in the tissues. The samples were counted using an automated gamma counter (PerkinElmer Wallac Wizard 1470-020 Gamma Counter; PerkinElmer Inc., Waltham, USA). The results were expressed as cpm/g of tissue.

### 2.5. Tight junctions

Fragments of ileum were collected for the analysis of gene expression using real-time quantitative reverse transcription-PCR, as previously described by Vieira et al. [18].

The collected ileum samples were stored in RNase-free micro tubes and stored in a freezer at −80 °C until analysis. Total RNA was extracted

from the sample using TRizol reagent (Invitrogen/Life Technologies, Grand Island, NY, USA), according to the manufacturer's protocol. The RNA extracted from the samples in the previous stage was transcribed into cDNA using the transcriptase reverse enzyme (MMLV RT). The cRNA levels were determined using SYBR Green reagent (Applied Biosystems/Life Technologies, USA) and specific primers in an ABiPrism 7900HT Sequence Detection System (Applied Biosystems/Life Technologies, Foster City, CA, USA).

Gene expression was normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase), and the results are expressed as the fold increase over control (2- ΔΔCT).

The primers used were: ZO-1: 5′CCAGCTTATGAAAGGGTTGTTC3′ and 5′CCAGCTTATGAAAGGGTTGTTC3′, occludin: 5′ATGTCCG GCCGATGCTCTC3′ and 5′TTTGGCTGCTCTTGGGTCTGTAT3′, GAPDH: 5′ACGGCCGCATCTTCTTGTGCA3′ and 5′ CGCCCAAATCCGTCCACAC CGA3′.

### 2.6. Histological, morphometric and mucus analysis

Ileum segments were processed for histological analysis, as described previously by Chen et al. [19]. The tissues were rolled and fixed in paraffin. Histological sections (4–μm thick) were stained with hematoxylin and eosin, and mucosal inflammation was assessed using the histopathological scores described previously [20]. Histopathological findings were determined using a microscope (Olympus BX51; Olympus, Tokyo, Japan).

Histological sections were also stained with periodic acid Schiff (PAS) to evaluate neutral mucins [21]. For the morphometric examination, villus high and crypt dept were measured (10 microscopic fields for each sample, and N = 8/group), and the measurements in the photomicrographs performed using the ImageJ program (v. 1.45S, Wayne Rasband/National Institutes of Health, Bethesda, MD, USA).

Goblet cell analysis was performed by evaluating the intensity of pixels of PAS staining of the ileum sections. The image capture was standardized using three random microscopic fields with an increase of 20×, for each mouse. Then, the images were converted to the 8-bit RGB format in the ImageJ software®, and with the aid of the color deconvolution plugin, positive PAS coloring enhancement was obtained. The areas corresponding to the goblet cells of the intestinal mucosa were selected and compared with the originally captured images. The intensity variation was evaluated on the pixel scale, at the count from 0 (darkest) to 255 (lighter). The results were expressed as the average intensity of pixels [21].

### 2.7. Myeloperoxidase (MPO) and eosinophil peroxidase (EPO)

Enzyme activities in the ileum were evaluated as described previously by Strath et al. [22]. The protein content in the samples was determined according to the Lowry method [23]. After protein quantification, the results obtained for the enzyme activities of MPO and EPO were corrected and expressed as per milligram of protein.

### 2.8. Short-chain fatty acids analysis

Animal feces were collected on the 10th day to determine the concentration of acetate, butyrate, and propionate. The analysis was performed in duplicate, as proposed by Smiricky-Tjardes et al. [24]. The analysis was performed in a gas chromatograph model CGMS-QP 5000 brand SHIMADZU, coupled to a microcomputer and equipped with a detector for recording the analysis of the chromatograms using the GC Solution program. The respective acids were separated and identified on a NUKOL capillary column (30 m × 0.25 mm × 0.01 mm). Helium gas was used as a carrier with linear velocity programmed to 38.5 cm/s. The injector and detector temperatures were 200 °C and 220 °C, respectively.

### 2.9. Secretory immunoglobulin A analysis

For sIgA analysis, the fluid in the ileum was weighed, homogenized in ice-cold phosphate-buffered saline (PBS) supplemented with an antiprotease cocktail (PBS; 1 mL/0.1 g), and centrifuged (5000 rpm, 30 min, 4 °C). The supernatant was collected and used to detect immunoglobulin by ELISA. The total sIgA level was determined by ELISA, as described by the kit (A-4789, *Sigma*).

### 2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad, Inc., La Jolla, CA, USA). The results were tested for outliers (Grubbs' test) and normality using the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test were used for all parameters except for the score histological (nonparametric distribution), which was analyzed with the Kruskal-Wallis and Dunn's tests. A P-value less than 0.05 was considered statically significant.

## 3. Results

### 3.1. Food consumption and weight variation

After mucositis induction, animals in the MUC, PT, and TT groups showed a significant reduction in food intake (Fig. 1A) and weight (Fig. 1B) compared with those in the CTR and FOS groups ($P < 0.05$).

### 3.2. Influence of FOS on intestinal barrier

Results showed that IP increased and the mRNA expression of ZO-1 and occludin decreased in the MUC group than those in the other groups (Fig. 2; $P < 0.05$). In the PT and TT groups, FOS supplementation aided in the maintenance of IP and mRNA expression of ZO-1 and occludin within physiologic levels (Fig. 2).

Table 1 presents the results of BT. The MUC group showed increased BT in the blood, liver, spleen, lungs, and MLN compared with the CTR group ($P < 0.05$). The animals in the PT and TT groups showed a significant reduction in BT in the blood, liver, and spleen ($P < 0.05$).

Regarding to histological parameters the results showed reduction in villus height, brush edge breakage, and loss of crypt architecture were observed in the MUC group (Fig. 3C) when compared with the normal histological aspects observed in the CTR (Fig. 3A) and FOS (Fig. 3B) groups. The PT group (Fig. 3D) showed partial improvement in mucosal integrity, with shortening of lower villi and greater conservation of crypt architecture. Animals in the TT group (Fig. 3E) showed greater preservation of the epithelium, including brush edge, than those in the PT and MUC groups.

Morphometric analysis demonstrated a significant reduction in all parameters (Fig. 4A–C) in the MUC group than in the CTR and FOS groups ($P < 0.05$). Pretreatment with FOS (PT group) was able to partially preserve the villous height, crypt depth, and villi/crypt ratio ($P < 0.05$). The TT group maintained the villous height, crypt depth, and villi/crypt ratio within the physiologic levels.

Histological score was used to evaluate the tissue damage caused by mucositis [20]. A score of 0 was observed in the CTR and FOS groups, indicating no alterations in the mucosa of these animals. The MUC group had a score 3, indicating the presence of inflammation, as observed by stunted villi, loss of crypt architecture, and brush edge disruption. The PT group presented an intermediate score between the CTR and MUC groups, and the TT group had a score of 1, indicating lesser tissue damage (Fig. 4D). In addition, we evaluated the effects of FOS supplementation on inflammatory infiltrate. The MPO and EPO enzymes were evaluated in the ileum for indirect determination of neutrophil and eosinophil infiltration, respectively. Result showed increase MPO (Fig. 4E) and EPO (Fig. 4F) activities in the MUC group compared with

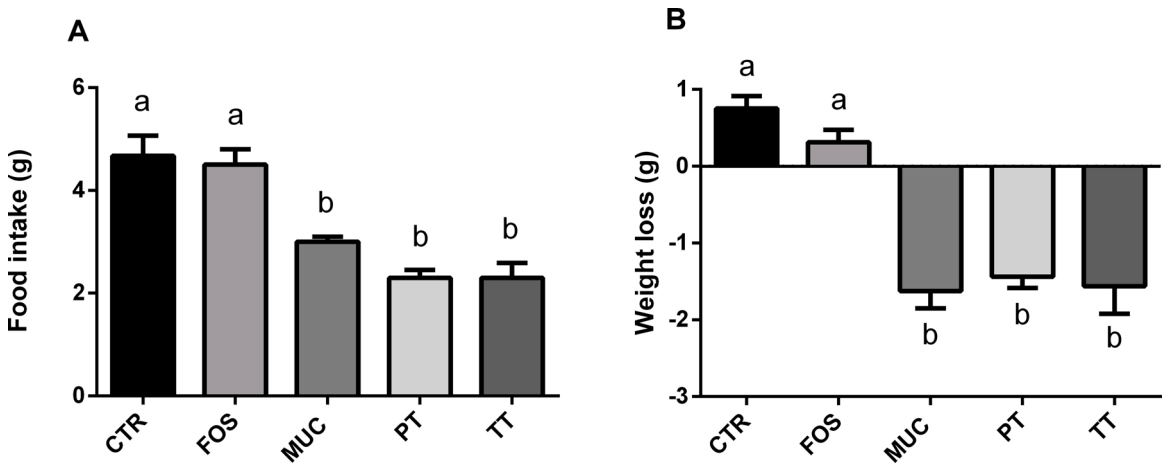


**Fig. 1. Variation in food consumption and weight after induction of mucositis.** (A) Food intake. (B) Weight loss. Data are expressed as mean ± SEM (N = 8). Different letters indicate statistical significance (P > 0.05; One-way ANOVA and Newman-Keuls Multiple Comparison Test). CTR = control, FOS = FOS control, MUC = mucositis, PT = pretreatment, TT = Total treatment.

those in the other groups (P < 0.05). In the PT and TT groups, MPO and EPO enzymes were maintained at physiological levels.

Finally, the mucus layer was quantified by PAS staining. Staining of the mucus layer staining (Fig. 5A–E) indicated reduced mucus production in the MUC, PT, and TT groups. These results were confirmed by the evaluation of the intensity of pixels by PAS staining (Fig. 5F).

### 3.3. Influence of FOS supplementation on short chain fatty acids and sIgA concentrations

Propionate (Fig. 6B) and butyrate (Fig. 6C) concentrations were higher in the FOS group than in the CTR, MUC, PT, and TT groups (P < 0.05). The MUC group showed a significant reduction in butyrate

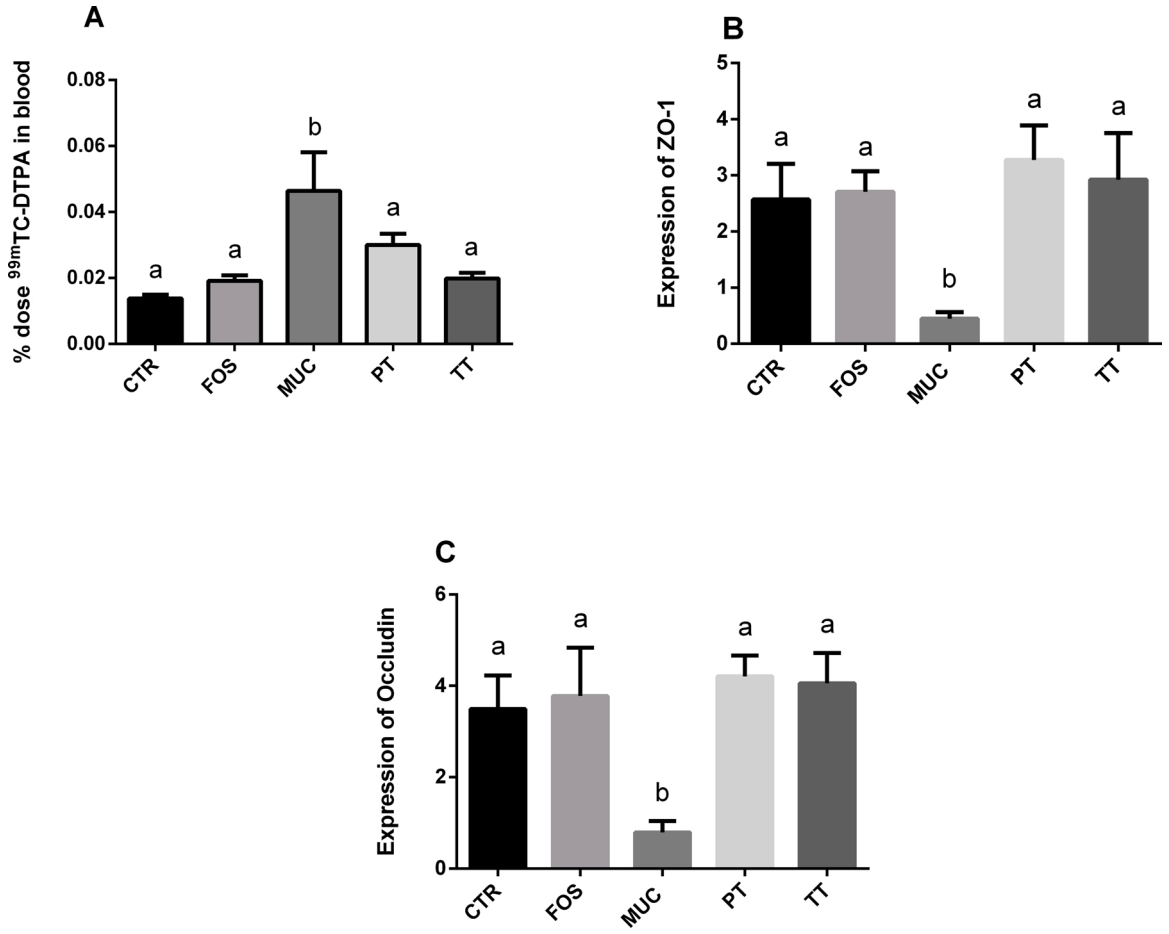


**Fig. 2. Intestinal permeability and tight junction mRNA expression.** (A) Intestinal permeability. Data are expressed as means ± SEMs (N = 6). (B) ZO-1 mRNA expression. (C) Occludin mRNA expression. Data are expressed as means ± SEMs (N = 8). Different letters indicate that there are statistically significant differences (P < 0.05; One-way ANOVA and Newman-Keuls Multiple Comparison Test). CTR = control, FOS = FOS control, MUC = mucositis, PT = pretreatment, TT = Total treatment.

**Table 1**
Biodistribution of $^{99m}$Tc-*Escherichia coli*.

| Group/ Tissue | CTR (cpm/g) | FOS (cpm/g) | MUC (cpm/g) | PT (cpm/ g) | TT (cpm/ g) |
|---|---|---|---|---|---|
| Blood | 1300 $^{a}$ ± 94.8 | 831 $^{a}$ ± 253.1 | 1988 $^{b}$ ± 393.4 | 597 $^{a}$ ± 127.6 | 904 $^{a}$ ± 110.7 |
| Liver | 1650 $^{a}$ ± 451.1 | 1913 $^{a}$ ± 614.5 | 6579 $^{b}$ ± 1639.0 | 3533 $^{a}$ ± 373.0 | 3273 $^{a}$ ± 478.2 |
| Spleen | 382 $^{a}$ ± 74.7 | 716 $^{a}$ ± 223.4 | 3235 $^{b}$ ± 380.3 | 1024 $^{a}$ ± 138.5 | 1532 $^{a}$ ± 288.1 |
| Lungs | 467 $^{a}$ ± 146.5 | 913 $^{a}$ ± 314.8 | 2964 $^{b}$ ± 481.1 | 2018 $^{a\ b}$ ± 305.8 | 2124 $^{a\ b}$ ± 650.5 |
| MLN | 461 $^{a}$± 55.9 | 508 $^{a}$ ± 133.1 | 1849 $^{b}$ ± 304.4 | 1301 $^{b}$ ± 246.1 | 1346 $^{b}$ ± 203.8 |

Data are expressed as means ± SEMs (N = 10). Different letters in the same line indicate statistically significant differences ($P < 0.05$; One-way ANOVA and Newman-Keuls multiple comparison test). CTR, control; FOS, FOS control; MUC, mucositis; PT, Pretreatment; TT, Total treatment; cpm, counts of radioactivity per minute.

concentration than that in the CTR group (Fig. 6C). In addition, when propionate concentration was compared between the CTR and MUC groups (Student's *t*-test), it was observed that mucositis reduced the propionate levels ($P < 0.05$; Fig. 6B). In the PT and TT groups, propionate concentration was maintained at a similar concentration as that in the CTR group. When butyrate concentration was compared between MUC vs. PT and MUC vs. TT groups (Student's *t*-test), it was observed that FOS led to an increased butyrate levels ($P < 0.05$; Fig. 6C). There was no difference in the acetate concentration among the MUC, PT, and TT groups (Fig. 6A).

There was a significant increase in the levels of sIgA in the MUC group compared with those in the CTR group ($P < 0.05$). There was no difference between the MUC and PT groups ($P > 0.05$). The total treatment (TT) was able to maintain sIgA levels similar to that of the CTR group ($P > 0.05$) (Fig. 6D). There was a significant increase in the levels of sIgA in the MUC group than in the CTR group ($P < 0.05$). There was no difference between the MUC and PT groups ($P > 0.05$). The TT group was able to maintain sIgA levels similar to that of the CTR group ($P > 0.05$) (Fig. 6D).

## 4. Discussion

Despite the higher prevalence of mucositis (approximately 50 %–80 % of patients) [25], cost ($3700 per cycle of chemotherapy leading to an incremental cost of $70,000 per patient) [26] and the advances in understanding its pathogenesis, there are still no effective therapies and protocols for its prevention and/or treatment.

In recent years, evidence has emerged that changes in the microbiota are involved in the pathogenesis and severity of mucosite. In this sense, the use of prebiotics because of their ability to improve intestinal function and repair intestinal damage could be a beneficial alternative

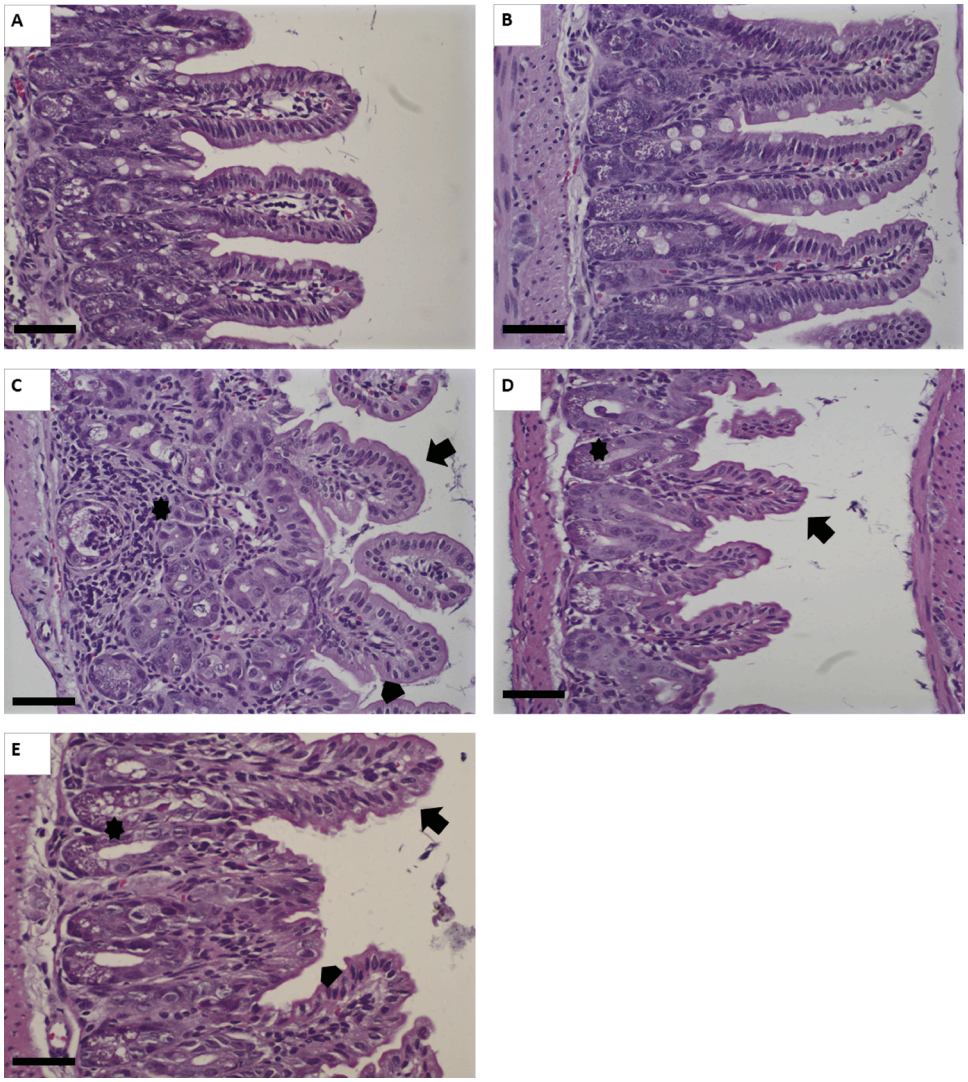


**Fig. 3. Histological analysis of the ileum.** (A) CTR group and (B) FOS group, normal tissue; (C) MUC group, shortened villi (arrows), brush edge breakage (arrowhead), and loss of crypt architecture (asterisks); (D) PT group, partial preservation of villi (arrows) and crypt architecture (asterisks); (E) TT group, preservation of villi (arrows) and crypt architecture (asterisks) as well as total preservation of the brush border (arrowhead). (N = 6); objective 40 ×. CTR = control, FOS = FOS control, MUC = mucositis, PT = pretreatment, TT = Total treatment.

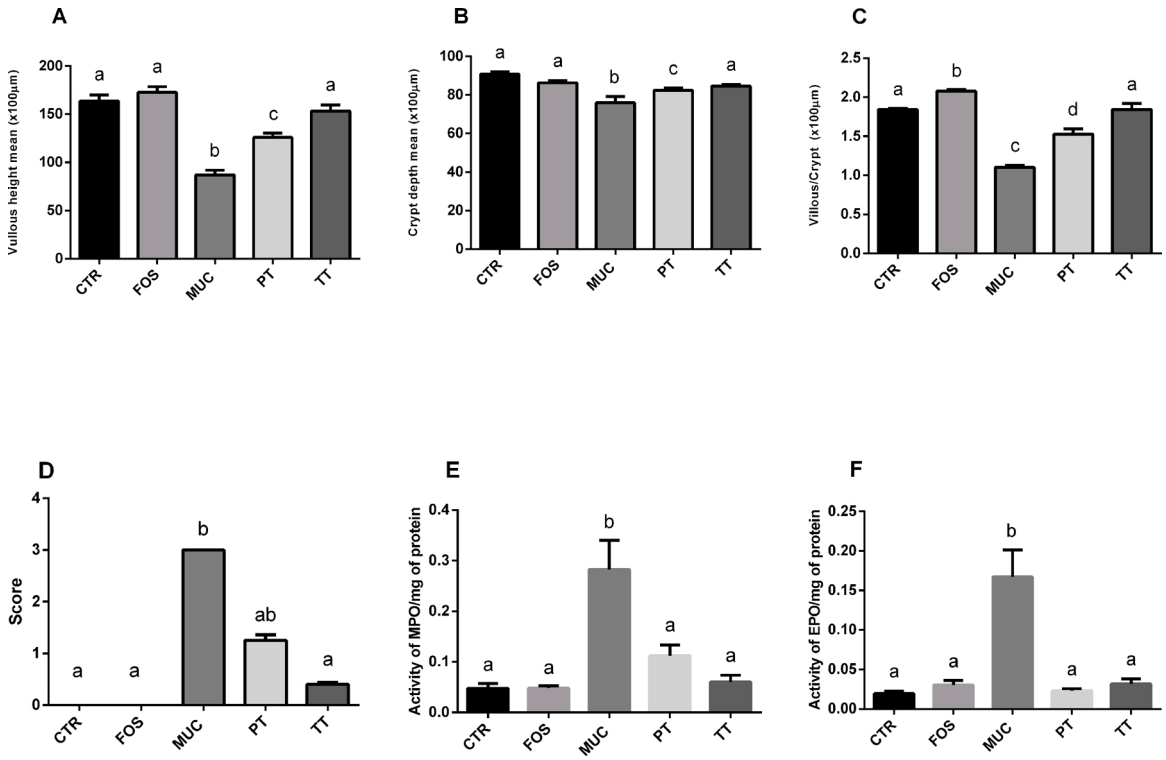


**Fig. 4. Morphometric analysis, histological score and inflammatory infiltrate.** (A) Villus height; (B) Crypt depth; (C) Villi/Crypt ratio; (D) Histological score; (E) Neutrophil infiltrate (MPO); (F) Eosinophil infiltrate (EPO). Data expressed in mean ± SEM (N = 6). Different letters indicate that there are statistically significant differences ($P < 0.05$; One-way ANOVA and Newman-Keuls Multiple Comparison Test). CTR = control, FOS = FOS control, MUC = mucositis, PT = Pretreatment, TT = Total treatment.

for the prevention or treatment of mucositis [10].

In the present study, we demonstrated the beneficial effects of total treatment and pretreatment with 6% FOS in intestinal mucositis induced by 5-FU. Our results showed that the total treatment was more effective than pretreatment in maintaining histological score, morphometric parameters, and sIgA production.

One of the features of mucositis is weight loss and decreased of food intake, probably due to the intestinal cell apoptosis induced by 5-FU, which leads to decreased nutrient absorption and odinophagy [27,28]. Our study showed significant weight loss and food intake reduction in animals that received 5-FU (MUC, PT, and TT groups; Fig. 1). We also observed that FOS supplementation, regardless of the timing of administration, was not able to prevent these alterations. Similarly, Galdino et al. [9], using the same dose of FOS (6%), observed no effect on weight loss and food intake. In contrast, Trindade et al. [29] showed that a higher dose of FOS (550 mg/day) was able to reverse these parameters in the same experimental model. Thus, it is possible that the effects of FOS on weight loss are dose dependent.

Chemotherapy and its side effects, such as mucositis, have been known to alter intestinal barrier decreasing tight junctions (TJs) increasing intestinal permeability which could lead to BT and subsequent activation of inflammatory response [13]. In addition, TJs are also essential for systemic anti-tumor responses [30], and alterations in their properties contribute directly to clinical symptoms of mucositis [31,32]. In the present study, we observed a reduction in the expression of TJs (ZO-1 and occludin; Fig. 2B, C) and increased intestinal permeability and BT in the animals of MUC group (Fig. 2A and Table 1). Here, we evaluated BT by measured of $^{99m}$Tc-*E. coli* uptake on blood and organs of mice. Animals of MUC group showed increased $^{99m}$Tc-*E. coli* in liver, spleen, lungs, and MLN when compared with CTR group. These alterations were probably related to villus height, brush edge breakage and loss of crypt architecture, which were also observed in the ileum of animals in the MUC group (Fig. 3C).

Other important aspect is that 5-FU administration stimulates the release of inflammatory mediators in response to tissue injury [33]. We observed Infiltrate increased neutrophil and eosinophil in the ileum of animals of the MUC group group when compared with the CTR group, similarly to the results found by Galdino et al. [9]. In line with these results, Min-Kyung Song et al. [34] also demonstrated that animals that received 200 mg/Kg of 5-FU showed decreased expression of occludin and claudin-1 when compared with the CTR group. Previous studies performed by our research group also demonstrated that 5-FU administration caused enhance of both IP and bacterial translocation.in mice corroborant with the present results [27,35].

One important finding in our study was that FOS supplementation (PT and TT groups) maintained the expression of TJs and IP within physiological levels (Fig. 2). In addition, a significant reduction in $^{99m}$Tc-*E. coli* uptake was observed in the blood, liver, and spleen (Table 1). These results are in accordance with the data observed by histological and morphometric analysis. FOS supplementation (PT and TT groups) improved mucosal architecture, crypt, and villous damage. In addition, we observed that FOS supplementation, regardless of the timing of administration, was able to reduce the inflammatory infiltrate in the intestine. In the PT and TT groups, there was a reduction in the level of both infiltrates, similar to that observed in the CTR group, indicating that FOS supplementation reduced neutrophil and eosinophil recruitment, as demonstrated by Galdino et al. [9]. These authors also observed IP and intestinal damage reduction with FOS supplementation.

In contrast, we provided combined treatment before and during mucositis (TT group). It is important to emphasize that only the TT group maintained the histological score, villous height, crypt depth, and villi/crypt ratio at physiologic levels. Therefore, this result reinforces our hypothesis that the effects of FOS are time and dose dependent [36]. We hypothesized that the beneficial effects observed with the use of FOS are related to the increased strength of the cytoskeleton promoted by TJs. There are three mechanisms involved in this regulation: activation

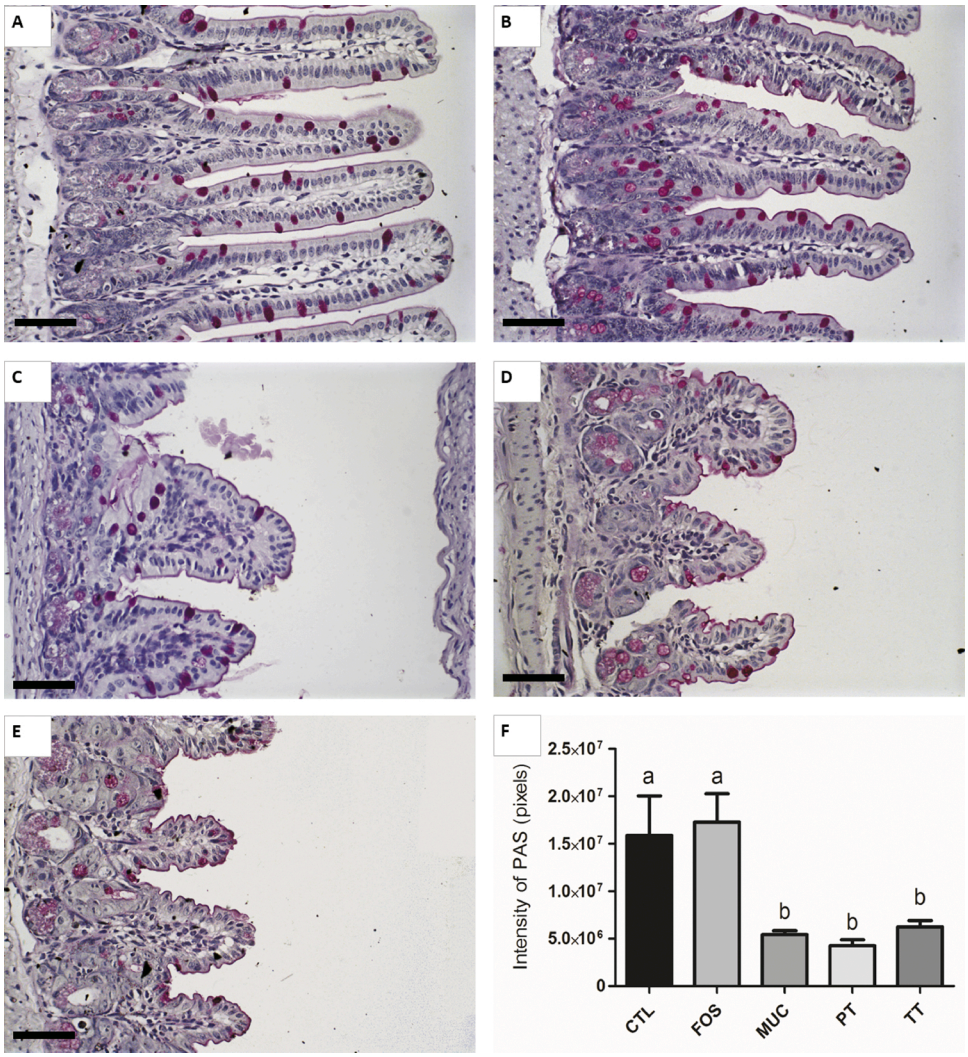


**Fig. 5. Mucus histological analysis.** (A) CTR and (B) FOS groups, Normal aspects of goblet cells and mucus content; (C) MUC and (D) PT group, Number of goblet cells reduced and mucus production reduced; (E) TT group, Preservation of the amount of goblet cells and amount of mucus. (N = 6); (objective 40×; bar =50 μm). (F) Analysis of mucus secretion. Data expressed in mean ± SEM of the intensity of pixels (N = 6). Different letters indicate that there are significant differences ($P < 0.05$; One-way ANOVA and Newman-Keuls Multiple Comparison Test). CTR = control, FOS = FOS control, MUC = mucositis, PT = pretreatment, TT = Total treatment.

of NF-κB via enhancement of inflammatory cytokine concentrations; alteration in post-translational phosphorylation induced by both inflammatory and oxidative stress; and the AMPK pathway, which signals the energetic status cellular [37]. There is evidence that FOS can act on these three pathways since it can modulate the immune system [38] and increase the catalase (antioxidant) levels [9]. In addition, butyrate, produced by fermentation of FOS, activates AMPK, leading to the regulation of TJs [39]. Muanprasat et al. [40] used an *in vitro* model with intestinal cells and showed that addition of prebiotic, chitosan oligosaccharide, was able to induce AMPK activation promoting tight junction assembly. Further analysis will be necessary to evaluate the TJs regulation mechanisms in order to better elucidate the outcomes obtained in the present work.

Other important components of the intestinal barrier are the mucus layer and sIgA production [41,42]. The animals in the MUC group showed a reduction in mucus production in relation to the CTR group. A similar result was observed by Hong et al. [43] in mucositis 5-FU induced in rats. Supplementation with FOS did not induce mucus production. This result is in accordance with Yazbeck et al. [44].

Mucositis induction increased the production of sIgA in the MUC group, as demonstrated by De Jesus et al. [45]. These results are explained by a physiological response of the organism seeking to maintain intestinal homeostasis in response to the loss of mucosal integrity caused by inflammation [46]. However, only TT group could maintain sIgA levels similar to those in the CTR group. Therefore, to obtain beneficial effects with respect to sIgA production, the prebiotic must be present before and after the disease. One probable explanation is that FOS is able to stimulate lactic acid production by bacteria that are related to the increase in sIgA production. Therefore, the supplementation of FOS during mucositis is also necessary to prevent the dysbiosis induced by 5-FU [47,48].

SCFAs, mainly acetate, propionate, and butyrate, have been shown to play important roles in intestinal integrity and the immune system. Another consequence of dysbiosis in mucositis condition is the substantial reduction in SCFA production. The results obtained in our study showed a reduction in the propionate and butyrate concentrations in the MUC group than in the CTR group (Fig. 6B and C).

In contrast, FOS supplementation (PT and TT group) maintained the production of propionate within physiological levels (Fig. 6B) and increased the production of butyrate when compared with MUC group (Fig. 6C). An interesting result was observed in healthy mice supplemented with FOS (FOS group). There was production increased of propionate and butyrate when compared with CTR group (Fig. 6B, C). It is known that FOS can stimulate the production of butyrate-inducing histological and functional changes in the intestinal mucosa, such as cellularity increased and also the number of crypts [49,50]. Furthermore, studies have shown that butyrate improves the intestinal barrier function by increasing the expression of tight junction proteins, such as claudin-1 and ZO-1 [51–54]

Our results showed SCFA concentration increased in the groups that received FOS, suggesting microbiota fermentation. It's known that FOS has a bifidogenic effect (ability to stimulate the growth of

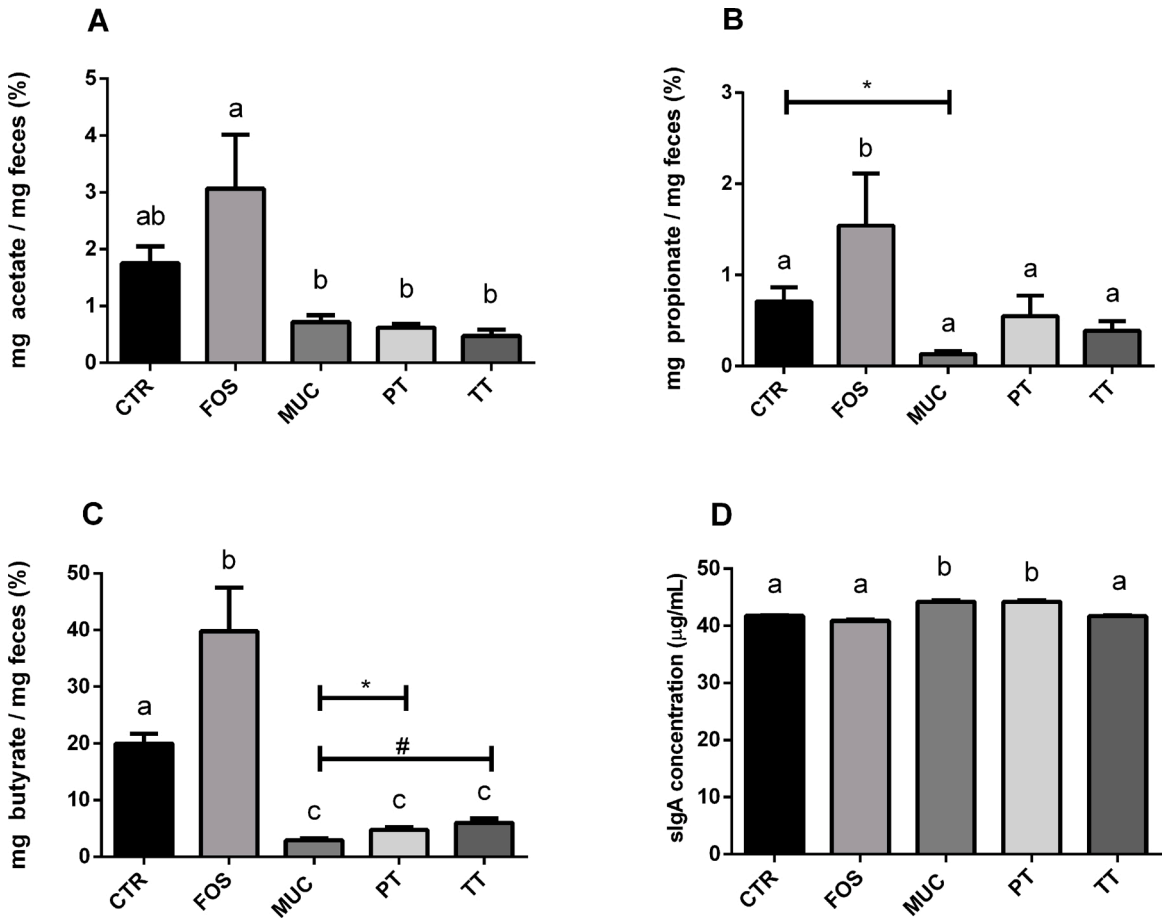


**Fig. 6. Dosage of short-chain fatty acids and sIgA.** (A) acetate concentration, (B) propionate concentration, (C) butyrate concentration, and (D) sIgA. Data expressed in mean ± SEM (N = 8). Different letters indicate that there are significant differences ($P < 0.05$; One-way ANOVA and Newman-Keuls Multiple Comparison Test). The bars represent statistically significant differences between specific groups performed by Student's *t*-test. CTR = control, FOS = FOS control, MUC = mucositis, PT = pretreatment, TT = Total treatment.

bifidobacteria) because of carbohydrates contain bonds that are degraded by the enzyme β-fructosidase that is produced by bifidobacteria, lactobacillus and bacterioidetes [48,55]. Thus, it is possible that FOS may modulate the intestinal microbiota, increasing the SCFA production and improve intestinal barrier function. Other mechanism postulated is that SCFAs bind to SCFAs receptors on GALT immune cells, activating G protein-coupled receptors (GPR), such as GPR41 and GPR43. This binding affects the recruitment of leukocytes to inflammatory sites and the production of proinflammatory cytokines and chemokines [37,38]. Finally, it is believed that one of the main mechanisms by which FOS protects the intestinal barrier in mucositis is related to an increase in the AGCC production, improving the intestinal barrier function and the expression of TJs.

In conclusion, our data showed that pretreatment and total treatment with FOS in mucositis induced by 5-FU had beneficial effects in mouse model of mucositis. These effects included maintenance of TJs expression with reduction in inflammatory infiltrate and histological score, and improvement in SCFA production. These effects contributed to the restoration and preservation of mucosal architecture and, consequently, led to the reduction of IP and BT. The novelty of this study is that total treatment with FOS was more effective in maintaining histological score, morphometric parameters, and sIgA production. In addition, we showed the role of FOS supplementation on TJs in the mucositis induced by 5-FU. In summary, our results showed that the total treatment presented more beneficial effects than the pretreatment, reinforcing the hypothesis raised in this work related to FOS time-dependent action after injury caused by chemotherapeutic agent.

## Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

## Declaration of Competing Interest

The authors report no declarations of interest.

## Acknowledgements

We would like to thank Editage (www.editage.com) for English language editing.

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