Pretreatment and treatment with fructo-oligosaccharides attenuate intestinal mucositis induced by 5-FU in mice

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ABSTRACT

Mucositis is a side effect observed in patients undergoing chemotherapy. Besides, this treatment also leads to the imbalance of the intestinal microbiota. This study evaluated the effects of pre and treatment with fructo-oligosaccharides (FOS) in intestinal mucositis induced by 5-fluorouracil (5-FU). Balb/c mice were divided into four groups: CTL (without mucositis + saline), MUC (mucositis + saline), PT (mucositis + 6% FOS before disease induction) and T (mucositis + 6% FOS after disease induction). Mucositis was induced with an intraperitoneal injection of 300 mg/kg 5-FU. After 72 h intestinal permeability (IP), inflammatory infiltrate, oxidative stress, histological analysis, and short-chain fatty acids (SCFA) production were evaluated. The MUC group showed lost weight, intense inflammatory infiltrate, increased oxidative stress and IP ($P < 0.05$). FOS supplementation attenuated all these parameters ($P < 0.05$). Only pretreatment was able to maintain the acetate and butyrate production. These results showed that FOS supplementation presented protective effects on intestinal barrier function.

1. Introduction

5-Fluorouracil (5-FU) is a chemotherapeutic agent used in the treatment of several carcinomas (Logan et al., 2009; Longley, Harkin & Johnston, 2003). However, this drug may cause damage to the gastrointestinal tract, leading to apoptotic cells as well as increased production of oxygen reactive species and pro-inflammatory cytokines. Consequently, the patients commonly develop side effects such as diarrhea, vomiting, and mucositis (Abdelouhab et al., 2012; Liu et al., 2012; Soares et al., 2008).

Mucositis occurs in approximately 50% of patients undergoing chemotherapy or radiotherapy (Yan Tang et al., 2017). This disease affects patient quality of life and can lead to discontinuation of treatment or reduction of chemotherapeutic doses in cancer therapies, contributing to an increase in health costs, prolonged hospital stays, and impaired nutritional status of patients (Ala et al., 2016; Azevedo et al., 2012).

Currently, there is no effective procedure for prevention or treat

mucositis. The options used, as laser therapy employed to treat oral mucositis, acts only in relieving symptoms, reducing the duration and severity of this pathological situation (Duncan & Grant, 2003). Thus, new therapeutic approaches are needed. It is known that the chemotherapeutic agents reduces the concentration of the commensal bacteria, leading to intestinal microbiota imbalance and, consequently dysfunction of intestinal barrier, events described as inductors of development and progression of mucositis (Yan Tang et al., 2017). Moreover, several studies have investigated the intestinal microbiota, modulation by probiotic, prebiotic, or symbiotic as strategy to improve several disorders affecting the gastrointestinal tract (Belorkar & Gupta, 2016; Flint, Duncan, & Scott, 2007; Rajkumar et al., 2015). In this sense, these biotherapeutic agents could be employed as an interesting strategy to prevent mucositis.

Prebiotics are defined as "selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (Roberfroid, 2007). Microbiota fermentation of prebiotics leads to the production of shorty chain fatty acids (SCFA) mainly, acetate, butyrate and propionate which stimulate the growth of beneficial bacteria's (Reichardt et al., 2017). Besides this, studies have shown trophic and immunological properties due SCFA actions (Comalada et al., 2006; Vieira et al., 2012). Fructo-oligosaccharides (FOS) are polysaccharides that have shown good prebiotic effects by selectively "feeding" some species of *Lactobacillus* and *Bifidobacterium* and reducing the number of other bacteria such as *Bacteroides* and *Clostridium* species and coliform bacteria (Bouhnik et al., 2007; Denipote, Trindade, & Burini, 2010). There are reports showing beneficial effects of FOS in the treatment of intestinal diseases; these effects include an increase in the production of SCFA, as well as reduction in mucosal damage, inflammatory cells infiltration, and body weight loss (Cherbut, Michel, & Lecannu, 2003; Goto et al., 2010; Koleva, Valcheva, Sun, Gänzle, & Dieleman, 2012; Smith et al., 2008).

Based on the above evidence, we hypothesize that pretreatment and/or treatment with FOS would prevent or attenuate inflammatory responses induced by 5-FU. In this context, the aim of the present study was to evaluate the effects of FOS in an experimental model of mucositis induced by 5-FU and investigate possible mechanisms of action involved in the process.

2. Materials and methods

2.1. Animals and experimental design

Thirty two male Balb/c mice weighing 20–25 g were provided by the Animal Care Center at Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais (UFMG). The animals were randomized into four groups: CTL group (without mucositis + saline), MUC group (mucositis + saline), PT group (mucositis + supplementation with FOS before the induction of the disease – 1st until 6th day) and T group (mucositis + supplementation with FOS after the induction of the disease – 7th until 10th day) (Fig. 1). The animals in the PT, and T groups received 240 mg of FOS (6% of total kilocalories), daily, diluted in saline by gavage. The FOS provided was NutraFlora®, GTC Nutrition LLC, Golden, CO, USA.

The animals were housed in cages, subjected to 12 h light–dark cycles and controlled temperature (22 °C ± 2 °C), and allowed free access to commercial ration and water. Body weights and food intake

Fig. 1. Experimental Design.

were recorded daily. This study was approved by the Ethics Committee in Animal Experimentation at the UFMG and complied with the institutional and national guidelines for the care and use of laboratory animals.

2.2. Mucositis induction

Intestinal mucositis was induced as proposed by Maioli et al. (2014) and Generoso et al. (2015). On the 7th day, animals in the MUC, PT, and T groups received an intraperitoneal injection of 300 mg/kg 5-FU (Faldfluor, Libbs, Embu das Artes, São Paulo, Brazil) to induce mucositis. The CTL group received intraperitoneal injection of the same volume of sterile saline. After 72 h, all animals were euthanized under anesthesia for the removal of blood and the small intestine for analyses.

2.3. Intestinal permeability

After 72 h of mucositis induction, all mice received 0.1 mL of diethylenetriamine pentaacetic acid solution labeled with technetium-99 m (99mTc-DTPA) solution containing 18.5 MBq of activity by gavage. After 4 h, all animals were anesthetized, and blood was collected, weighed, and placed in appropriate tubes for radioactivity determination. Blood radioactivity levels were determined using an automatic gamma counter (PerkinElmer Wallac Wizard 1470–020 Gamma Counter; PerkinElmer, Waltham, MA, USA). The data are expressed as % dose, using the following equation: % dose/g = (cpm in g of blood/cpm of standard) × 100, where cpm represents the counts of radioactivity per minute (Andrade et al., 2015).

2.4. Histologic analysis

Ileum segments were processed for histologic analysis, as described previously (Arantes & Nogueira, 1997). The tissues were rolled up and fixed in Bouin's solution. Histological sections (4 μm) were stained with hematoxylin and eosin and mucosal inflammation was assessed using histopathological scores described by Soares et al. (2008). This score evaluates alterations of the mucosal architecture (general structure, cell distribution, mucosa and submucosa aspect), ulcerations and inflammatory infiltration and villus height. The score ranged from 0 (no alteration) to 3 (severe alteration). The histopathological aspects were documented using a microscope (Olympus BX51; Olympus, Tokyo, Japan) and Image-Pro Express 4.0 for Windows software (Media Cybernetic, Bethesda, MD, USA).

2.5. Inflammatory infiltration

The enzyme activities of myeloperoxidase (MPO); N-acetylglucosaminidase (NAG), and eosinophilperoxidase (EPO) were evaluated in the ileum, as described previously (Leonel et al., 2013). The protein content of the samples was determined according to the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). After protein quantification, the results obtained for the enzyme activities of NAG, MPO, and EPO were corrected and expressed as per mg protein.

2.6. Oxidative stress

Fragments of ileum were homogenized in a high-speed homogenizer with 1 mL of cold phosphate-buffered saline and centrifuged at 9000g for 10 min at room temperature. The supernatant was separated for analysis of thiobarbituric acid-reactive species (TBARS), hydroperoxide concentration, superoxide dismutase (SOD), and catalase activity (Leocádio et al., 2015; Leonel et al., 2013). All results were normalized to total protein concentration. Protein concentration was analyzed using the Lowry method (Lowry et al., 1951).
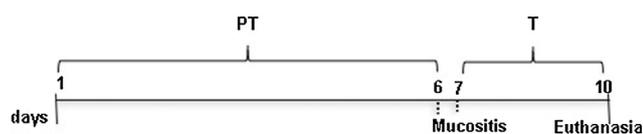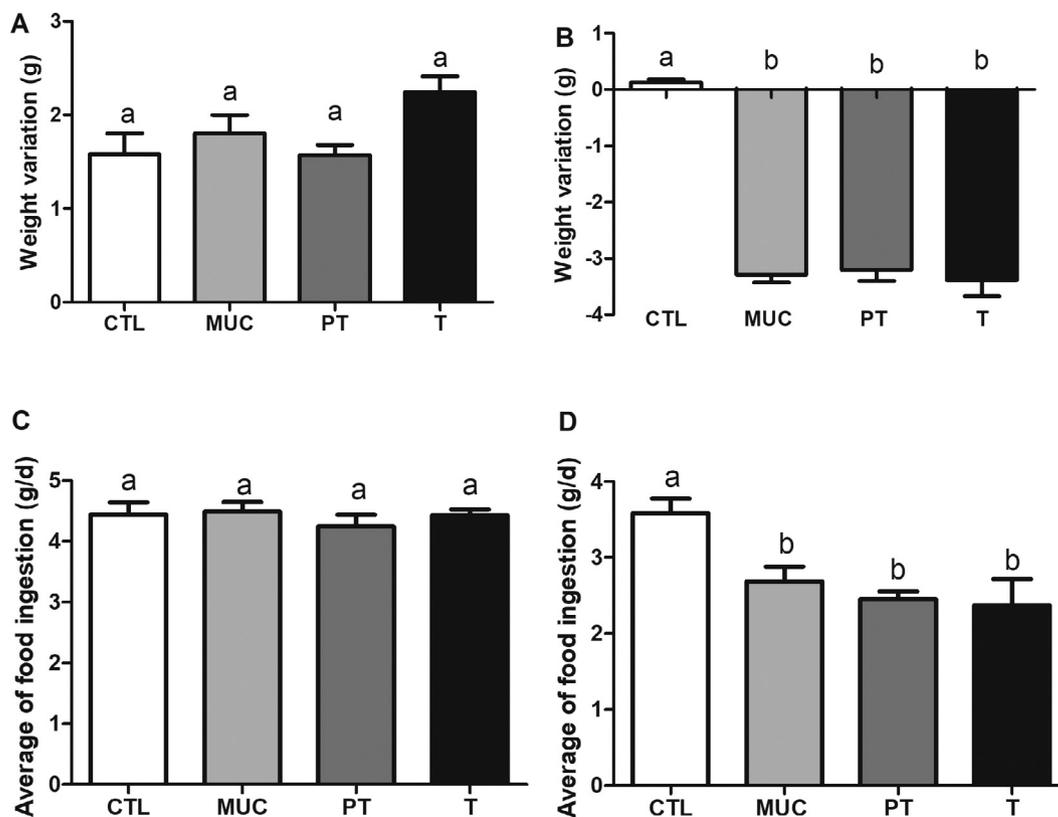
Fig. 2. Variation of food consumption and weight before induction of mucositis (A; C) and after induction of mucositis (B; D). Data are expressed as mean ± SEM (n = 10). Different letters indicate statistical significance ($P < 0.05$).
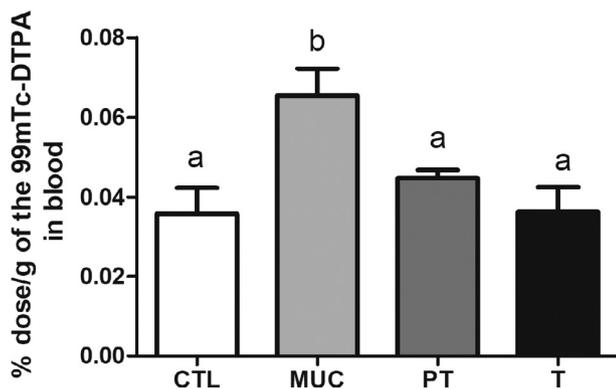

Fig. 3. Intestinal permeability 72 h after induction of mucositis. Data are expressed as mean ± SEM (n = 8). Different letters indicate statistical significance ($P < 0.05$).

2.7. SCFA analysis

Animal feces were collected on 10th day to evaluate acetate and butyrate. The analyses were performed in duplicate, as proposed by Smiricky-Tjardes, Grieshop, Flickinger, Bauer & Fahey (2003). The analyses were performed in a gas chromatograph (CGMS – QP 5000, Shimadzu ®, Japan) coupled to a microcomputer equipped with a detector for recording the analysis of the chromatograms using the GC Solution program (Shimadzu ®, Japan). The respective acids were separated and identified on a Nukol capillary column (30 m × 0.25 mm × 0.01 mm; Sigma-Aldrich ®, USA). For a chromatographic retreat, 1 mL of sample was injected with a 10 mL syringe (Hamilton®) in a Splitless system in the SIM mode (Ion Monitoring System). Helium gas was used as carrier with linear velocity programmed to 38.5 cm/s. The injector and detector temperatures were

200 °C and 220 °C, respectively. The mass was scanned from 40 to 400 m/z. The flow of the mobile phase in the column was 1.1 mL/min.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad, Inc., La Jolla, CA, USA). 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 histological score (nonparametric distribution), which was analyzed with the Kruskal–Wallis and Dunn's tests. The level of significance was set at < 0.05.

3. Results

3.1. Food consumption and weight variation

After mucositis induction, animals in the MUC, PT, and T (2.69 ± 0.19 g/day; 2.37 ± 0.35 g/day; 2.45 ± 0.10 g/day, respectively) groups showed a significant reduction in food intake compared to the CTL group (3.59 ± 0.19 g/day) ($P < 0.05$; Fig. 2). Furthermore, the weight loss was increased in the MUC (− 3.29 ± 0.14 g), PT (− 3.20 ± 0.20 g) and T (− 3.37 ± 0.28 g) groups compared to the animals in the CTL (0.13 ± 0.05 g) ($P < 0.05$; Fig. 2).

3.2. FOS enhances intestinal permeability and ileum histology

Results showed increased intestinal permeability (IP) (Fig. 3), reduction in the villus height, presence of tissue damage, inflammatory cells and ulcerations in the ileum of animals of MUC group compared with animals of all groups (Fig. 4; $P < 0.05$).

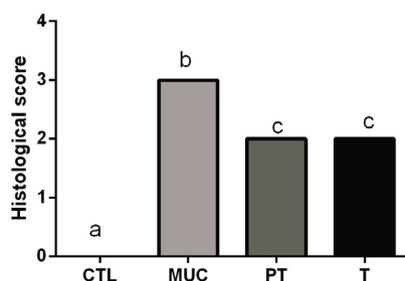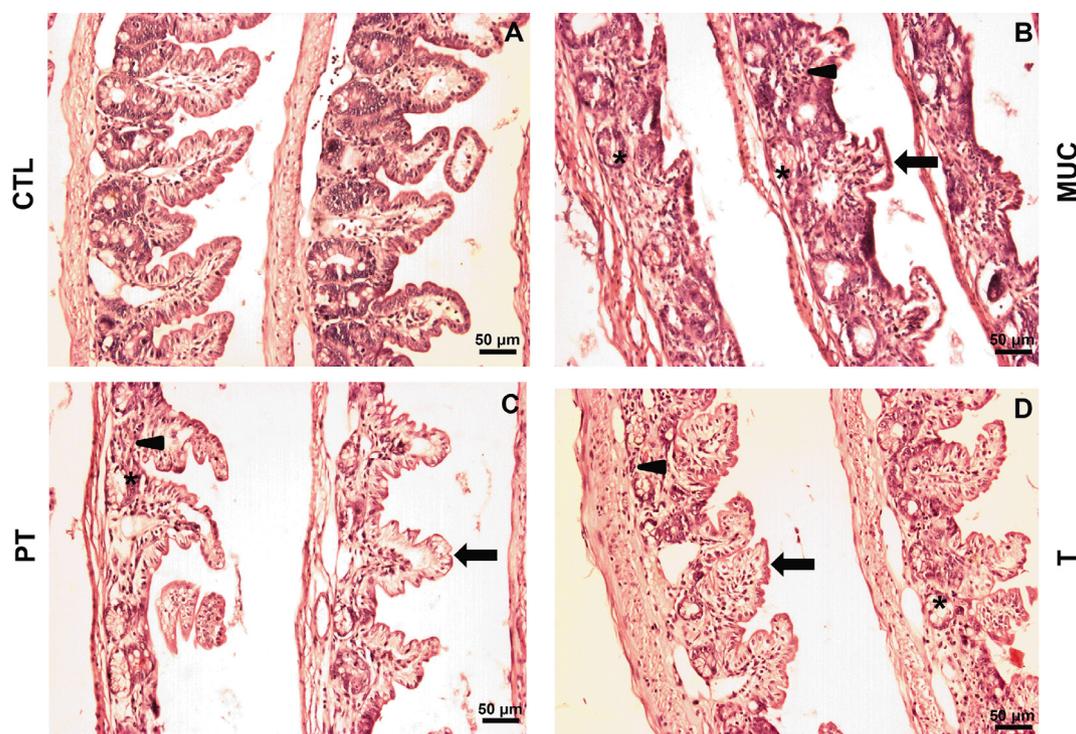In the groups with mucositis who received FOS (PT and T), the data

Fig. 4. Histological analysis of ileum. Normal aspects for CTL (A) group. Shortened villi (arrows), intense infiltration of inflammatory cells (arrowhead), and necrosis of crypt (asterisks) are present in the mucositis (C) groups. Partial preservation of villi (crypts) and crypts (asterisks) and presence of inflammatory cells (tips of arrows) are observed in the PT or T groups (D and E, respectively). Bar = 50 μM, hematoxylin and eosin staining. In the histological score, the data are expressed as mean ± SEM (n = 5). Different letters indicate statistical significance ($P < 0.05$).

showed that FOS supplementation maintained the IP at physiologic levels (Fig. 3). In addition, less tissue damage and better preservation of the villus height were observed in the PT and T groups (Fig. 4). The histopathological changes were confirmed by the scoring system; the MUC group presented a score of 3, indicating severe alteration. The PT and T groups showed an intermediate score (score of 2) between the CTL and MUC groups (Fig. 4).

3.3. Effects of FOS on inflammatory infiltrate and oxidative stress

MPO and EPO activity increased in the MUC group ($P < 0.05$) and was significantly reduced by FOS in the PT and T groups ($P < 0.05$). There was no difference observed in the NAG activity ($P > 0.05$) (Fig. 5).

Lipid peroxidation and the hydroperoxide concentration in the ileum were similar in animals of all groups ($P > 0.05$). The activity of the SOD enzyme was lower in the MUC, PT, and T groups when compared with the CTL group ($P < 0.05$). FOS supplementation increased the activity of the catalase enzyme in the PT and T groups ($P < 0.05$) when compared with the MUC group (Fig. 6).

3.4. Influence of FOS on SCFA concentration

The acetate and butyrate concentrations were higher in the PT group (5.47 ± 0.61 mg and 6.07 ± 0.26 mg, respectively) than in the MUC group (3.01 ± 0.20 mg and 2.75 ± 0.38 mg, respectively) ($P < 0.05$). However, the acetate and butyrate concentrations in the T group (2.79 ± 0.40 mg and 2.10 ± 0.34 mg, respectively) were not significantly different from the MUC group ($P > 0.05$) (Fig. 7).

4. Discussion

In the present study, we demonstrated the beneficial effect of FOS in an experimental model of 5-FU-induced mucositis. The results showed decreased IP and inflammatory infiltrate, partial preservation of the intestinal epithelium, and increased antioxidant action in animals supplemented with this prebiotic.

Chemotherapy-induced mucositis is a limiting factor in cancer therapy. The mucositis reaches the cells of the gastrointestinal mucosa, causing ulcerations and debilitating symptoms as the reduced intake and lose weight (Soares et al., 2008; Sonis, 2004). In the current study, we observed significant weight loss and reduction in food intake in the MUC, PT, and T groups after disease induction. The similar results were observed by Leocádio et al. (2015), Generoso et al. (2015), and Barros
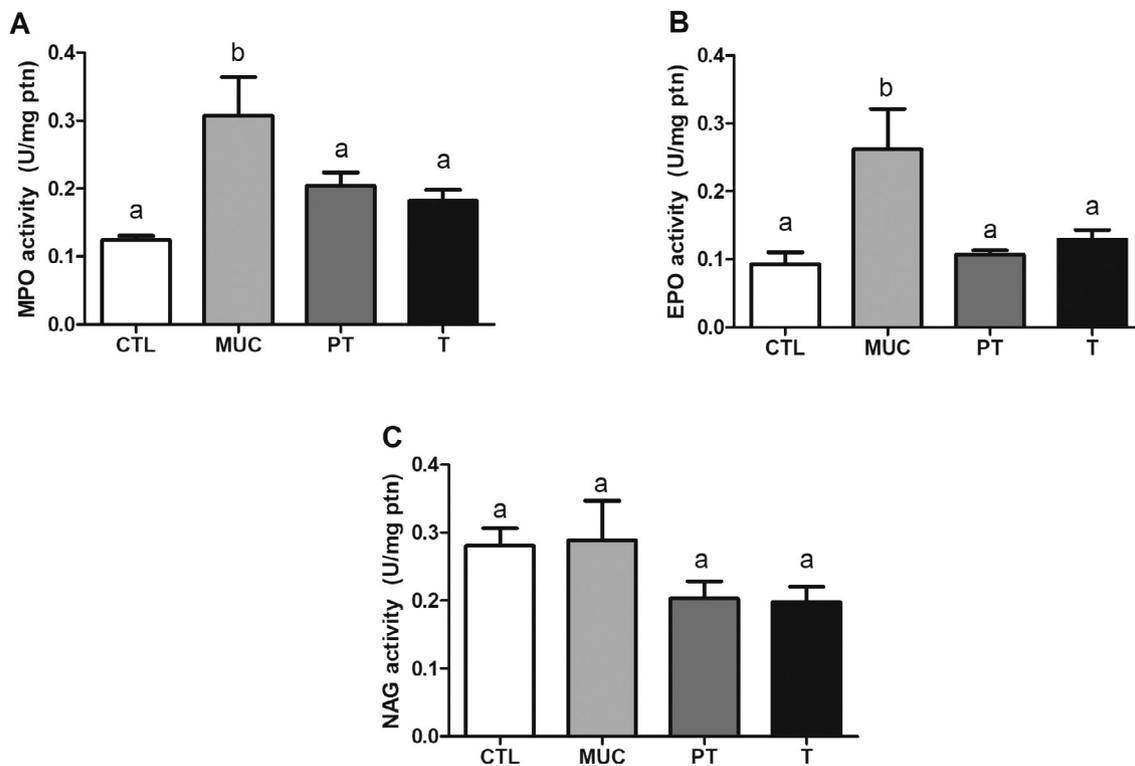
Fig. 5. Inflammatory infiltrate in the ileum. (A) Neutrophil Infiltrate (MPO), (B) Eosinophil infiltrate (EPO), and (C) Macrophages infiltrate (NAG). Data are expressed as mean ± SEM (n = 6). Different letters indicate statistical significance (*P* < 0.05). MPO, myeloperoxidase; EPO, eosinophilperoxidase; NAG, N-acetylglucosaminidase.
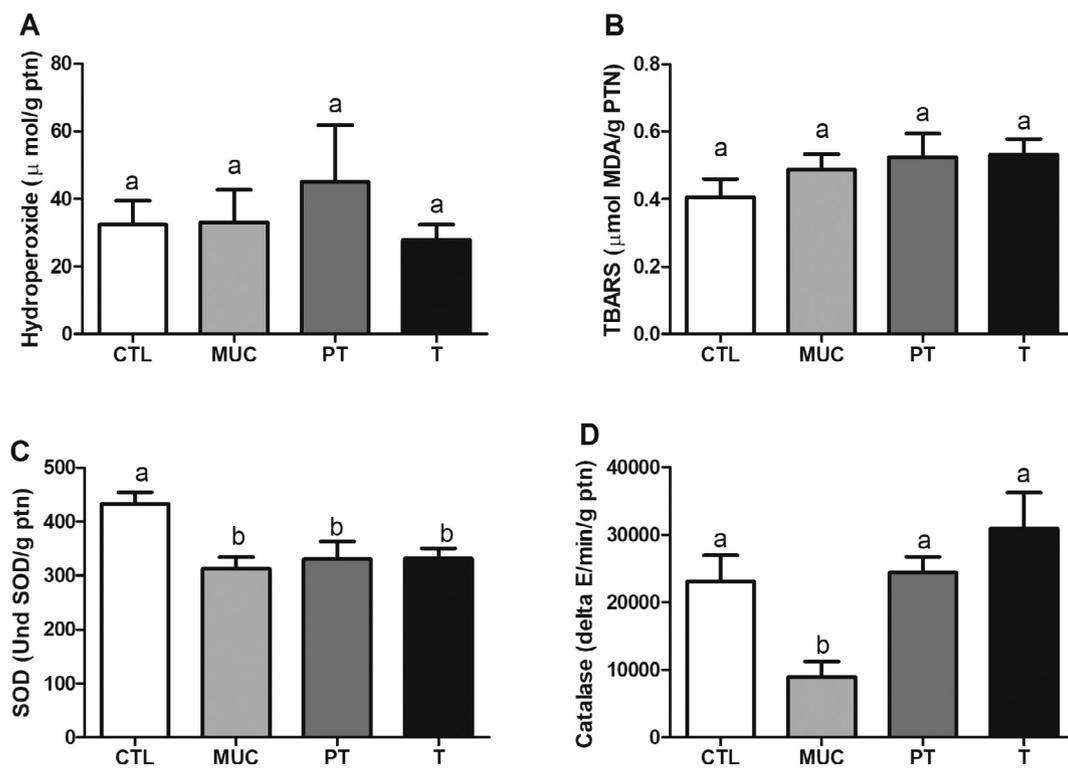

Fig. 6. Evaluation of oxidative stress in the ileum. (A) Concentration of hydroperoxides, (B) Concentration of TBARS, (C) Concentration of SOD, (D) Concentration of catalase. Data are expressed as mean ± SEM (n = 6). Different letters indicate statistical significance (*P* < 0.05). TBARS, thiobarbituric acid-reactive species; SOD, superoxide dismutase.

et al. (2016) in animals that received 5-FU. However, the pretreatment and treatment with FOS employing 240 mg per animal/day did not improve food intake and weight loss. A study performed by our research group showed that 550 mg of FOS per animal/day was able to attenuate weight loss indicating that this effect is dose dependent (Trindade et al., 2018).
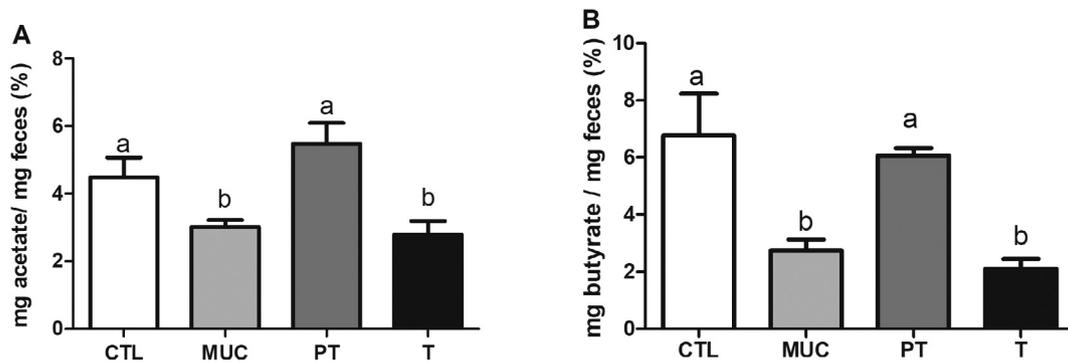
Fig. 7. Dosage of short-chain fatty acids in feces. (A) Concentration of acetate in feces, (B) Concentration of butyrate in feces. Data are expressed as mean $\pm$ SEM (n = 8). Different letters indicate statistical significance ($P < 0.05$).

Mucositis also can cause changes in intestinal mucosal integrity and create barrier dysfunction, resulting in increased IP to toxins and pathogens, leading to bacterial translocation and several inflammatory reactions (Rao & Samak, 2013). In this study, IP was evaluated by measuring blood radioactivity after the oral intake of ^{99m}Tc-DTPA. ^{99m}Tc-DTPA is a hydrophilic macromolecule that rarely crosses the intestinal barrier under physiological conditions; however, when the paracellular IP is increased because of the mucosal lesion affecting the tight junctions' proteins, the presence of DTPA in the bloodstream can be detected in greater quantity (Andrade et al., 2015). Our results showed increased percentage of ^{99m}Tc-DTPA in the blood of animals in the MUC group compared with other groups. Previous studies performed by our research group also demonstrated an increase in IP after administration of 5-FU (Antunes et al., 2015; Generoso et al., 2015). Increased IP correlates with intestinal architecture impairment, crypt necrosis, intense inflammatory infiltrate, and edema as observed in the MUC group. It is known that the inflammatory infiltrate, characterized by the presence of neutrophils and eosinophils, can promote changes in the tight junctions' proteins and consequently increasing IP (Maeda et al. 2010). In this sense, we observed a greater neutrophil and eosinophil infiltration in the MUC group. The same was observed by Azevedo et al. (2012) in mice with 5-FU-induced mucositis. Leocádio et al. (2015) also observed significantly higher EPO in a model of 5-FU-induced mucositis.

On the other hand, the supplementation with FOS (PT and T groups) showed reduced of IP and also of the MPO and EPO enzymes activity. This improvement was likely due to the observed reduction in the intensity and extent of inflammation and also the preservation of mucosal architecture, which contributed to the reduction of IP in the PT and T groups. Considering the participation of these cells in the increase of inflammation and oxidative stress, these results suggest anti-inflammatory effects of FOS.

In addition, the oxidative stress was also evaluated, due to its importance in the initiation of mucositis (Maeda et al., 2010; Sonis, 2004). In this study, there were no differences observed to lipid peroxidation and hydroperoxide concentration in all investigated groups. Maeda et al. (2010) reported that the increase in lipid peroxidation occurs in the period between 24 h and 48 h after the induction of mucositis. Thus, the results obtained in the present study might be partially explained by the experimental design that assessed mucositis at 72 h after its induction.

The enzymes SOD and catalase also were analyzed once these are part of the body's antioxidant system, acting as important regulators of reactive oxygen species (ROS) (Afonso, Champy, Mitrovic, Collin, & Lomri, 2007; Powers & Jackson, 2008). In the analysis of the SOD enzyme, MUC, PT, and T groups showed decreased activity compared with the CTL group. These results are in agreement with the findings of Justino et al. (2014), who demonstrated that 5-FU can reduce the activity of antioxidant enzymes. In addition, the analysis of the catalase

enzyme showed reduced activity only in animals of the MUC group. Catalase has several biochemical functions, but its main objective is to catalyze the reaction of transformation of hydrogen peroxide into water and oxygen (Afonso et al., 2007; Powers & Jackson, 2008). FOS supplementation increased the activity of the catalase enzyme in the PT and T groups, and these results corroborated with the histological analysis, which showed more preserved intestinal crypts and a lower presence of inflammatory cells than the MUC group. The results suggest that FOS supplementation in mucositis can improve the cellular metabolism, preserving catalase content and exerting antioxidant properties. In agreement with our results, Yen, Kuo, Tseng, Lee, & Chen (2011) demonstrated that FOS supplementation presented antioxidant action in constipated elderly patients, associating this effect with its bifidogenic action. In addition, Franco-Robles and Lopez (2015) reported that fructans, such as FOS, have the ability to eliminate ROS.

Studies have shown that SCFA, mainly acetate, propionate, and butyrate, can restoring the intestinal mucosa after injury (Ferreira et al., 2012; Vieira et al., 2012). SCFA improves mucin production in the colon, contributing to trophic effects on the large intestine through the direct contact of acids in the colonic mucosa, and stimulating DNA, RNA, and protein synthesis (Comalada et al., 2006; Franco-Robles & Lopez, 2015). Our data showed physiological levels of acetate and butyrate in PT group compared to MUC group, indicating that the pretreatment was capable to maintain the microbiota balance like to control. It is known that FOS has potential benefits in the prevention and treatment of intestinal diseases and can act modulating the intestinal microbiota, stimulating the commensal bifidobacteria. In several studies in humans and animals, FOS supplementation increased the number of beneficial bacteria, such as lactobacilli and bifidobacteria, and decreased potential pathogens (Bali, Panesar, Bera, & Panesar, 2015; Bornet, Brounsl, Tashiro, & Duvillier, 2002; Caetano et al., 2016; Koleva, Ketabi, Valcheva, Gänzle, & Dieleman, 2014). In a study conducted by Bouhnik et al. (1999) with healthy volunteers that received FOS supplementation for 7 days showed bifidobacteria increased counts compared with volunteers that received placebo on 8th day. In contrast to the PT group, the T group showed decreased of SCFA levels when compared with CTL group. The production of SCFA is determined by different factors, including the number and composition of the microbiota, substrate type, intestinal transit time (Wong & Jenkins, 2007) and initial pH (Reichardt et al., 2017). It is possible that these factors contributed to the different values found of SCFA. Another factor that could influence these results is the time of supplementation between PT and T groups. Le Blay, Michel, Blottière, & Cherbut (1999) demonstrated in a study with rats fed with diet added with 9% FOS that the fermentation products and microbiota population differed considerably depending on the period of ingestion. Another factor to be considered is that when the T group animals received the 5-FU administration, their intestinal microbiota had not yet been modulated by FOS as possibly occurred in the PT group. In addition, 5-FU substantially decreases the

number and diversity of the microbiota (Fijlstra, Ferdous, & Koning, 2015).

5. Conclusion

The present results showed that pre and treatment with FOS, in experimental mucositis, reduced inflammatory infiltrate and IP, preserved the intestinal mucosa and increased the catalase levels. However, only pretreatment was able to maintain the acetate and butyrate production at physiological levels. Therefore, the results suggest that FOS supplementation could be an important adjuvant in the prevention and treatment of mucositis.

6. Ethics statements file

This study was approved by the Ethics Committee on Animal Use at Federal University of Minas Gerais (CEUA/UFMG) and complies with the institutional and national guidelines for the care and use of laboratory animals.

Conflicts of interest

None declared.

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