The importance of oxygen free radicals in the etiopathogenesis of diversion colitis in rats[1]

A importância dos radicais livres de oxigênio na etiopatogenia da colite de exclusão em ratos

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ABSTRACT
Purpose: Quantify the levels of oxidative DNA damage of epithelial colon cells comparing segments with and without fecal stream. **Methods**: Sixty Wistar rats were subjected to deviation of fecal stream by proximal colostomy and a distal mucosal fistula. Animals were divided into three experimental groups that were sacrificed 6, 12 and 24 weeks after surgery. In each experimental group, five animals underwent laparotomy without intestinal deviation (sham subgroup). The diagnosis of colitis was made by histopathological analysis and the inflammatory activity index by graduated scale. The neutrophil infiltration was determined by myeloperoxidase tissue levels and the intensity of oxidative DNA damage by comet assay. The Mann-Withney and Student t test were used to compare the results among experimental subgroups and the Kruskal-Wallis test for variance analysis, adopting a significance level of 5% ($p \leq 0.05$). **Results**: Colon segments without fecal stream was shown higher histological inflammatory score of the colon wall after 12 and 24 weeks ($p=0.001$) that increased with the time of diversion ($p=0.01$). The activity of myeloperoxidase in segments without fecal stream decreased with the time ($p=0.001$). Oxidative DNA damage levels were significantly higher in the segments without fecal stream, ($p=0.0001$), independent of time of colon diversion, and increase with the time ($p=0.0007$). **Conclusions**: Colon segments without fecal stream showed high levels of oxidative DNA damage related to histological alterations observed in diversion colitis. The levels of oxidative DNA damage in segments devoid of the fecal stream increase with the time of intestinal exclusion.
Key words: Colitis. Colostomy. DNA Damage. Oxidative Stress. Comet Assay. Fatty Acids, Volatile. Rats.

RESUMO
Objetivo: Quantificar os níveis de dano oxidativo ao DNA em células epiteliais da mucosa cólica comparando segmentos com e sem trânsito fecal. **Métodos**: Sessenta ratos Wistar foram submetidos à derivação do trânsito intestinal por colostomia proximal e fístula mucosa distal. Os animais foram divididos em três grupos experimentais segundo terem sido sacrificados 6, 12 e 24 semanas após a cirurgia. Em cada grupo experimental, cinco animais foram submetidos à laparotomia isolada sem derivação fecal (grupo sham). O diagnóstico de colite foi estabelecido por análise histopatológica e o índice de atividade inflamatória por escala graduada. A infiltração neutrofílica foi determinada pelos níveis teciduais da mieloperoxidase e a intensidade do dano oxidativo ao DNA pelo ensaio em cometa. Utilizaram-se os testes de Mann-Withney e o teste t de Student para comparar os resultados encontrados entre os subgrupos experimentais e o teste de Kruskal-Wallis para análise de variância, adotando-se nível de significância de 5% ($p \leq 0,05$). **Resultados**: Os segmentos cólicos, sem trânsito fecal apresentaram maior escore histológico de inflamação após 12 e 24 semanas ($p=0,001$), que aumentou com o tempo de derivação ($p=0,01$). A atividade da mieloperoxidase nos segmentos sem trânsito fecal diminuiu com o progredir do tempo ($p=0,001$). Os níveis de dano oxidativo ao DNA foram significativamente maiores nos segmentos sem trânsito fecal ($p=0,0001$), independente do tempo de exclusão considerado, aumentando com o progredir do tempo de exclusão ($p = 0,0007$). **Conclusões**: Segmentos cólicos desprovidos de trânsito fecal apresentam níveis elevados de dano oxidativo ao DNA relacionados às alterações histológicas observadas na colite de exclusão. Os níveis de dano oxidativo ao DNA nos segmentos desprovidos de trânsito fecal aumentam com o decorrer do tempo de exclusão.
Descritores: Colite. Colostomia. Dano ao DNA. Estresse Oxidativo. Ensaio em Cometa. Ácidos Graxos Voláteis. Ratos.

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Introduction

Glotzer *et al.*[1], were the first to describe the development of an inflammatory process in the mucosa of colon without a fecal stream This new form of inflammatory bowel disease (IBD) is called diversion colitis (DC). Various theories regarding the pathogenesis of DC have been proposed but most of authors believe that DC arises due to the absence of short chain fatty-acids (SCFAs) in the intestinal lumen due to the diversion of the fecal stream[2,3,4,5]. However, the molecular mechanism by which SCFAs deficiency causes epithelial aggression has been not been studied in experimental models of DC.

Studies have demonstrated the importance of SCFAs in the prevention and treatment of patients with DC[6,7,8,9,10]. SCFAs are formed by the fermentation of dietary fiber by anaerobic bacteria in the intestinal lumen and represent the main energy substrate for colonic epithelial cells[7,8]. The diversion of the fecal stream prevents the passage of SCFAs through the excluded segment. The epithelial cells in the segment are deprived of their main nutritional fuel and begin to use the glutamine offered by the systemic circulation, as an alternative energy source[11]. However, this alternative supply seems to be unable to provide all of the energy necessary to sustain the metabolism of the colonic epithelial cells, causing significant changes in the oxidative metabolism of the mitochondrial respiratory chain of these cells.

Epidemiological and experimental studies support the involvement of SCFAs in colon physiology and a protective role for butyrate in IBD and colorectal carcinogenesis[12,13]. Antioxidant activity has recently been suggested as a possible mechanism by which SCFAs, mainly butyrate, may exert its anti-carcinogenic effects[12,13]. The reported protective effect of butyrate might be important in pathogenetic mechanisms mediated by reactive oxygen species (ROS), and helps to explain the apparent ability of dietary fiber to protect against IBD and colorectal cancer[12,13].

Recently, the radical induction theory was proposed to explain the phenomena preceding the initial trigger and infiltration of inflammatory mucosa in patients with ulcerative colitis (UC)[14]. This theory proposes that the initial attack on the intestinal mucosa is due to oxidative stress in the epithelial cells caused by changes in their oxidative energy metabolism. ROS are toxic to cells and their overproduction causes breakage of the various lines of defense that make up the mucosal barrier, represented by the layer of mucus covering the epithelium, the cytoplasm membrane, the intercellular junction's complex and the basal membrane[14]. This allows bacteria of the colon lumen to migrate to the sterile submucosa[15]. In an attempt to combat this bacterial infiltration, polymorphonuclear (PMN) cells, mainly neutrophils, migrate to the intestinal wall, leading to the inflammatory process characteristic of the disease. Reinforcing the possibility that ROS can damage the colonic epithelium, it has already been shown that the administration of enemas with hydrogen peroxide (H_2O_2), a potent donor of ROS, can cause the appearance of severe colitis in the mucosa of the normal colon[16].

Studies have shown that oxidative stress and oxidative DNA damage are hallmarks of UC and probably play key roles in the pathogenesis of this disease and the associated carcinogenesis[12,14]. DC has clinical, macroscopic and microscopic features similar to those of UC. Despite being regarded as clinically distinct entities, DC and UC have several similar aspects in terms of endoscopic and histological features as well as therapeutic approaches[17]. The common denominator of both diseases is an intense chronic inflammatory process in the colonic mucosa that is aggravated with the duration of the disease. It is possible that the etiopathogenic mechanisms of the two diseases are also similar, since the chronic inflammation of the intestinal mucosa is common feature of both diseases. More strikingly, the infusion of antioxidant drugs such as 5-aminosalicylic (5-ASA), nutritional solutions and SCFAs is effective in treating both diseases[18,19].

Although some studies have suggested a relationship between oxidative stress and the initial stages of chronic inflammatory injury to intestinal mucosa in patients with UC, to the best of our knowledge, only one experimental study evaluated the presence of oxidative stress comparing TNBS-induced colitis with DC[20]. In the light of these considerations, it seems prudent to confirm whether the diversion of the fecal stream can enhance overproduction of ROS and inflammatory changes to the colonic epithelium similar to those found in UC. Thus, the aim of the present study was evaluated the importance of the oxidative stress in the damage of colon epithelium in experimental model of DC.

Methods

This study was conducted in accordance with Federal Law No. 11,794 and the guidelines of the Brazilian College for Animal Experimentation (COBEA). The Research Ethics Committee of São Francisco University, Bragança Paulista, approved this experimental study (Process nº 22-11/2007).

Sixty SPF (specific pathogen-free) male Wistar rats (*Rattus norvegicus Bakenhouff*) weighing between 300 and 350 grams were used. The rats were obtained from the Multidisciplinary Center for Biological Investigations of the State University of Campinas (CEMIB-UNICAMP). The animals were distributed randomly into three groups of 20 rats each, according to the sacrifice date: 6, 12 or 24 weeks after the operation. Each group was divided into two subgroups. The first (experimental) subgroup consisted of 15 animals that underwent deviation of the colonic transit; the second subgroup (sham operated) consisted of five animals that underwent laparotomy and intestinal manipulation for 20 minutes without transit deviation.

Surgical technique: diversion of the fecal stream

Rats were placed under general anesthesia by intra-muscular administration of 0.1 ml/100 g of a 1:1 (v/v) of a ketamine (50 mg/ml) and xylazine (20 mg/ml) solution mixture. The abdomen was shaved and a 5-cm-long midline incision was made. In animals submitted to diversion of the fecal stream (15 rats in each group), the left colon was sectioned and exteriorized in its mid portion, corresponding to the sigmoid and approximately 3 cm above the Peyer's lymphoid patch. Two circular skin pellets 3 mm in diameter and 2 cm apart were made in the left flank of the animal at the same vertical level. The proximal end of the colon was exteriorized through the anterior cutaneous orifice. The distal segment of the sectioned large intestine was catheterized with a 12F polyvinyl catheter attached by ligature with 3-0 cotton thread,

after which it was irrigated with 0.9% physiological serum until the effluent drained through the animal's anus no longer presented fecal material. After irrigation, the catheter was removed and the distal colon was brought to the exterior as a colostomy on the lower left lateral face of the abdominal wall. The proximal end and the distal stoma were fixed to the skin with full-thickness sutures of polypropylene 5-0. The abdominal incision was closed in two layers: the peritoneum and aponeurosis were closed with polyglycolic 4-0 and the skin with nylon 4-0. In essence, two colostomies were performed, the second of which is a mucus fistula. In animals of the control group (sham operated), the abdominal wall was closed with the same technique after colon manipulation. The rats were maintained in individual cages without particular care for the stomas or abdominal incisions.

Sample collection

Animals were anesthetized as described above at 6, 12 and 24 weeks after the surgical procedure and the colons were quickly removed to obtain samples. Animals were shaved again, and the midline incision was repeated. In the control group, 40 mm of sigmoid colon was removed. In the colostomy group, specimens were taken from the excluded (distal colon) and non-excluded segments (proximal colon). The specimens, measuring approximately 4.0 cm, were dissected and longitudinally opened through the anti-mesenteric border. Longitudinal fragments of the intestinal wall were obtained and randomly distributed to different assays.

Histological analysis

The colon segments for histological analysis were always collected from standardized locations from all animals in the experimental and control subgroups. The colon segments were laid out on a flat cork surface, secured with the mucosal surface facing up and fixed in a 10% formaldehyde solution for three days. After fixation, the tissues were dehydrated using successively increasing concentrations of alcohol and clarified in xylene. The material was embedded in paraffin and cut into 5 µm thick longitudinal sections. The slides were stained with hematoxylin-eosin and subjected to histological analysis under an optical microscope at a final magnification of 200× to confirm the presence of colitis. A pathologist with experience in diagnosing colorectal diseases but unaware of the objectives of this study performed the analysis of the slides. The diagnosis of diversion colitis was based on the presence of erosions or ulcers, crypt atrophy, edema, vascular congestion, PNM infiltration in the colonic mucosa and presence of nodular lymphoid hyperplasia. The histological inflammatory activity index (HAI) was graduated using a previous proposed scale adapted to DC[21]. The degree of inflammation of each slide was scored as follow: absent (0), no crypt atrophy, no epithelial infiltration by PMN, no erosions or ulcers; low (1-3), no epithelial erosions or ulcers, slight atrophy of crypts and PMN of <50% five contiguous crypts or <50% of three different analyzed fields; moderate (3-6), no epithelial erosions or ulcers, severe atrophy of colon crypts and PMN infiltration of >50% five colonic crypts or >51% of three different analyzed fields; severe (7-8) epithelial erosion or ulceration, irrespective of the other features.

Myeloperoxidase (MPO) activity in the colon tissues

The presence of PMN infiltration was evaluated by determination of the tissue MPO levels[22]. Colon samples were homogenized in 0.5% (w/v) hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0. For the MPO assay, 50 mL of each sample was added to 200 mL of o-dianisidine solution (0.167 mg/mL o-dianisidine dihydrochloride, 0.0005% hydrogen peroxide in 50 mM phosphate buffer, pH 6.0). Immediately after the samples were mixed, the change in absorbance was read at 460 nm over 5 min using a microplate reader. MPO was assessed as an index of neutrophil infiltration.

DNA damage assessed by comet assay

The levels of ROS in the tissue were determined indirectly by the alkaline electrophoresis of an isolated cell (comet assay). Cells from colon biopsies were isolated as described[23]. Briefly, the biopsies were pooled and incubated with 5.5 mg proteinase K (Sigma-Aldrich) and 4 mg collagenase I (Invitrogen Life Technologies, Grand Island, N.Y., USA) in 3 ml Hank's balanced salt solution (HBSS) for 45 min at 37ºC to free the cells; the cells were then resuspended in 10 ml HBSS. The resulting suspensions were centrifuged at 750 × g for 5 min and the supernatant was discarded. Since the comet assay is only accurate for samples with a cell viability of more than 75%, cell viability was determined using the fluorescein-diacetate/ethidium bromide (FDA/EtBr) assay[24]. The alkaline comet assay was performed according to Singh *et al.*[25] with some modifications. Briefly, a 15 ml single-cell suspension (2 x 10^4 cells) was mixed with molten 0.5% low-melting-point agarose and spread on agarose-precoated microscope slides. The slides were immersed overnight at 4ºC in freshly-prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium salt N-Lauryl sarcosine, pH 10, with 1% Triton X-100 and 10% DMSO). Subsequently, the cells were exposed to alkaline buffer (1 mM EDTA and 300 mM NaOH, pH 13.4) at 4ºC for 40 min to allow DNA unwinding and exposure of alkali-labile sites. Electrophoresis was then performed on the same solution. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with 40 ml EtBr (20 mg/ml) and analyzed with a fluorescence microscope equipped with an image analysis system (Komet 5.5; Kinetic Imaging, Nottingham, UK). Four hundred randomly selected cells (200 from each of two replicate slides) were evaluated from each sample and the mean of the tail moment was determined. Tail moment (*TM*) is defined as the product of DNA in the tail and the mean distance of migration in the tail and is calculated by multiplying tail intensity and the sum comet intensity, according to the tail center of gravity-peak position (Figure 1).

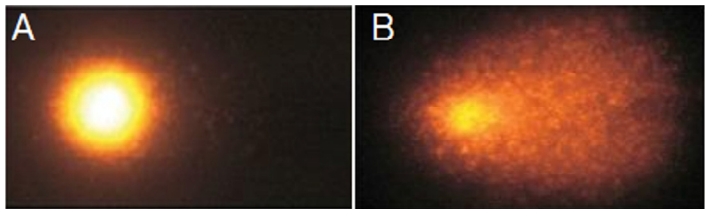

FIGURE 1 – Oxidative DNA damage (*TM)* by alkaline electrophoresis of a single cell (comet assay). **A.** Cell without oxidative DNA damage; **B.** Cell with severe oxidative DNA damage.

The comet assay was only performed in samples that showed cell viability greater than 75%. Cell viability was determined with FDA/EtBr staining. Briefly, a cell staining solution containing 30 ml of FDA in acetone (5 mg/ml), 200 ml of EtBr in phosphate buffer (PBS, 200 mg / ml) and 4.8 ml PBS was prepared immediately before use. The suspension containing isolated cells was then mixed with 25 ml of dye solution, placed on a slide and covered with laminula. Slides were then examined with an immunofluorescence microscope. The nuclei of viable cells are stained green, while the unviable cells (dead cells) are stained red. After examination of the slides, we selected samples of tissues that contained more than 75% viable cells.

Statistical analysis

Means and standard deviations with a significance level of 5% ($p<0.05$) were calculated for all results. The Student's t test and Mann-Whitney test were used to compare results between the experimental and control groups for each of the periods of fecal transit exclusion (6, 12 and 24 weeks). The Mann-Whitney test was used to compare the results of HAI between the segments with and without transit for each of the exclusion periods and Student's t test to compare the results of MPO tissue activity and *TM* between the segments with and without transit for each of the exclusion periods. The Kruskal-Wallis test was used to analyze variance in the oxidative stress levels in the segments with and without transit between all three different exclusion periods. All data obtained were analyzed using SPSS® for Windows (version 13.0).

Results

Histological inflammatory activity index (HAI)

Figure 2 shows the HAI in each intestinal segment and a comparison of control animals with animals subjected to fecal deviation (colon segments with and without fecal stream) for different lengths of time. We also verify that the HAI in segments without a fecal stream at 6, 12 and 24 weeks was 2±0.35, 3±0.25 and 3±0.25. The HAI was higher in segments without fecal stream after 12 and 18 weeks than in segments with a fecal stream or from the control group. (p=0.01).

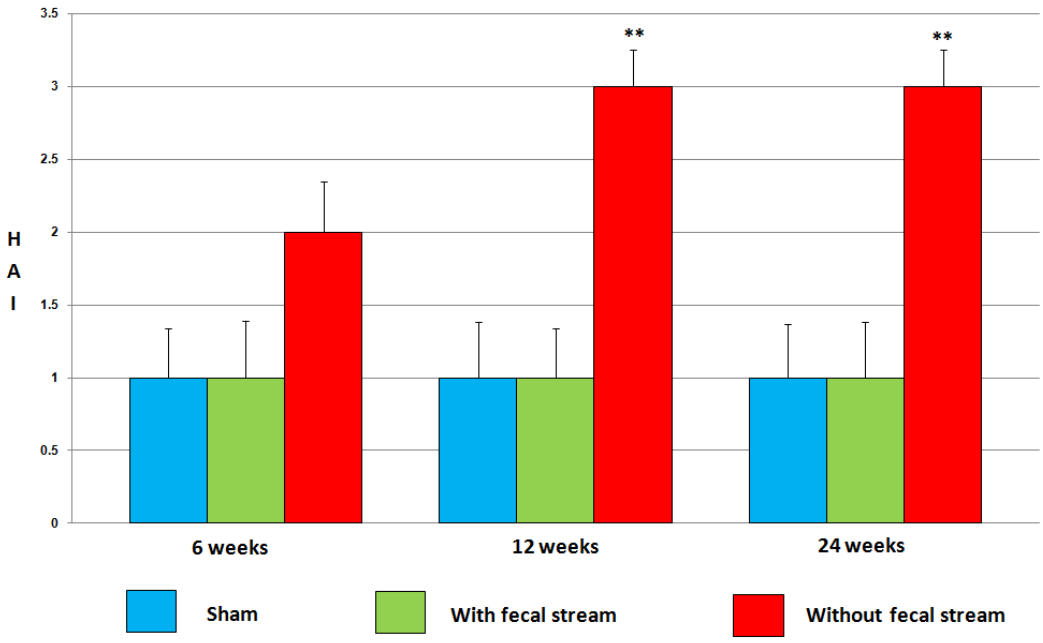

FIGURE 2 – HAI in sham and experimental subgroups (colon segments with and without fecal stream) for different durations of fecal stream diversion. ** = significant difference (without fecal stream × control and with fecal stream). Mann-Whitney test.

MPO activity

The level of MPO activity at 6, 12 and 24 weeks in segments without fecal streams was 1.96±0.51, 1.07±0.12 and 0.68±0.12. As shown in Figure 3, the samples obtained from excluded colons at 6 and 12 weeks after surgery had higher MPO activity than did non-excluded and control samples (p=0.0001). After 24 weeks, the MPO level of all samples was the same. We found that the MPO activity in segments without a fecal stream decreases significantly with the time of fecal stream diversion (p=0.001). MPO activity did not vary in segments with fecal stream and from animals in the control group.

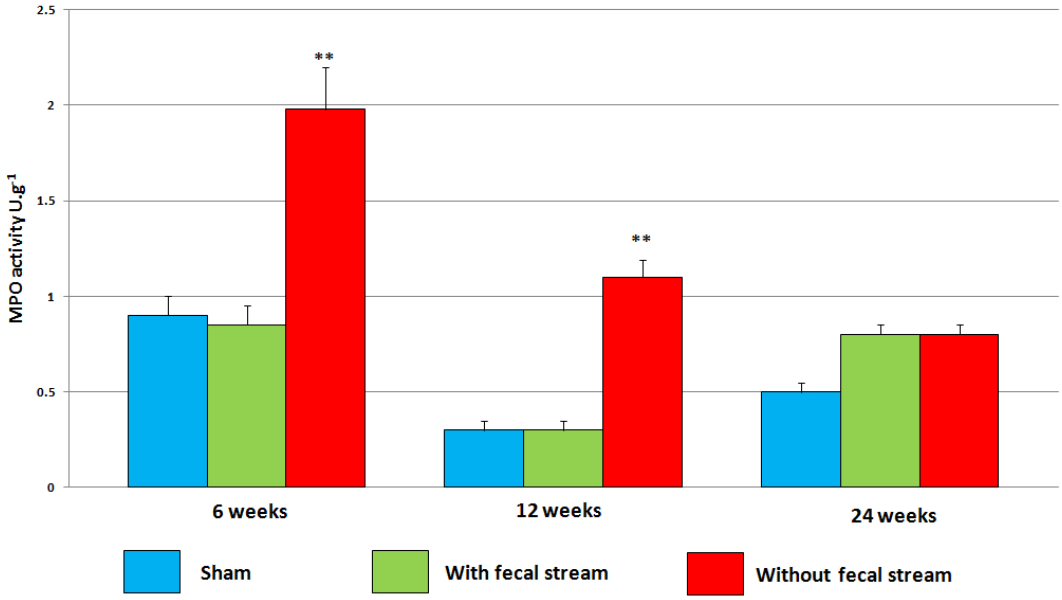

FIGURE 3 – MPO activity in sham and experimental subgroups (colon segments with and without fecal stream) for different durations of fecal stream diversion. ** = significant difference (without fecal stream × control and with fecal stream). Student t test.

Figure 4 shows the levels of oxidative DNA damage in animals from the control and experimental groups (segments with and without fecal stream) after 6, 12 and 24 weeks of fecal stream diversion. In colon segments without a fecal stream the levels of Tail Moment (oxidative DNA damage) were 3.24±0.44, 3.74±0.40 and 4.39±0.32, respectively. We show that in colons without a fecal stream, the levels of oxidative DNA damage were significantly higher than the values obtained from a sham group and segments with a fecal stream, independent of the time of the exclusion considered (p=0.0001). The levels of oxidative DNA damage in the segments without a fecal stream increase with the duration of the diversion time (p=0.0007).

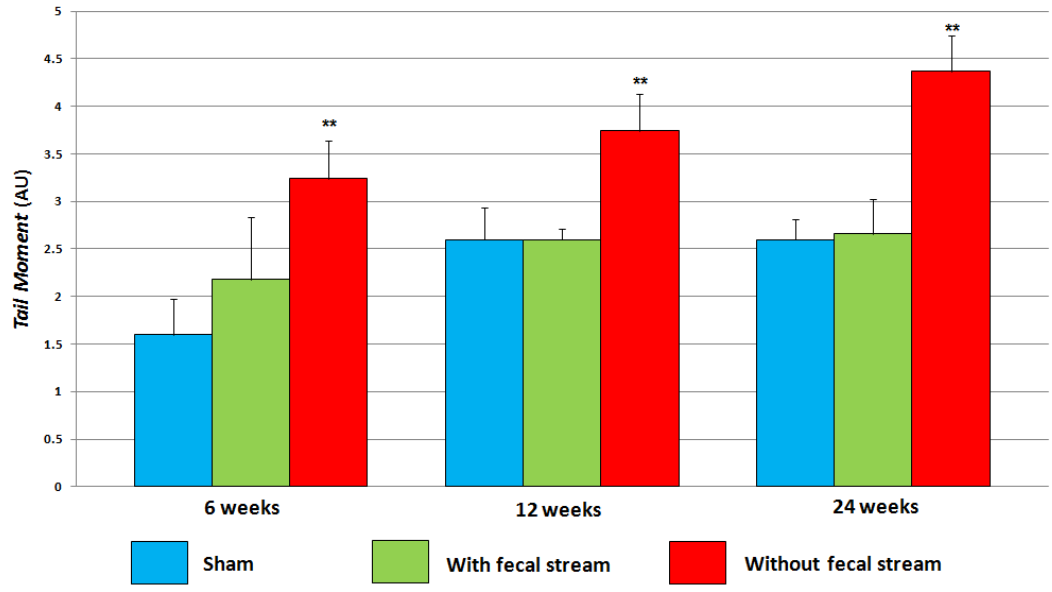

FIGURE 4 – Mean *TM* values in control and experimental subgroups (colon segments with and without fecal stream), for the different durations of fecal stream diversion. ** = significant (without fecal stream × control and with fecal stream). Student t test.

Discussion

A variety of theories have been proposed to explain the etiopathogenesis of DC[26,27]. Prominent among these theories is the hypothesis that DC is a nutritional deficiency disease caused by decreased quantities of SFCAs in the intestinal lumen[2-7,28,29]. The SCFAs, represented by butyrate, acetate and propionate, are the principal energy substrates for colonocytes, providing 80% of the energy needs of the colon epithelial cells[28]. It has been demonstrated that 70% of the oxygen consumption in colonic cells comes from the oxidation of SFCAs, particularly butyrate[29]. In segments without a fecal stream, the absence of dietetic fibers and changes to the normal bacterial flora decrease the production, absorption and use of SFCAs by the epithelial cells, leading to the overproduction of ROS[30].

The importance of adequate oxidation of SCFAs in the prevention of DC is known through clinical and experimental studies showing that rectal instillation with SCFAs in diverted colon segments reduces the inflammatory process[14,16]. Reinforcing this hypothesis, experimental studies have shown that the administration of substances that inhibit the oxidation of the AGCC colonic mucosa, such as 2-bromo-octanoate sodium, provokes histological changes characteristic of chemically-induced colitis, and similar to those found in DC. This observation suggests that energy deficiency is an early event in the pathogenesis of the disease[31,32]. The results of the present study strongly support this hypothesis, showing that an increase in HAI occurs in segments without fecal stream concomitant with the time of colon exclusion.

SCFAs exert potent effects on a variety of colonic mucosal functions including the inhibition of inflammation and carcinogenesis, reinforcement of various components of the colonic defense barrier and the reduction of oxidative stress[13,14,33]. When SCFAs are no longer supplied to the epithelial cells, the oxidative mechanisms of the mitochondrial respiratory chain are disrupted and even greater ROS formation occurs[27,30,34]. Confirming this possibility, study show that butyrate in the intestinal lumen is required to prevent and control ROS aggression in the healthy colonic mucosa[34]. It has already been shown that the oxidative metabolism of butyrate is impaired in individuals with UC, causing the formation of high levels of ROS[35]. The protective effect of butyrate might be important in pathogenetic mechanisms mediated by ROS, and identifying this effect aids our understanding of the apparent protective effect SCFAs have toward colorectal cancer[12,13]. Further, SCFAs can modulate the expression of the catalase and COX-2 genes[36,37]. This point to a dual role of SCFAs in chemoprotection, since more catalase and less COX-2 enhances the detoxification of H_2O^2, one of the most important compounds that cause oxidative stress[36,37]. The results of the present study showed that in colon segments with an adequate supply of SCFAs, the levels of oxidative DNA damage were significantly lower than segments without fecal stream. The results showed that the absence of SCFAs could increase the levels of oxidative DNA damage as well as inflammatory activity.

Oxidative stress occurs when there is an imbalance between the generation of ROS and the action of antioxidant defense systems. The control of ROS overproduction and the loss of chemical antioxidant systems, like catalase and superoxide dismutase, provide a strong rationale for developing novel antioxidant therapies to treat IBD. Studies have demonstrated that antioxidant substances such as n-acetylcystein and deferoxamine are capable of reducing the oxidative aggression in DSS-induced colitis[38,39]. Likewise, the use of drugs with a powerful antioxidant activity, such as 5-aminosalicylate, has been shown to be effective in treating DC[18]. Authors have similarly used experimental models of DSS-induced colitis to demonstrate that the therapeutic use of antioxidant substances, like superoxide dismutase and catalase, is effective in ameliorating colitis through decreasing the colonic level of ROS[40].

The theory that UC is induced by ROS proposes that the excess of un-neutralized H_2O_2 that is produced by the epithelial cells of the colon mucosa due to aberrant cell metabolism can spread through the membranes to the extracellular space[41]. There, it is converted to the hydroxyl radical (OH.), which is capable of causing extensive oxidative damage to the structures responsible for maintaining the function of the epithelial barrier of the colon mucosa[14]. In cases of colitis, it is believed that the basal membrane, proteins of the extracellular matrix and proteins that form tight and adherens junctions between cells become damaged by the action of ROS, causing alterations in selectivity of colon mucosa barrier. These suppositions are corroborated by evidence showing that, in patients with colitis, the basal membrane may be destroyed before PMN infiltration into the intestine wall occurs[14]. This reaffirms the possibility that the initial tissue damage may be due to substances produced by the modified metabolism of the epithelial cells themselves[14]. Once the epithelial barrier has become damaged, it is no longer capable of preventing fecal antigens from invading the submucosal tissue, which is normally sterile. The exposure to fecal antigens provokes an initial immune response consisting of infiltration of PMN into the colon mucosa in an attempt to impede and prevent systemic bacterial invasion[14]. When present within the mucosa, these cells are capable of producing larger quantities of ROS and further damaging the colonic wall[42]. This progressive inflammatory activity leads to the ulceration of the colon mucosa, with the consequent bleeding and mucus loss that are the clinical characteristics of the disease[14].

The importance of ROS in the initial events of colon mucosa inflammation becomes more evident in a case series that correlated rectal instillation of H_2O_2 with the inflammation of the colon mucosa, similar to what is seen in cases of UC. For many decades, H_2O_2 enemas were routinely used to eliminate impacted feces from the rectum. However, from 1930 onwards, reports of the development of severe and even fatal forms of colitis subsequent to the use of H_2O_2 with this objective started to appear[16]. Even small quantities of this substance, such as used for cleaning colonoscopes, may cause colitis in humans[43]. Through the observation that H_2O_2 causes damage to the cells of the colon mucosa and that epithelial cells of the colon mucosa with modifications of their oxidative metabolism can produce H_2O_2, it is reasonable to speculate that excessive production of ROS by these cells may cause inflammatory aggression, thereby provoking the appearance of DC[14].

Since the first description of DC, the main clinical, endoscopic and therapeutic aspects of this disease have been demonstrated. Nevertheless, there is still some controversy regarding the initial phenomena involved in aggression against

segments of colon mucosa without fecal transit[2,11,44]. The resemblance between the histological findings from patients with DC and from patients with exacerbation of IBD, particularly UC, suggests that the two conditions have similar etiology. This is especially true with regard to the initial mechanisms of inflammatory aggression to the cells of the colon mucosa.

Until the last decade, the measurement of oxidative stress involved sophisticated biochemical techniques that require a considerable amount of tissue to achieve accurate quantification[45]. Due to limitations related to obtaining sufficient material for analysis, these techniques could not be applied in small amounts of tissue, such as those obtained during endoscopic procedures. For this reason, few studies have assessed oxidative stress in individuals with UC, comparing the normal and inflamed tissues[46]. With the advent of alkaline gel electrophoresis (comet assay), it has become possible to quantify the level of oxidative stress in a single cell. The comet assay allows the measurement of the ROS in small fragments of tissue, enabling the comparative study of cells in normal and inflamed colonic mucosa, and even cells in different stages of colorectal carcinogenesis[23,45]. The comet assay is a rapid, sensitive and relatively simple method for detecting oxidative DNA damage at the level of individual cells. It combines the simplicity of biochemical techniques for detecting DNA single strand breaks (strand breaks and incomplete excision repair sites), alkali-labile sites, and cross-linking[12,13]. The method recognizes oxidized bases and has been used to determine levels of oxidative DNA damage, which has been implicated in several health conditions[12,13,20,23,24,25,45].

The results of this study confirmed that a change in cellular energy substrate is able to cause the development of colitis. This supports the theory that the induction of colitis by ROS by demonstrating that epithelial cells devoid of AGCC are able to undergo oxidative stress. We found high levels of ROS formation in colon segments devoid of fecal stream, similar to what occurs in models of chemically-induced colitis[20,47]. The association between the increased presence of oxygen free radicals and inflammatory changes in diverted colon demonstrates the importance of ROS in the development of DC. In a previous study, our group evaluated the levels of oxidative DNA damage, comparing animal's subjected to 2,4,6-trinitobenzene sulfonic acid (TNBS)-induced colitis to colon segments without fecal stream through six, 12 and 18 weeks[20]. We found that in animals subjected to TNBS-induced colitis, the levels of DNA damage were elevated only in the acute phase of inflammation, but the levels were always higher in segments without when compared to the segments with the fecal stream, regardless of the time considered[20]. However, in this initial study, we used a small number of animals in each experimental subgroup and we diverted the fecal stream for a maximum of 18 weeks. In order to verify if the levels of DNA damage could increase after 18 weeks of diversion of the fecal stream, in the present study, we derive the fecal stream for a longer period of time (24 weeks) and increase the number of animals in each group as well the number of cells analyzed by comet assay in each sample[21]. With a greater number of animals in each group we found that the levels of oxidative damage in the segments without transit after 18 weeks continued rising, reaching its highest levels after 24 weeks. These findings demonstrate that, although a non-chemical strategy was used to undermine the colonic mucosa, higher levels of ROS were found in segments devoid of fecal stream than in segments in which transit was preserved, regardless of time of exclusion and confirm that changes in substrate supply can cause changes in intracellular metabolism, forming large amounts of ROS.

In the present study we could see higher levels of oxidative DNA damage in the colon of animals subjected to fecal stream exclusion for 6 weeks than in colons that retained transit. These findings show that epithelial cells can produce high levels of oxidative damage from the beginning of the process of fecal exclusion, confirming the importance of SFCAs in maintaining normal energy metabolism. It should be noted that in this group of animals, a lower HAI was found despite the already-high oxidative tissue damage. Nevertheless, these animals had high MPO activity, suggesting that the presence of PMN infiltration could explain the increase in the production of ROS in these first weeks. In animals subjected to 12 weeks of exclusion of transit, the levels of oxidative DNA damage were also higher in the portion of the colon without a fecal stream. In this experimental group, reduced levels of MPO are observed, suggesting that tissue inflammatory aggression could be maintained by the excessive production of ROS in cells deprived of SCFAs. In animals subjected to exclusion of the fecal stream for 24 weeks, oxidative DNA damage is also higher in the colonic segments that lack a fecal stream. In this group of animals, we found higher HAI and increased levels of DNA damage. Interestingly, the levels of MPO in segments devoid of the fecal stream were similar to those found in segments with fecal stream, suggesting that the high levels of ROS in this moment could not be related to PMN infiltration. It is possible that the increased production of ROS is maintained due to a deficient supply of SCFAs to the colonic mucosal cells.

These results founded confirm that oxidative stress may be responsible not only for the initial aggression but also for the maintenance of aggression of the colonic mucosa throughout the period of fecal exclusion. Suggest that AGCC deficiency causes changes in the energy metabolism of epithelial cells of the colonic mucosa, leading to the production of large amounts of ROS and oxidative tissue stress. The results showed that highest levels of ROS in segments devoid of fecal stream are related to the histopathological changes found in DC. Thus, it's suggested that ROS play an important role in the initiation and maintenance of inflammatory aggression to the DNA of the mucosa colon cells in diversion colitis.

Conclusions

The results from the present study allow it to be concluded that there is a significant increase in the levels of oxidative DNA damage in colon segments without transit, in comparison with segments with fecal stream, independent of the duration of exclusion. It is also concluded that, despite the reduction in the levels of MPO in colon segments without fecal stream, there is an increase in HAI, related with time of intestinal exclusion, suggesting that ROS maybe the molecules responsible by damage of the colon epithelium in DC.

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