Original Article

Evaluation of topical n-acetylcysteine in diversion colitis

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ABSTRACT: **Introduction:** Diversion colitis (DC) is a benign condition characterized by the appearance of inflammation in the mucosa of the colon or rectum devoid of fecal stream. Oxidative stress has been associated with the etiopathogenesis of the disease. N-acetylcysteine (NAC) is a substance with antioxidant properties, used in different treatments of inflammatory diseases. The purpose of this study was to evaluate the effects of topical applications of NAC in an experimental model of DC. **Methods:** Thirty-six Wistar rats were submitted to deviation of fecal stream by proximal colostomy and a distal mucosal fistula. They were distributed into 3 experimental groups of 12 animals according to the daily application of enemas containing 0.9% saline or 2 doses of NAC, 25 mg/kg and 100 mg/kg, respectively. In each group, half of the animals were sacrificed after two weeks of irrigation and half after four weeks of irrigation. The diagnosis of colitis was assessed by histopathological analysis and the grade of inflammation by inflammatory grading scale. The results were evaluated with the Mann-Whitney test, adopting significance level of 5% ($p<0.05$). **Results:** Daily enema of NAC improved the inflammatory alterations in colon without fecal stream. In colonic segments without fecal stream, the inflammatory score was significantly lower in the animals treated with 100mg/kg, compared with those treated with saline solution or 25 mg/kg of NAC, regardless of the duration of intervention ($p<0.05$). The inflammatory score in colon segments without fecal stream was significantly lower in the animals treated with both concentration of NAC, compared with those treated with saline, regardless of the concentration and duration of irrigation ($p<0.01$). **Conclusions:** Enemas containing NAC improve the inflammatory process and constitute a beneficial tool in the treatment of DC.

Keywords: acetylcysteine; colon; colitis; fatty acids, volatile; histology; rats.

RESUMO: **Introdução:** Colite de exclusão (CE) é uma condição benigna caracterizada pelo desenvolvimento de inflamação na mucosa do cólon desprovida de trânsito fecal. O estresse oxidativo tem sido implicado na patogênese da doença. A n-acetilcisteína (NAC) é uma substância com efeitos antioxidantes, sendo utilizada no tratamento de várias doenças inflamatórias. **Objetivo:** Avaliar os efeitos da aplicação tópica de NAC em modelo de CE. **Método:** Trinta e seis ratos Wistar foram submetidos ao desvio do trânsito por meio de colostomia proximal e fístula mucosa distal. Os animais foram distribuídos em três grupos experimentais de igual tamanho segundo a aplicação de enemas diários contendo soro fisiológico 0,9% ou NAC nas concentrações de 25 mg/kg ou 100 mg/kg. Em cada grupo, metade dos animais foi sacrificada após duas ou quatro semanas de irrigação. O diagnóstico de CE foi feito por estudo histopatológico e a graduação por escala inflamatória. Na avaliação dos resultados, utilizou-se o teste de Mann-Withney, adotando-se nível de significância de 5% ($p<0,05$). **Resultados:** A aplicação intrarretal de NAC melhorou a inflamação no cólon sem trânsito intestinal. Nos segmentos sem trânsito, o escore inflamatório foi menor nos animais tratados com NAC na concentração de 100 mg/kg, quando comparado aos tratados com 25 mg/kg ($p<0,05$). O escore inflamatório nos segmentos sem trânsito fecal foi menor nos grupos tratados com NAC, quando comparados ao tratado com soro fisiológico, independente da concentração e do tempo de intervenção ($p<0,01$). **Conclusão:** A aplicação de enemas contendo NAC melhora o processo inflamatório demonstrando-se estratégia benéfica para o tratamento da CE.

Palavras-chave: acetilcisteína; colo; colite; ácidos graxos voláteis; histologia; ratos.

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INTRODUCTION

Short-chain fatty acids (SCFAs) play an important role in the metabolism of epithelial cells of the gastrointestinal tract[1-3]. They represent the main substrate for the cells of colonic mucosa, using more than 80% of the oxygen consumption required to produce energy. Reduction in SCFAs supply or metabolism alterations have been associated with inflammatory bowel diseases (IBDs), diversion colitis (DC), pouchitis, formation of adenomas and even colorectal cancer (CRC)[2,3].

DC is characterized by the development of an inflammatory process in colonic mucosa segments without intestinal stream[1,4]. This disease was discovered in the 1970's, but it was not well understood until1981, when Glotzer et al.[5] described ten cases of a form of colitis that developed in colonic segments without fecal stream, in patients that did not present prior evidence of IBDs[4,5]. Subsequent studies attributed inflammation to reduced supply of SCFAs to mucosa cells of the segment without fecal stream[6-10]. The application of SCFAs in the excluded colon was experimentally demonstrated to improve the inflammatory process, while the interrupted application promoted the disease dissemination[10]. The deficient supply of SCFAs as a factor that triggers DC was confirmed with the results of clinical and experimental studies showing that the restoration of fecal stream or the application of butyrate-rich solutions, the main subtype of SCFAs metabolized by colonocytes, reverted the clinical symptoms and the characteristic endoscopic and histological alterations of the disease[11-19].

However, despite these evidences, the molecular mechanisms through which the deficient supply of SCFAs determines the tissue inflammation, have not been fully explained[20]. With the purpose of evaluating the molecular mechanisms involved in the DC etiopathogenesis, the epithelial cells of colonic mucosa without fecal stream were recently demonstrated to produce high amounts of oxygen free radicals (OFRs). The resulting oxidative stress causes histological and biochemical alterations similar to those found in experimental models of chemically induced colitis or human colitis. The oxidative stress causes alterations in the colonic mucosa that aggravate according to the time of diversion[20-27].

Based on these evidences, the use of substances with antioxidant activity was suggested to be effective in the prevention and treatment of this disease[20,27].The benefits of these substances in the treatment of DC were recently confirmed with the application of enemas with 5-aminosalicylic acid (5-ASA), a powerful antioxidant, was demonstrated to reduce the levels of tissue oxidative stress and improve the inflammatory process in the mucosa without fecal stream, even after the ex-vivo exposure to hydrogen peroxide (H_2O_2)[27]. However, the use of 5-ASA in the treatment of DC is expensive and presents side effects that do not allow to use it for extended periods.

N-acetylcysteine (NAC) is a substance with recognized antioxidant activity both in vitro and in vivo[28-30], which has been used in the treatment of different inflammatory diseases. Studies that analyzed forms of chemically induced colitis showed that NAC improves the inflammatory process by removing OFRs created during the cellular energy metabolism[28,30]. The application of enemas with NAC was able to reduce inflammation in colitis induced by dextran sulfate (DSS) and acetic acid after removing OFRs and increasing the antioxidant activity of colonic mucosa[28-31]. However, despite the therapeutic effectiveness of NAC in chemically induced colitis, its effects have never been tested in forms of DC. If demonstrating therapeutic effectiveness, it could become a new option for the treatment of DC, improving the quality of life of patients who have to experience the limitations involving the presence of a stoma. Then, the purpose of this study was to evaluate the effects of topical application of enemas with NAC in colonic mucosa without fecal stream in an experimental model of CE.

METHOD

This study was approved by the Animal Ethics Committee (CEUAP) at the Universidade São Francisco and all experimental phases were conducted following the principles defined by the Colégio Brasileiro de Experimentação Animal (COBEA) and Law 11.794, of October 2008, which regulates the use of animals in research.

Animals

The study used 36 Wistar male rats weighting from 300 to 350 g, from the Central Biotery at the Universidade São Francisco. The animals were kept in individual cages, in an acclimatized environment,

with controlled temperature, lighting, moisture and noise. On the day before the surgical intervention, they remained on a 12-hour fast, receiving only water. The cages were identified with number, experimental group and subgroup where they belonged, and these data were tattooed, using China ink, on each animal's tail. The rats were fed always with the same food, suitable for rodents, and their weight was checked weekly.

Surgical technique

Intestinal stream bypass in all animals was performed after general anesthesia, with the intramuscular administration of 0.1 mL/100 g of 1:1 (v/v) ketamine (50 mg/mL) and xylazine (20 mg/mL) solution in their left rear foot. After anesthetized and fixed to the operating table, their abdominal cavity was opened with a 3-cm median longitudinal incision. After the Peyer's patch identification using a caliber, the distance between the patch and the site selected for left colon incision was measured, 4 cm above the upper edge of the patch. After the ligation of marginal vascular arcade, the colon was incised at the selected site, placing the proximal segment out, as a terminal colostomy at the left hypochondrium, fixing the colostomy to the skin using separated stitches of absorbable monofilament suture 4-0 at the 4 cardinal points, and between them. After the proximal colostomy was fixed, the caudal segment of the left colon was catheterized and irrigated with 40 mL saline solution (0.9% SF) at 37ºC, until the fluid drained through the anus did not present fecal residues. After the irrigation was concluded, the catheter was removed and the distal colon was placed out as a colostomy (distal mucous fistula), in the lower left lateral face of the abdominal wall. The distal stoma was fixed using the same technique as for the proximal stoma. The abdominal wall synthesis was performed at two suture planes: peritoneum and aponeurosis, with continuous polyglycolic acid suture 4-0 (and the skin with separated nylon suture 4-0).

Experimental groups

Figure 1 shows the algorithm for establishing experimental groups. All 36 animals were randomly distributed into 3 experimental groups with 12 rats each. The first group received daily enemas with saline solution at 0.9% (control group). The second and third groups (experimental groups) received daily enemas with NAC (Sigma-Aldrich Brasil Ltda., São Paulo, Brazil), at two different concentrations (25 mg/kg and 100mg/kg, respectively). In each group, six animals were killed two weeks and six were killed four weeks after the intervention.

Sample collection

Two or four weeks after the intervention with the proposed substances, the animals were anesthetized with the same technique describe above. The abdominal cavity was reopened, removing two 4-cm fragments from each colon submitted or not to irrigation with the proposed solutions. The specimens were opened longitudinally through the antimesenteric side, rinsed with SF and divided into two 2-cm fragments for the histological study.

Histological analysis

The fragments removed for histological study were submersed in buffered formalin solution at 10% (Sigma, St. Louis, MO, USA) for 24 hours, dehydrated by exposure to increasing alcohol concentrations and embedded in paraffin. Histological cuts of 5 μm thick were made for the preparation of slides. After that, they were bleached, hydrated and stained through the hematoxylin-eosin technique for the study. The analysis of slides was conducted with an optical microscope (Eclipse DS-50, Nikon Inc., Osaka, Japan) with final 200x enlargement, performed by a pathologist with experience in IBDs with no access to the material origin and study objective. Histological photographs were taken using a digital video capture system (DS-Fi-50; Nikon Inc., Osaka, Japan) previously coupled to the microscope. The diagnosis of colitis and the tissue inflammatory grading score were determined according to the criteria previously described by Akgun et al.[29] with modifications (Table 1).

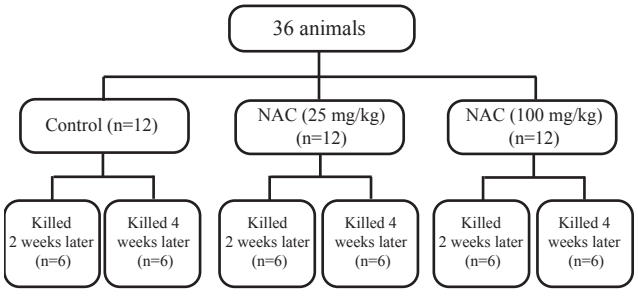


Figure 1. *Algorithm of experimental groups used in the study.*

Statistical analysis

Data obtained were described according to the median values. The comparison between the groups was evaluated through the Mann-Whitney test. At the statistical analysis of results, the level of significance of 5% ($p<0.05$) was adopted, using SPSS® (SPSS Inc., Chicago, USA) version 13.0 for Windows.

RESULTS

Histological evaluation

Figures 2A and 2B show the colonic mucosa of segments without fecal stream of the animals from the control group (irrigated with SF 0.9%), two and four weeks after the intervention, respectively.

Figures 3A and 3B show the colonic mucosa of segments without fecal stream of the animals submitted to irrigation with NAC, at the concentration of 25 mg/kg for 2 and 4 weeks, respectively. The colonic mucosa without fecal stream after irrigation with NAC was intact, without erosion or ulcer. Colonic crypts were intact, with preserved goblet cells.

Figures 4A and 4B show the colonic mucosa of segments without fecal stream of the animals submitted to irrigation with NAC, at the concentration of 100 mg/kg for 2 and 4 weeks, respectively. In the animals that received topical intervention with NAC, the

Table 1. *Variables used in the classification of the inflammatory histological score.*

Findings	Score	Criteria
Epithelial loss	0	No mucosa inflammation
	1	Loss of<5% of the epithelial surface
	2	Loss between 5 and 10% of the epithelial surface
	3	Loss of >10% of the epithelial surface
Integrity of crypts	0	Intact crypts
	1	Loss of <10% of crypts
	2	Loss between 10 and 20% of crypts
	3	Loss of>20% of crypts
Inflammatory infiltrate	0	None
	1	Mild
	2	Moderate
	3	Severe
Stress to goblet cells	0	Absent
	1	Present

Modified from Akgun et al.[29].

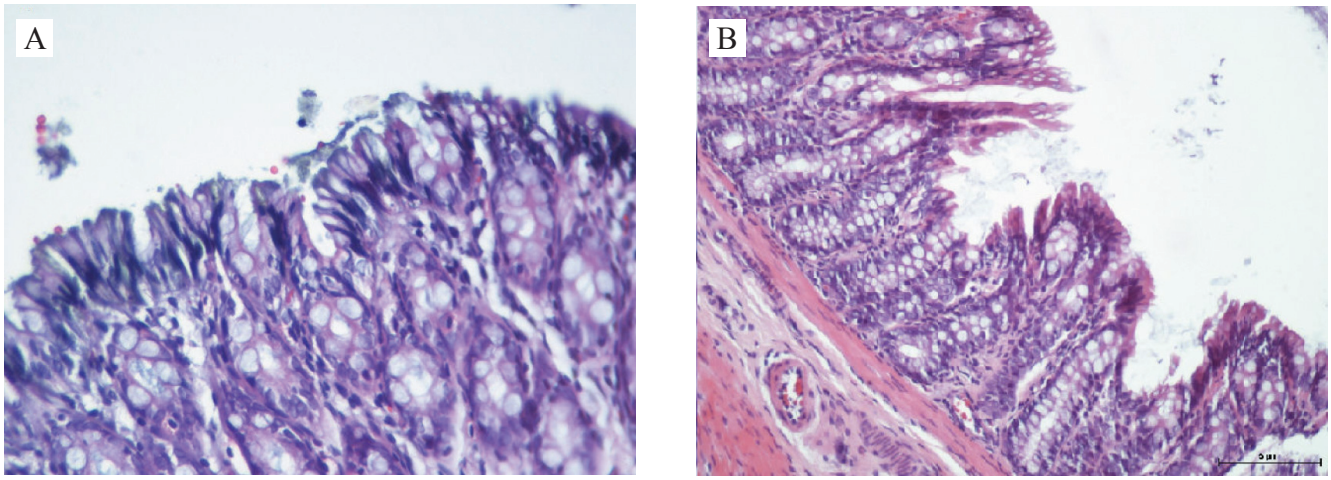
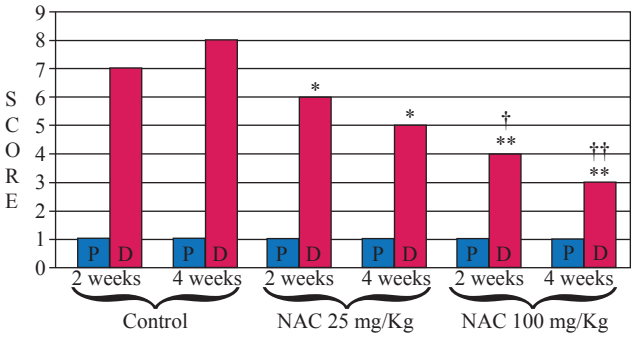


Figure 2. *(A) Surface of the epithelium of the segment without fecal stream irrigated with saline solution at 0.9% for 2weeks (HE 400x). (B) Erosion and ulceration formations in the colonic epithelium without fecal stream 4 weeks after the irrigation with saline solution at 0.9% (HE 200x).*

segments without fecal stream had more regular apical surface of cells, without ulcer or erosion, with lower edema between intact crypts, no alteration in the number of goblet cells and lower inflammatory infiltrate.

Figure 5 presents the microscopic inflammatory score, comparing segments with and without fecal stream in the animals from the control group and experiment groups (NAC at 25mg/kg and 100mg/kg) two and four weeks after the intervention. The histological score in the segments with fecal stream of all groups did not show significant differences. The administration of enemas with 25 mg/kg or 100 mg/kg of NAC in the segments without fecal stream reduced the histological inflammatory score when compared to the segments without fecal stream irrigated with sa-



**Significant (NAC 25 mg/kg x control, p<0.05); **NAC 100 mg/kg x control, p<0.01; †Significant (NAC 100 mg/kg x NAC 25 mg/kg, p<0.05); ††Significant (NAC 100 mg/kg x NAC 25mg/kg, p<0.01).*

Figure 5. *Inflammatory grading score in colon with fecal stream (P) and without fecal stream (D) after irrigation with saline solution 0.9 or NAC at the concentrations of 25 mg/kg and 100 mg/kg for 2 and 4 weeks.*

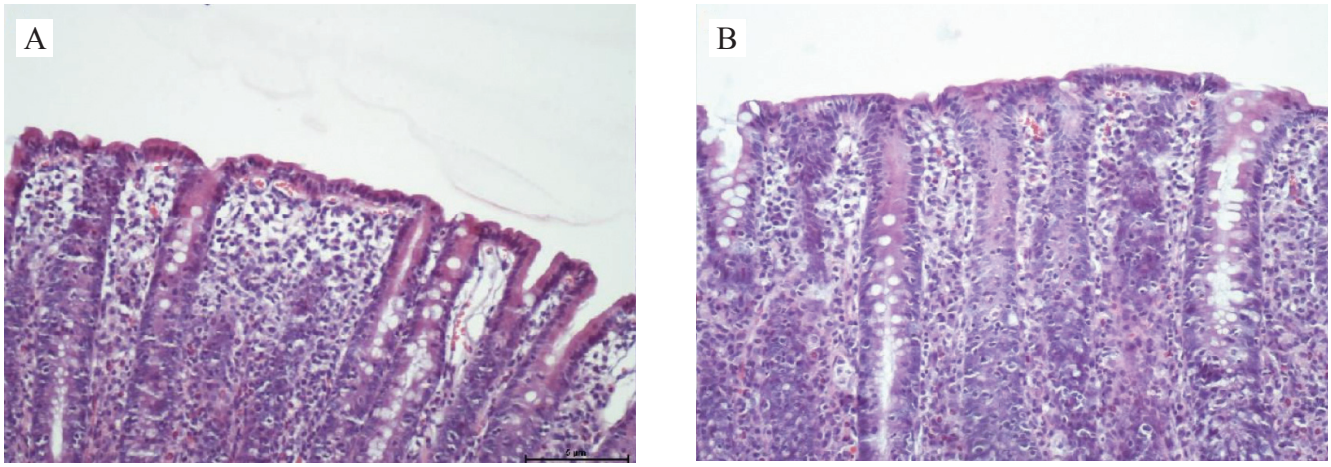


Figure 3. *(A) Surface of the epithelium of the segment without fecal stream irrigated with NAC for 2 weeks (HE 200x). (B) Segment without fecal stream 4 weeks after the irrigation with NAC (HE 200x).*

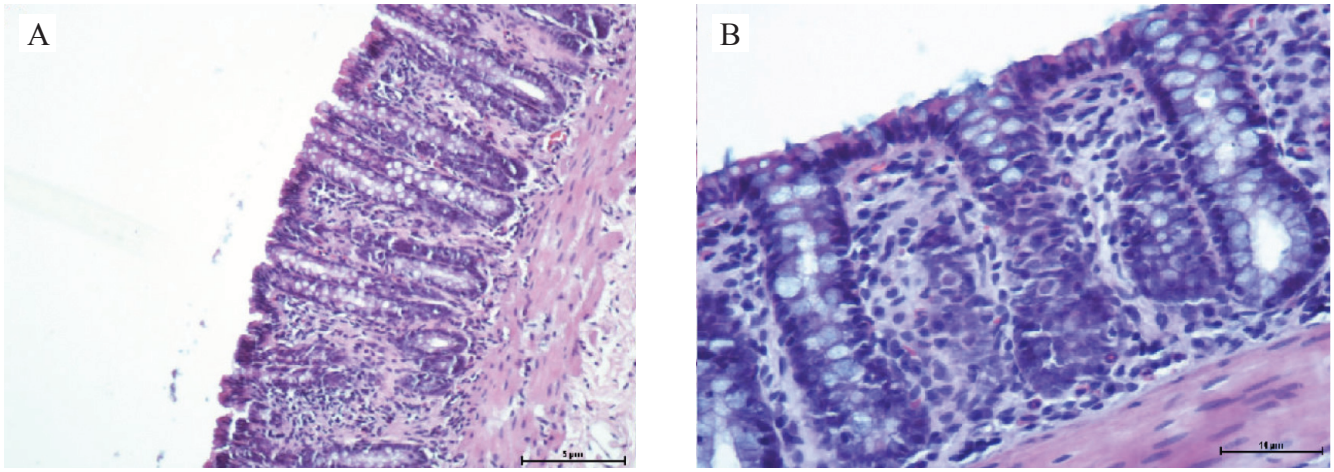


Figure 4. *(A) Surface of the epithelium of the segment without fecal stream irrigated with NAC for 2 weeks (HE 100x). (B) Segment without fecal stream 4 weeks after the irrigation with NAC (HE 400x).*

line solution ($p<0.05$ and $p<0.01$, respectively). The inflammatory score was improved when the higher concentration of NAC (100 mg/kg) was used, in relation to the time after the intervention ($p<0.05$ and $p<0.01$, respectively).

DISCUSSION

DC is a chronic inflammatory disease developed in 100% of the patients 36 months after the intestinal stream interruption[5,6,9-20]. Recent studies have demonstrated that the increased OFRs production by epithelial cells with deficient SCFAs supply and the recognized deficiency of colonic mucosa in antioxidant enzymatic systems, which makes the intestinal epithelium particularly vulnerable to oxidative stress, are mechanisms related to the pathogenesis of DC[20,27]. Studies have demonstrated that oxidative stress reduces the production of mucins that cover the epithelium, promotes peroxidation of phospholipids that constitute the cytoplasmic membrane of colonocytes and breaks the intercellular connections and the basement membrane, the main components of the colonic mucosal defense systems[22-26]. Rupture of this defense system allows the migration of intestinal lumen bacteria into the sterile layers of colon wall. In an attempt to fight against bacterial infiltration, inflammatory cells migrate from the systemic circulation, promoting and keeping the inflammatory system that characterizes the disease[21]. Neutrophil infiltration causes increased production of OFRs, which further aggravates the epithelium harm[20,21]. The relation between stress and the development of DC is evident with the result of experimental studies, showing that the application of enemas with H_2O_2, a powerful producer of OFRs, in segments without fecal stream, causes serious conditions of colitis, similar to those found in human ulcerative colitis[22,32].

When considering that the deficient supply of SCFAs causes the disease, the best treatment strategy is to avoid the inflammatory process development[20]. Then, the early restoration of fecal stream, reestablishing the supply of SCFAs, prevents the disease development[9,10]. Studies have shown that fecal stream restoration reverts the disease symptoms in most cases, making it the most effective therapy for the prevention of DC[13].However, fecal traffic recovery is not always possible, which promotes the evaluation of other therapeutic strategies[20]. As the pathogenesis of DC is related to deficient supply of SCFAs, the most obvious strategy is to apply enemas with SCFAs in the segment without fecal stream[18,19,33,34]. A series of studies confirmed that the irrigation of segments without fecal stream with SCFAs-rich solutions, particularly butyrate, can revert endoscopic and histological alterations related to the disease and reduce the levels of tissue oxidative stress[9-13,15,17,18,33,34]. The main action of SCFAs in the segment without fecal stream would be to restore homeostasis in the reactions that promote energy production by the cells, preventing the excessive formation of OFRs and, consequently, the tissue oxidative stress[34].

When considering that the use of substances with antioxidant activity can neutralize the harmful effects of OFRs, it becomes an interesting strategy for the treatment of diseases related to oxidative stress. Studies conducted in experimental models of chemically induced colitis confirmed the beneficial effects of antioxidants, improving the inflammatory process of colonic mucosa[27-31]. Several drugs have been tested with effective results in experimental conditions, including 5-ASA, glucocorticoids, L-carnitine, L-glutamine, melatonin and antioxidants found in the aqueous extract of *Ilex paraguariensis* and *Curcuma longa*[33-39]. Unfortunately, although they are effective in the treatment of this disease, some of these substances cause side effects when used at higher doses and for longer periods[27,36]. Then, the search for new effective drugs to control the disease, but involving fewer side effects, is still the purpose of further research.

NAC is a small molecule that has a thiol group at one of its ends. It is filterable and easily crosses the cellular membranes, with easy access to different intracellular compartments[29]. NAC has been used as an antioxidant both in vivo and in vitro, in different situations[29,30] and it has been used in clinical practice to treat several diseases, including pulmonary disorders caused by excess oxygen, against the paracetamol toxic effects, infection of human immunodeficiency virus, and the treatment of disorders caused by ischemia in liver, lungs and skin flaps[37,40,41]. The possibility of different pharmacological applications is regulated by the chemical properties of the group of thiol-cysteine molecules to eliminate OFRs[30]. NAC increases intracellular concentrations of cysteine and, consequently,

reduces the concentration of glutathione and removes excess OFRs[29,30]. With its antioxidant activity, it increases biosynthesis and the tissue supply of reduced glutathione (GSH) to reactions catalyzed by the antioxidant system of peroxidase glutathione (GPx)[29,30].

Just like other clinical situations have described, studies have shown that the administration of NAC, in experimental models of induced colitis, can reduce the extension of colonic epithelial stress, attenuate the neutrophil infiltration, reduce lipid peroxidation of cellular membranes and restore the antioxidant enzymatic systems (superoxide dismutase, catalase), especially with early administration[29,30,37]. Long-term treatment with high concentrations of NAC significantly reduces the degree of infiltration of polymorphonuclear cells (evaluated by the myeloperoxidase levels in tissues), the levels of tissue oxidative stress (evaluated by the malondialdehyde and GSH levels in tissue) and colonic mucosa inflammation in models of chemically induced colitis[29,37]. Besides its anti-inflammatory effects, NAC hinders the formation of OFRs (inhibition effect) and removes the radicals formed in the tissue (scavenger effect). Thus, it decreases the oxidative stress in nuclear DNA of colonic mucosa cells, reducing the possibility of genetic mutations related to the development of CRC in patients with ulcerative colitis[28-30,41]. Another advantage is that this substance presents topical effects, and for this reason, it can be used in the form of suppository or enema, with insignificant side effects, even at high concentrations.

The results of this study confirmed the benefits of NAC in the proposed experimental model of DC. We found improvement of the inflammatory process in animals irrigated with NAC when compared to the animals from the control group, regardless of the concentration used. Like other authors have described, who evaluated the effects of NAC in models of induced colitis, our study found that reduced inflammatory grading score is associated with the substance concentration and period of utilization[28,29]. Akgun et al.[29], when studying the effects of NAC in a model of chemically induced colitis, found that the use of the substance, regardless of the used concentration, for a short period (two days), it was not able to improve the degree of tissue inflammation. On the other hand, the use of higher concentrations (100 mg/kg) for a longer period (7 days) significantly reduced the degree of tissue inflammation. In our study, we also obtained a greater reduction of inflammatory grading score in the animals treated with higher concentrations of NAC, suggesting a dose-response relationship. We observed that, regardless of the concentration used, the inflammatory score reduction was more evident in the animals submitted to irrigation for four weeks, showing a relation with the intervention period. However, it should be noted that, unlike the result reported in chemically induced colitis, we observed that, even with lower concentrations (25 mg/kg), NAC was able to reduce the inflammatory score[29].

These findings suggest that, in the model of DC used, the drug administration period is more important than the concentration used. As the score reduction was significantly higher with greater concentrations of NAC used for a longer period, we believe that the best therapeutic effect is obtained with greater concentrations and for a longer period.

Regarding the administration method, a study comparing the effects of intraperitoneal and intrarectal NAC in a model of colitis induced by acetic acid showed significant improvement in different histological and biochemical parameters used to classify the inflammatory process intensity, regardless of the administration method[30,36].The authors noticeably demonstrated that the application of intrarectal NAC presented more evident improvement of the inflammatory process when compared to the intraperitoneal method. The authors also suggest that, although NAC can be administered through both methods, the topical treatment ensures greater therapeutic effectiveness. This study confirmed such findings when observing that the application of intrarectal NAC reduced the inflammatory score in the proposed model of DC. When recalling that most patients that develop DC are submitted to left colon bypass and that inflammation occurs especially in the rectal segment without fecal stream, these findings become relevant. The greater effectiveness of the topical application of NAC in the form of enema or suppository becomes the ideal option for the treatment of patients that develop DC.

The antioxidant activity of NAC has also been effective in the reduction of oxidative stress of cellular DNA, reducing the risk of mutations related to the development of CRC in patients with ulcerative colitis and in experimental models of CRC associated with

ulcerative colitis[41].Although the development of CRC in patients with DC that did not present prior IBD or CRC is an exceptional finding, the oxidative stress in the mucosa without fecal stream may also cause mutations in these patients. In a recent study that has not been published yet, in which we evaluated the effects of topical application of NAC against the oxidative stress to DNA, we found a significant reduction in the levels of stress to colonic mucosa cells without fecal stream, confirming the anticarcinogen potential of NAC.

The results found in this study justify the use of enemas with NAC as a valid therapeutic strategy for the treatment of local DC, especially in the distal segments of the colon and rectum. However, only after studies are conducted in human beings, with an expressive number of patients, it will be possible to extrapolate the results demonstrated in this study.

CONCLUSION

The intrarectal application of enemas containing NAC improves the inflammatory alterations found in segments without fecal stream in an experimental model of DC.

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