Original Article

Oxidative stress and changes in the content and pattern of tissue expression of β-catenin protein in diversion colitis

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ABSTRACT: **Objective:** The aim of this study is to verify if oxidative stress is related to changes in content and pattern of β-catenin protein expression in an experimental model of diversion colitis. **Methods:** Sixty Wistar rats were submitted to intestinal bypass. The animals were divided into three groups according to the sacrifice to take place in six, 12 and 18 weeks. For each group, five animals only underwent laparotomy (control). The presence of colitis was diagnosed by histological study, and its severity, by inflammation grading scale. Cellular oxidative stress was measured by comet assay. Tissue expression of β-catenin protein was analyzed by the immunohistochemistry and quantification of its tissue content by computerized morphometry. Statistical analysis was performed with the Student's t-test, median, Mann-Whitney, ANOVA and Kruskal-Wallis, adopting a significance level of 5% ($p < 0.05$). **Results:** Colon segments without fecal stream developed colitis, which worsened with time of exclusion. Segments without fecal stream suffer higher levels of oxidative stress when compared to those with stream, and it worsens with time of exclusion. The levels of cellular oxidative stress are directly related to the degree of inflammation. The total content of β-catenin in segments without fecal stream reduces after six weeks, and does not vary thereafter. The content of β-catenin in the apical portion of the colon crypts decreases with time, whereas in the basal region, it increases. The total content of β-catenin is inversely related to the degree of inflammation and levels of tissue oxidative stress levels. **Conclusion:** There are changes in tissue content of E-cadherin and increased expression of β-catenin in proliferative regions of colonic crypts, related with oxidative tissue stress.

Keywords: colon; colitis; oxidative stress; adherens junctions; cell adhesion molecules; catenins; comet assay; immunohistochemistry; fatty acids, volatile; rats.

RESUMO: **Objetivo:** O objetivo do presente estudo é avaliar a relação entre estresse oxidativo e conteúdo tecidual de β-catenina em modelo experimental de colite de exclusão. **Métodos:** Sessenta ratos Wistar foram submetidos à derivação intestinal e divididos em três grupos experimentais segundo o sacrifício ser realizado em 6, 12 e 18 semanas. Para cada grupo, cinco animais foram submetidos apenas a laparotomia (controle). A colite foi diagnosticada por estudo histológico, enquanto sua intensidade por escala de graduação inflamatória. Os níveis de estresse oxidativo foram mensurados pelo ensaio cometa, enquanto a expressão e o conteúdo tecidual de β-catenina por imunoistoquímica e morfometria computadorizada, respectivamente. Os resultados foram analisados pelos testes *t* de Student, Mann Whitney, ANOVA e Kruskal-Wallis, estabelecendo-se nível de significância de 5% ($p<0,05$). **Resultados:** Nos segmentos sem trânsito fecal ocorre desenvolvimento de colite que piora com o tempo de exclusão. Segmentos sem trânsito sofrem maiores

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níveis de estresse oxidativo quando comparados àqueles com trânsito, piorando com o tempo de exclusão. Os níveis de estresse oxidativo encontram-se diretamente relacionados a piora da inflamação. O conteúdo total de β-catenina no cólon sem trânsito reduz após seis semanas de exclusão. O conteúdo de β-catenina no ápice das criptas cólicas diminui com o tempo, enquanto na região basal, aumenta. O conteúdo total da β-catenina encontra-se inversamente relacionado ao grau de inflamação e aos níveis de estresse oxidativo. **Conclusão:** Existe redução no conteúdo de β-catenina, principalmente no ápice das glândulas cólicas e aumento nas regiões basais, relacionadas à piora do estresse oxidativo.

Palavras-chave: colo; colite; estresse oxidativo; junções aderentes; moléculas de adesão celular; cateninas; ensaio cometa; imunoistoquímica; ácidos graxos voláteis; ratos.

INTRODUCTION

The colonic mucosa is one of the most perfect functional barriers of the human body[1]. It is formed by a single cell layer, separating the intestinal content, which is rich in bacteria, from the internal sterile intestinal wall. The maintenance of this efficient functional barrier is determined by a series of defense mechanisms. When they act together, they protect the internal intestinal wall from bacterial invasion[1]. The main components of this defense system are represented by the mucus that covers the mucosal surface, the colonocyte apical and basolateral membranes, the adhesion systems formed by intercellular junctions, desmosomes, hemidesmosomes and, finally, the basal membrane[1,2].

From the defense systems, the intercellular junctions (ICJ) represent one of the most efficient mechanisms[1,3]. They are formed by three types of junctions: occluding (OJ), adherens (AJ) and communication (CJ). The OJs are located in the apical portion of the intercellular space and seal the space between neighboring cells, thus preventing the migration of small molecules[3-6]. The AJs, located right below the OJs, connect the cytoskeleton of a cell to its neighbor, and they also play a relevant role in the mechanisms of cellular signalling[7]. They are formed by a transmembrane protein called E-cadherin and by cytoplasmatic proteins of the catenin family (α, β, γ)[8]. Finally, the CJs control the transmission of electrical and chemical signals from one cell to the other, making a complex cell communication network[9-11].

For the development of colitis, it is necessary that epithelial barrier mechanisms be compromised[12-15]. However, the rupture of these defense systems should be the first stage, which comes before the bacterial invasion of the submucosa and the subsequent inflammatory response[1]. Among the ICJs, AJs are the most compromised ones in patients with inflammatory bowel diseases (IBD)[16]. Important changes in content and chemical structure of proteins that form JA were described in patients with ulcerative rectocolitis (URC)[5,8,17,18].

Studies demonstrated that the tissue content and location of the proteins that constitute AJs are modified in the tissue that is chronically inflammed in IBD, URC, colorectal cancer (CRC), CRC associated with URC, and in models of experimental colitis[13,14,19-23]. A strong relation was found between the reduced tissue expression of β-catenin protein and the worsening of URC[24]. The importance of β-catenin protein in the early stages of the development of colitis is more evident when it is demonstrated that knockout mice (*Min*$^{-/-}$), for the genes that translate the AJ proteins, develop severe forms of colitis at early stages[25]. These findings present the existing relation between β-catenin protein tissue changes and the worsened colitis[24,26,27]. However, the molecular mechanisms that determine the ruptured ICJs in patients with URC are not yet enlightened. Among the possibilities, it has been recently shown that oxygen free radicals (OFR) can be the molecules responsible for the initial damage to the mucosal barrier[1]. Since they are toxic radicals, its excessive production is able to damage the ICJs, allowing the migration of bacteria through the intercellular space[1]. Experimental studies confirmed the harmful role of OFR by demonstrating that the exposure of colonic mucosa to hydrogen peroxide (H_2O_2), which is a strong OFR producer, enables the appearance of colitis[28,29]. When demonstrated that OFR can cause damage to the epithelial barrier, it is reasonable to suppose that they can also damage the AJs in the early stages of colitis[1,30,31]. However, most experimental models of colitis do not allow the analy-

sis of this possibility, since it causes damage to the colonic barrier due to the exposure of the mucosa to harmful substances, such as trinitrobenzene sulfonic acid (TNBS), acetic acid or dextran sulfate sodium (DSS)[26,30,32]. Actually, these models do not reproduce the initial molecular mechanisms that cause the mucosal barrier to rupture in the different forms of colitis. However, they confirm that the integrity of defense mechanisms is indispensable to stop bacterial infiltration, thus keeping the local immune response at a quiescent state[33]. Therefore, the ideal experimental model to confirm the initial stages of colitis should cause damage in the defense systems, and among them, in ICJs, just with changes in the metabolism of epithelial cells, without damaging the functional barrier artificially[32].

Glotzer et al.[34], in 1981, described the development of an inflammation in the colonic mucosa without fecal stream, similar to what happens with URC, and this condition is called diversion colitis (DC). DC appears due to the interruption in the supply of short-chain fatty acids (SCFA), main energetic substrate for the oxidative metabolism of epithelial cells in the colonic mucosa[32,35,36]. It has been demonstrated that cells from the colonic epithelium without fecal stream produce increasing amounts of OFR with time of exclusion, and that the resulting oxidative stress is related to the epithelial lesion[31-33,37-40]. It is possible that the lesion in the colonic mucosa, which triggers DC, is related to the ruptured ICJs, and especially AJs, which is a result of the increased production of OFR by the changes in the oxidative cellular metabolism.

Although it has been demonstrated, in models of chemically induced colitis, that there are changes in the expression of β-catenin protein in the sore mucosa, the evaluation of content and changes in the pattern of the protein expression has not been studied in experimental models of DC. Thus, the objective of this study is to check if there is a relation between the oxidative stress and the changes in content and pattern of β-catenin protein expression in an experimental model of DC.

METHOD

This study obeys the Federal Law 11,794, from October 8, 2008, and the guidelines of *Colégio Brasileiro de Experimentação Animal* (COBEA). This project was approved by the Animal Research Ethics Committee of *Universidade São Francisco*, Bragança Paulista (SP).

Animals used for experimentation and experimental groups

Sixty SPC male Wistar rats, whose weight varied from 300 to 350 g and with mean age of 4 months were used. Three experimental groups with 20 animals were randomly constituted and divided according to the sacrifice to take place in 6, 12 and 18 weeks after surgical intervention. Each group was then divided into two subgroups called experiment and control. In the experiment group, with 15 animals, bowel stream was bypassed in the left colon, while in the control group, composed of five rats, there was only laparotomy, without stream bypass. Animals were kept in individual cages during the experiment, in a climatized environment, with temperature, light, humidity and noise control. For surgery, they were anesthetized with 2% xylazine hydrochloride (Anasedan®) and ketamine chloride (Dopalen®), 0.1 mL/100 g, administered via intramuscular in the left back paw.

Surgical technique

After the anesthesia, a trichotomy of the anterior abdominal region was performed, followed by a medial longitudinal 3 cm long incision and posterior opening of the abdominal wall by layers. The left colon was identified and cut 4cm above the Peyer's patch, and the proximal segment was exteriorized as terminal colostomy in the left hypocondrium, fixated to the skin with separate stitches made with absorbable monofilament thread. The caudal segment of the large intestine was catheterized and irrigated with 40 mL of 0.9% physiological solution at 37ºC, until the effluent drained by the anus did not present with stool. After the irrigation, the distal colon was exteriorized, like a distal mucus fistula in the left iliac fossa. Afterwards, the abdominal wall was closed with two suture layers (aponeurosis and skin). After the surgery, the rats were kept warm in an incubator, and after anesthesia recovery, they were placed in individual cages previously identified with the number of the animal and the experimental group to which they belonged. The intake of standardized water and rat food was allowed

after they regained consciousness. They remained in individual cages until the day of sacrifice, and no additional care was taken in relation to the surgical wound and stomas. No antibiotics or analgesic were administered, and during postoperative follow-up it was necessary to sacrifice one animal, which was replaced, due to bowel obstruction caused by internal hernia.

Material collection

The day before the date scheduled for material collection, the animals were fasting for 24 hours, except for water. For the removal of colonic fragments to be analyzed, they were under anesthesia with the same technique previously described. After the cavity was reopened, the whole colon with stream was removed, including colostomy, and the caudal segment, without fecal stream, involving the anus. In the animals of the control subgroup, the whole large intestine, including the anus, was resected. Immediately after removal, the colonic segments were opened longitudinally by the anti mesocolic border and washed carefully with warm physiological serum to remove fecal residue. Two fragments were taken from each colonic segment, and each of them were 20 mm long, being one of them sent to histological and immunohistochemical analysis, and the other one to determine the tissue levels of cellular oxidative stress by the comet assay. In the animals of the control group, the same number of fragments was collected from the left colon, also 20 mm long, 10 mm above the Peyer's patch. For those addressed to measuring the levels of cellular oxidative stress, the mucosa was separated from the other layers of the wall by microdissection. The removed part was immediately stored in *eppendorf*, with freezing solution and at -80ºC.

Histological analysis

For the histological analysis, the fragments of colons with and without stream of each animal in the experiment group, and of the left colon of animals in the control group, were fixated onto a flat cork surface with the mucus side facing up. After identification, they were stored in a 10% buffered formaldehyde solution, and remained there for 72 hours. After this period, they were washed in running and distilled water, and then dehydrated in successive increasing concentrations of alcohol and clarified in xylene. Afterwards, they were included in paraffin blocks and submitted to two longitudinal cuts, 5 μ thick, for the histological and immunohistochemical studies. The slides destined to diagnose colitis and to grade the inflammation score were stained with hematoxylin-eosin (HE). For the evaluation of colitis severity, the grading system for inflammation was used, being previously proposed and validated, which considers the presence of erosions and ulcers on the colonic mucosal surface and the intensity of the inflammatory cell infiltrate[41].

Immunohistochemical technique

For the research of tissue β-catenin protein expression, histological cuts obtained from all the samples were analyzed (colons with and without stream and left colon of the control group) in the three periods of exclusion proposed. After being deparaffinized, the cuts were rehydrated in alcohol at decreasing concentrations and washed in distilled water. Afterwards, they were submersed in PBS (0.05 M, pH 7.2) for 10 minutes and the dry slides at ambient temperature. The endogenous peroxidase activity was blocked with 3% H_2O_2 at ambient temperature for 10 minutes, followed by another wash with PBS for more 10 minutes. Afterwards, the antigen recovery with 10 mM sodium citrate was performed (pH 6.0) in bath water at 95ºC for 45 minutes. For the study of tissue β-catenin expression, the anti-β-catenin primary antibody was used (Dako — Denmark A/S, Glostrup, DE — Ref. M3539, Lot 10025022) diluted at 1:50 in bovine albumin (1%). The slides were covered with 100 μL of the solution containing the primary antibody and stored at 4ºC for 24 hours. After the conclusion of this stage, they were washed with PBS, incubated with the secondary antibody and submitted to the complex biotin-streptavidin peroxidase staining for 45 minutes, prepared with 1:100 dilution in PBS. The slides were developed with a solution of diaminobenzidine tetrahydrochloride (DAB, 10 mg in 10 mL of PBS with 3 mL H_2O_2), which was dropped over the blades and incubated for 3 minutes. Afterwards, they were washed and counterstained with methyl green, and again washed in distilled water. After the counterstaining, there was dehydration by immersion in increasing solutions of ethanol and xylene. Finally, they were set, labeled and stored in the horizontal position for 24 hours.

Measuring the content and evaluating the pattern of β-catenin expression

The presence of β-catenin was considered as positive when the brownish coloration was diffusely present, with variable intensity regions and fine granular distribution in the apical and basolateral membrane, cytoplasm and cell nucleus. According to the recommendation of the manufacturer, the negative control of immunocoloration was performed without the addition of the primary antibody, and the positive one used the neoplastic colonic tissue, which is positive for the protein. The analysis of the β-catenin expression was performed with a common optical microscope, Nikon Eclipse DS-50 (Nikon Inc., Osaka, Nippon), with final 200x magnification, by an experienced pathologist in immunohistochemical techniques. He did not know the origin of the material and objectives of the study. The photographic documentation was obtained with a video capture camera DS-Fi-50 (Nikon Inc., Osaka, Nippon), previously attached to the microscope, and the images were digitized, identified and filed in a computer.

The pattern of expression of β-catenin protein was evaluated according to the place of greater expression along the colonic crypts (apex or base), classifying the intensity of immunocoloration in each of the sites into crosses: + mild expression; ++ moderate expression; and +++ intense expression. The pattern of final tissue expression for each slide was the median found after reading three different fields with at least three full and contiguous crypts. The grading intensity expressed into crosses was performed by two independent observers, and the conflicting results were analyzed afterwards.

The total tissue content of β-catenin was measured by computer assisted image analysis (computerized morphometry) in three fields, which showed, in a longitudinal cut, three contiguous and full crypts. The selected image was captured by the camera, processed and analyzed by the software *NIS-Elements* (Nikon Inc., Osaka, Nippon). For the quantification of β-catenin content in each chosen Field, in 100 micrometers rgb wavelength filter was selected containing the brown color (which identified the tissue immunoexpression of β-catenin). Afterwards, the software transformed the captured content into a binary image in which the white color represented the presence of protein, and black represented the rest of the field. The values found for the total content of β-catenin were expressed as percentage per field. The final number adopted for the animals of subgroups control and experiment (segments with and without intestinal stream) was always represented by median, with the respective standard deviation.

Figure 1A exemplifies the expression of β-catenin protein in the mucosal layer of the colon with fecal stream after 18 weeks of bypass, while Figure 1B shows the quantification of protein in the same field shown by Figure 1A, in binary image, using the computer-assisted image processing.

Evaluation of oxidative stress levels

The quantification of oxidative stress levels by gel electrophoresis of isolated cells (comet assay) was performed according to the previously described technique[42]. Briefly, all the samples from animals in the control and experiment groups, with colon with or without intestinal stream, underwent triplicate analysis. The specimens were incubated in 3 mL of Hank's buffered solution (Invitrogen, Carlsbad, CA, USA), with 5.5 mg of proteinase K (Sigma Chemical, CO, St. Louis, MO, USA) and 3 mg collagenase for 45 minutes at 37ºC for the isolation of colonic mucosal cells. Parts were removed and the cellular viability was assessed. Finally, the fluorescein diacetate (FDA) / ethidium bromide (EtBr) method (Sigma-Aldrich, St. Louis, MO, USA) was used. The solution of cell coloration was prepared immediately before its use, and it contained 30 mL of FDA in acetone (5 mg/mL), 200 mL of EtBr in phosphate buffered solution (PBS; 200 mg/mL), and 4.8 mL of PBS.

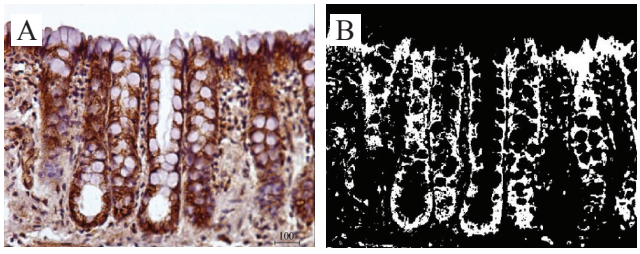


Figure 1. *(A) Pattern of β-catenin expression in the mucosa of the colonic segment with fecal steram after 18 weeks of intestinal bypass (immunohistochemistry 400x). (B) Tissue β-catenin content (white color) by binary analysis of computer assisted image in the same field described in Figure 1A (400x).*

The suspension containing isolated cells was isolated, and then mixed to 25 mL of stain solution, placed over the slide, covered with glass slides and read in fluorescence microscope. The nuclei of viable cells were stained green, and those of unviable cells were stained red. After the analysis of the slides, only tissue samples that presented more than 75% of the viable cells were selected. For the alkaline version of the comet assay in the viable samples, 15 ml of the previously obtained cell suspension were mixed to the 0.5% low melting point agarose placed onto a blade and covered with a glass slide. Finally, they were immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1º SDS, pH10 with 1% Triton X-100 and 10% DMSO) and remained at 4ºC for 12 hours. Subsequently, they were exposed to an alkaline buffer (1 mM EDTA and 300 mM NaOH, pH 13.4) for 40 minutes at 4ºC. Electrophoresis was performed in this buffer, inside the refrigerator, at 4ºC, for 30 minutes at 25 V and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with *Sybr Safe* (Invitrogen, Carlsbad, CA, USA) and analyzed at the fluorescence microscope. The whole material was processed and verified at the same time to avoid technical variations. Two hundred cells were randomly selected (100 of each intestinal segment, with and without stream and of animals in the control group), which were analyzed with the Komet 5.5 software (Kinetic Imaging, NY, USA). So, the value of tail moment (TM) was obtained, and the means were determined. According to the manufacturer's manual, TM is defined as the product between the fragments of tail DNA and the mean distance of migration of the comet tail, which reflects the extension of the rupture of DNA helix (oxidative stress). The value can be quantified by the intensification of image and computational analysis. For each animal, the mean obtained from the reading of 100 cells of each colonic segment performed by the same technician, was used. The technician did not know about the origin of the material and the objectives of the study.

Statistical method

The results were described by mean with the respective standard deviation. The Student's t-test was used to assess the total content of oxidative stress, and the Mann-Whitney test analyzed the total content of β-catenin, comparing animals from the control and experiment groups. The median test was used to analyze the intensity of expression of β-catenin protein in the apical and basal regions of the colonic glands in control and experiment groups. The inflammation grading scale in the different times of exclusion was assessed by the Main-Whitney test. We applied the ANOVA test for the analysis of variance in relation with time of exclusion of stream and total tissue protein content. Kruskal-Wallis was used to evaluate the variation in the protein expression in apical and basal regions of the colonic glands in relation to the time of experiment.

The established significance level was 5% ($p<0.05$) for all the tests. Statistical analysis was performed with the software SPSS (SPSS Inc., Chicago, USA version 13.0).

RESULTS

Figure 2A shows the epithelial surface of the colonic mucosa with fecal stream, while Figure 2B shows the colon without stream after 18 weeks of exclusion. It was possible to observe that in segments without stream the colonic glands were dilated, with a great amount of mucus in the lumen. The caliciform cells are dilated and replace the cells with absorptive function in the apical surface, which no longer present the same juxtaposition by the edema in the stroma, thus configuring an aspect similar to a "brush border".

Figure 3 indicates, by mean and the respective standard deviation, the values found for the inflammation score, comparing animals in the control and experiment subgroups (segments with and without fecal stream) in the different proposed periods of exclusion. We found higher score for those without stream, regardless of considered time of exclusion. The inflammation score in the colon without stream after 6 weeks was 3±0.40, while after 12 and 18 weeks of bowel exclusion these values were 8±0.37, presenting statistical significance when compared to the colon with stream and to the animals in the control group ($p<0.01$). The colon without stream presented increased inflammation score in sacrificed animals in 12 and 18 weeks in relation to those sacrificed after 6 weeks ($p<0.05$).

Figures 4A and 4B show, respectively, show the tissue expression of β-catenin protein in the colonic mucosa with and without fecal stream after 12 weeks

of fecal exclusion, while Fgures 4C and D present the protein expression in segments with and without fecal stream after 18 weeks of exclusion. It is observed that in the colon with stream (Figures 4A and C), the greater protein expression is concentrated in cells of the apical surface of colonic glands, while the cells of deeper regions present with lower expression. On the contrary, in segments without fecal stream (Figures 4B and D), the expression of β-catenin protein is more intense in the deep portions of the Lieberkühn glands, exactly in the proliferative regions of colonic glands.

Figure 5 indicates, in average, with the respective standard deviation, the total tissue content of β-catenin, comparing the control and experiment subgroups (with and without fecal stream) in the different periods of fecal stream exclusion, measured by computerized morphometry. In colonic glands, after six weeks of intestinal exclusion, the mean percentage of β-catenin tissue content was 23.30±3.00%, while in animals submitted to bypass from 12 to 18 weeks it was 26.79±4.85% and 25.52±3.08%, respectively. When the total β-catenin tissue content was considered in the glands of the colonic epithelium, a reduction in the segments without fecal stream was observed in comparison to the colon with stream and the animals of the control group after only six weeks of fecal exclusion. The total protein content in the colon without stream did not range with the time of exclusion (Table 1).

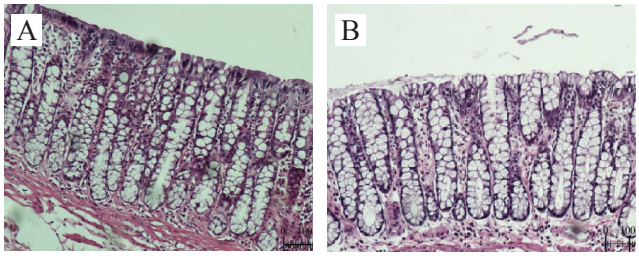
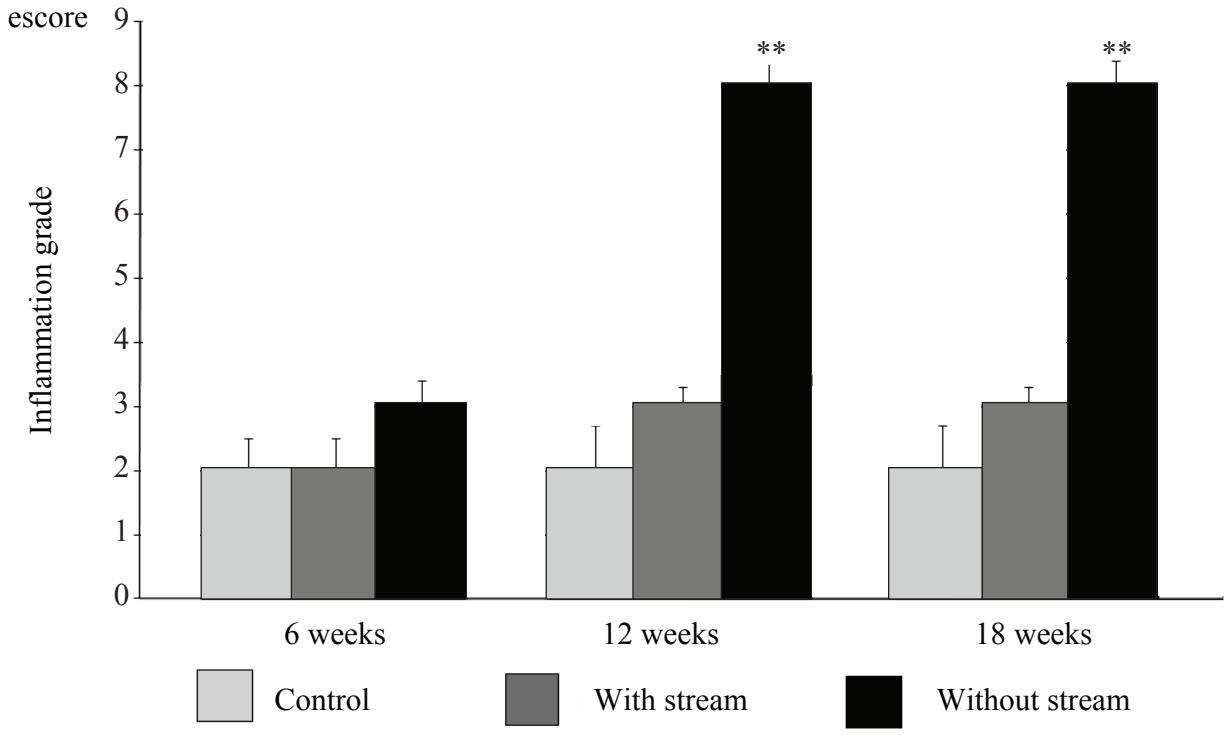


Figure 2. *(A) Colonic mucosa with stream after 18 weeks of fecal stream diversion (hematoxylin-eosin 200x); (B) Colonic mucosa without fecal stream after 18 weeks of intestinal diversion (hematoxylin-eosin 200x).*



*** Significant (without stream X control and with stream) (p<0.01). Mann-Whitney test.*

Figure 3. *Inflammation score comparing the control and experiment subgroups (colons with and without stream) in the different periods of exclusion.*

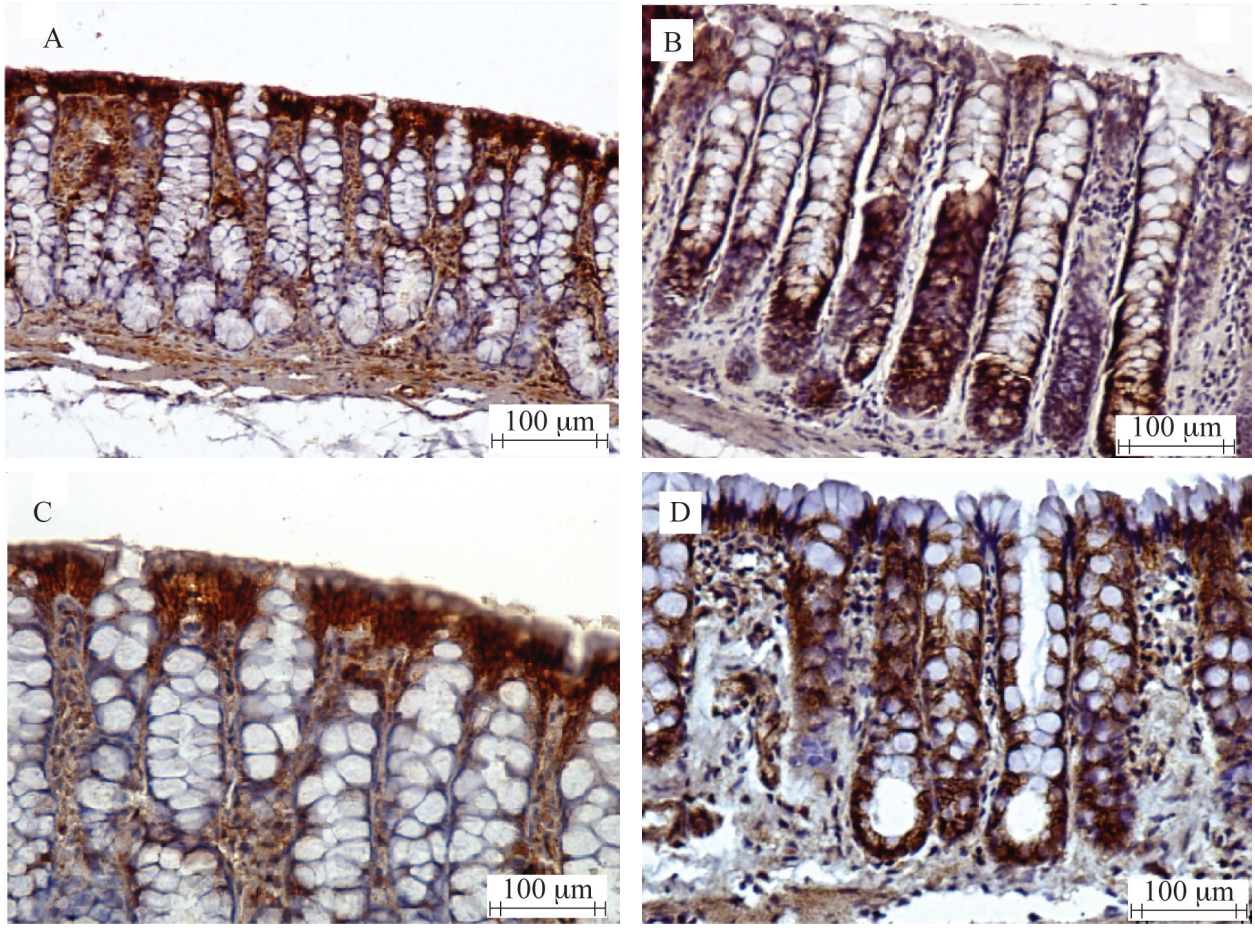


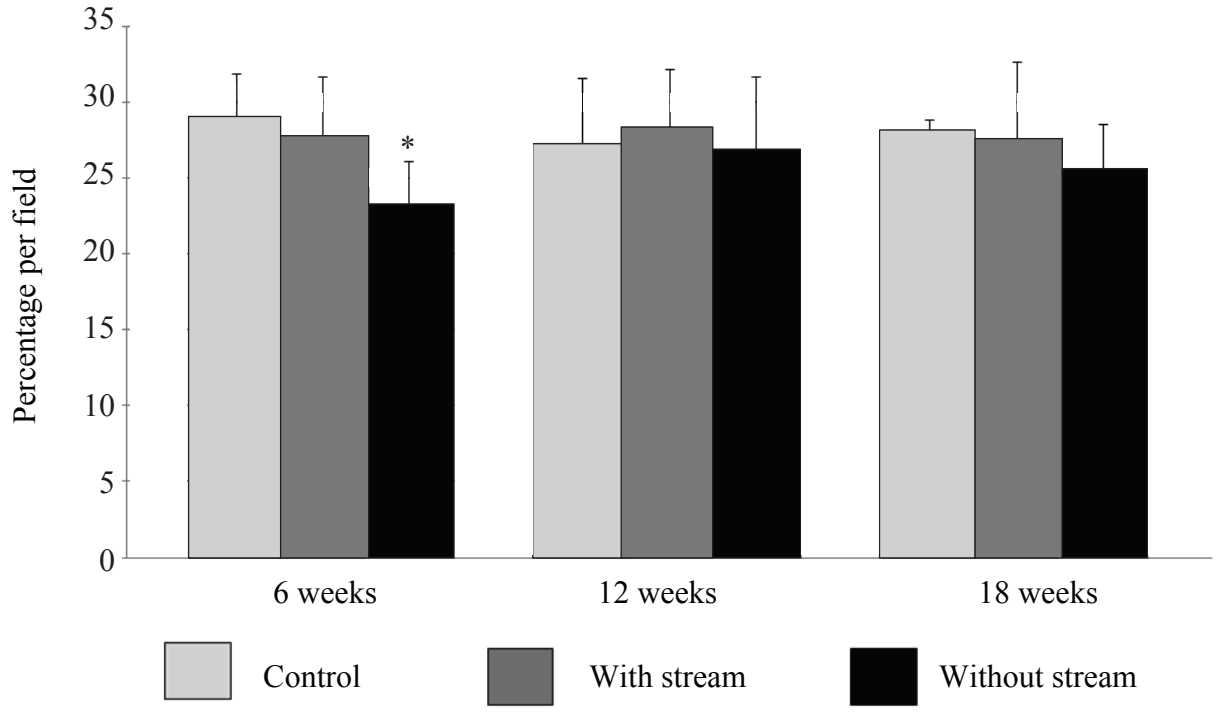
Figure 4. *(A) β-catenin expression in the mucosa of the colonic segment with fecal stream after 12 weeks of intestinal bypass (immunohistochemistry 200x); (B) Protein expression in the mucosa of the colonic segment without fecal stream after 12 weeks of intestinal bypass (immunohistochemistry 400x); (C) β-catenin protein expression in the mucosa of the colonic segment with fecal stream 18 weeks after surgery (immunohistochemistry 400x); (D) Expression in the colonic mucosa without fecal stream 18 weeks after intestinal bypass (immunohistochemistry 400x).*

Table 1. *Variation of cellular oxidative stress levels and β-catenin content in the colon without fecal stream in relation to the different times of exclusion.*

	Colon without stream			
	Mean±SD.			
	6 weeks	12 weeks	18 weeks	p-value
Oxidative stress (TM)	3.24±0.44	3.74±0.40	4.37±0.32	0.0007*
β-catenin	23.13±3.02	26.79±4.95	25.52±3.08	0.49

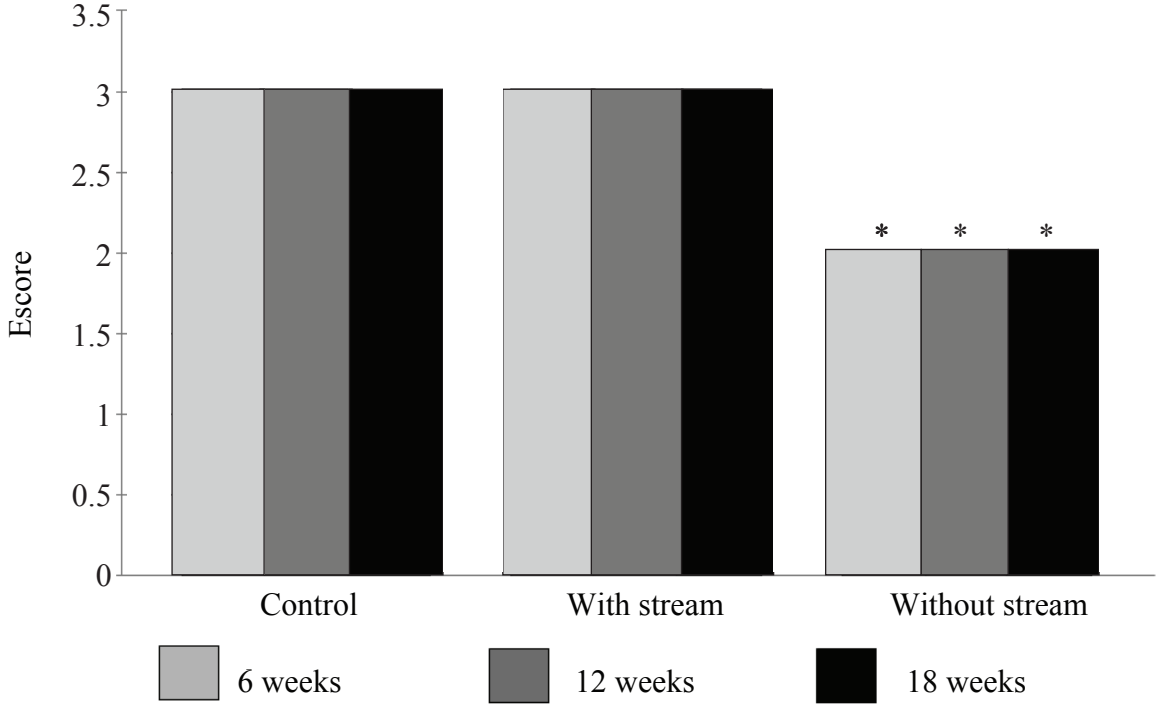
*TM: Tail moment; SD: standard deviation; *Significant; ANOVA test.*

Figure 6 shows, in median, the intensity of the variation of β-catenin protein expression in the apical regions of the colonic mucosal crypts in animals of control and experiment groups (segments with and without fecal stream) in different times of exclusion. It was observed that, regardless of the time of exclusion, there was a significant reduction in the content of β-catenin in the apical regions of colonic crypts, especially after 12 and 18 weeks of exclusion ($p<0.05$). The protein content in the apical portions of the colonic glands in the segments without stream did not show variation according to the time of exclusion.



**without stream<Control and Proximal. (p<0.05). Mann-Whitney test.*

Figure 5. *Total β-catenin tissue content measured by computerized morphometry comparing animals from the control and experiment subgroups (colons with and without stream) in the different periods of intestinal stream exclusion.*



**Without stream 6, 12 and 18 weeks<control and with stream (p<0.05). Median test.*

Figure 6. *Variation of β-catenin tissue content in the apical region of the crypts, comparing the animals in control and experiment subgroups (colons with and without stream) in the different periods of intestinal stream exclusion.*

On the other hand, Figure 7 shows, in median, the variation of intensity in the β-catenin protein expression in the basal regions of the colonic mucosa glands in animals from the control and experiment groups (with and without fecal stream) in the different considered times of exclusion. The increased β-catenin contentin the deeper regions of colonic glands was observed, regardless of time of exclusion, and such increase was even more significant after 12 and 18 weeks of exclusion. The increased protein content in the basal regions of the crypts was also observed after six weeks of exclusion, when these values started to stabilize.

Figure 8 shows, in mean, with the respective standard deviation, the values found for the levels of tissue oxidative stress, comparing the animals in the control and experiment subgroups (with and without fecal stream) in the different times of fecal exclusion. The levels of oxidative stress were similar in the subgroup control and in segments with stream, regardless of the considered time of exclusion. In the colon without stream for 6 weeks, these values were 3.24±0.44 TM, while, after 12 and 18 weeks of exclusion, they were 3.74±0.40 TM and 4.37±0.32 TM, respectively. The results showed that the levels of tissue oxidative stress were higher in the segments without stream when compared to those with stream and to the subgroup control, and time of exclusion was not relevant (p=0.0001). Table 1 indicates that levels of oxidative stress in the colon without stream increase with time of exclusion (p=0.0007).

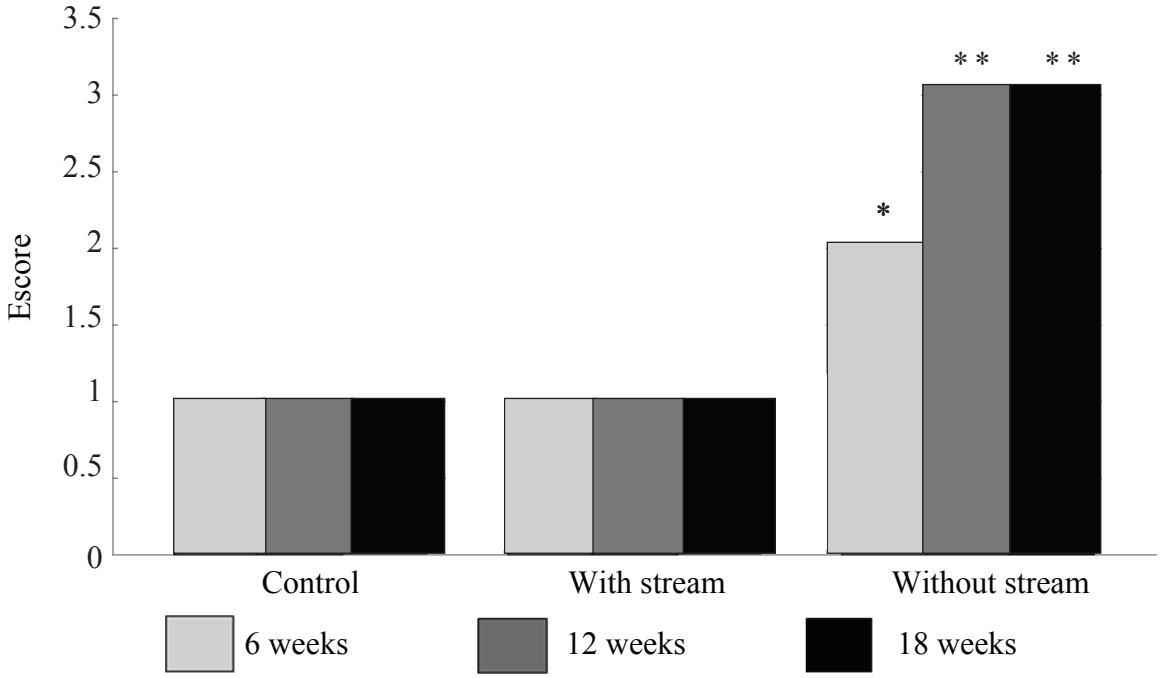
Table 2 presents the variation of β-catenin content between the apical and basal regions of the colonic crypts in segments without fecal stream, in relation to time of exclusion. The variation in the pattern of expression of the tissue protein in the basal region of colonic glands was observed, in comparison to colons without stream for 6 weeks and those for 12 and 18 weeks (p=0.04 and p=0.04, respectively).

DISCUSSION

Cell adhesion is a primary characteristic of the architecture of most of the tissues in the human body. The epithelium that covers the digestive tube is formed by an isolated layer of specialized cells, with absorption and secretion functions, intimately adhered with each other and to the basal blade[11,43]. These cells are gathered to one another by systems of cell-cell adhesion, which support most of the mechanical stress and also work as a functional barrier. The specialized ICJs are located in places where there is no contact between two cells, or between one cell and the extracellular matrix. In order for the cells to function in an integrated manner in a compact set, the already mentioned specialized ICJs, formed by group systems, are needed[43]. There are three functional groups of ICJ: The OJs or zonula occludens, the AJs or the zonula adherens, and the communicating or electrotonic ones. There are also specialized junctions in the adhesion of the cell with the extracellular matrix or basal membrane, represented by hemidesmosomes. All of these ICJs are composed by different types of proteins, which have specific roles inside the complex that form them.

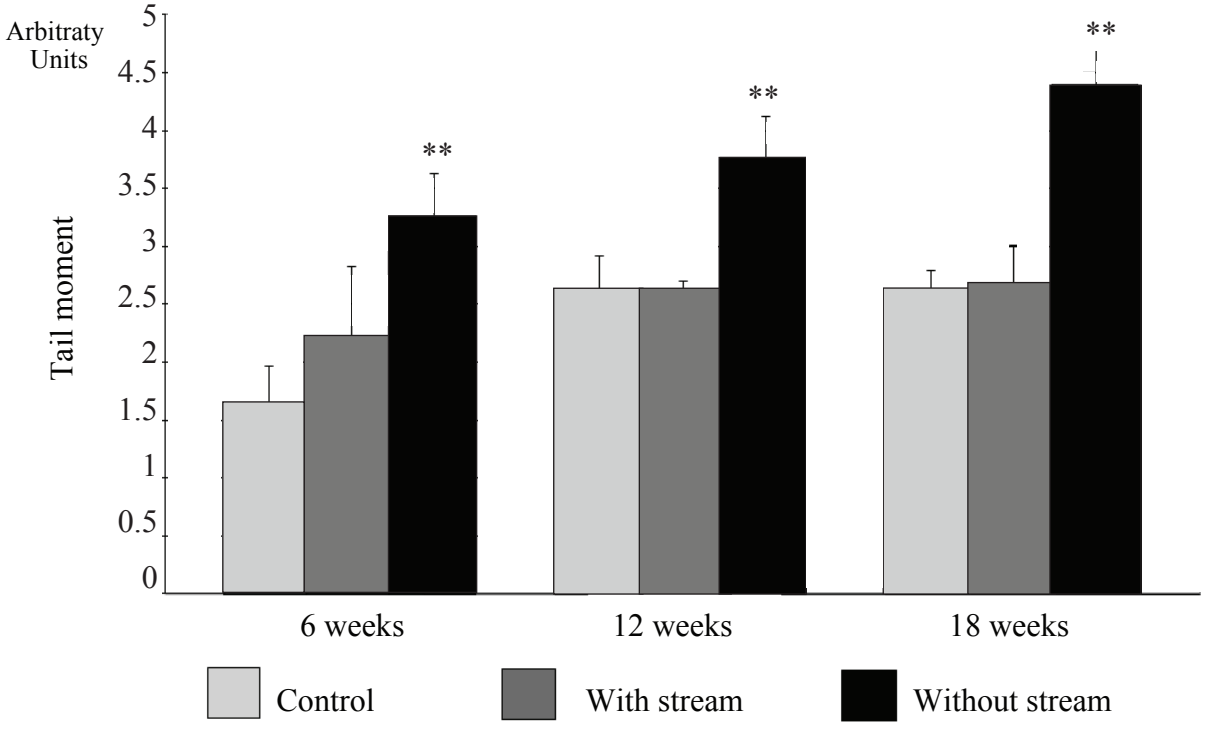
The AJs connect the internal cytoskeleton of a cell to its neighbor, by means of a protein complex formed by the proteins in the cadherin, catenin, vinculin and actin families[11]. The AJs are also related to the proteins of the intracellular signaling pathway, enabling them to participate in the communication mechanisms that are present inside the neighboring cells, and the cadherins are the main proteins that form AJs[11]. These are proteins dependent on calcium that play an important role in intercellular adhesion, tissue differentiation, polarization and epithelial stratification[44]. The cytoplasmic domain of E-cadherin joins one or more intracellular anchor protein, represented especially by β-catenin, while the extracellular domain interacts with the domain of another homologous molecule, from the neighbor cell[11,43].

In order for the occurrence of the anchor between the E-cadherin and the actin protein, which is the most important component of the cellular cytoskeleton, proteins from the catenin family (α-catenin, β-catenin e γ-catenin) are essential[11]. β-catenin is translated from the transcription of the *CTNNB1* gene, located on the chromosome 8q32, with molecular weight of 88 kDa. The β-catenin isoform presents double cell function, because besides participating in the mechanisms of adhesion between two neighboring cells, it is important for the Wnt signaling[11]. When the protein system that form the AJs in the intercellular space breaks, there is the accumulation of free β-catenin in the cell cytosol. With the increased concentration of the protein inside the



**Without stream 6 weeks > with stream 12 weeks (p<0.05); **Without stream 12 and 18 weeks > with stream 12 and 18 weeks (p<0.01). Median test*

Figure 7. *Variation of β-catenin tissue content in the deep region of the crypts, comparing the animals in control and experiment subgroups (colons with and without stream) in the different periods of intestinal stream exclusion.*



***Significant (without stream X control and with stream) (p<0.01). Student's t-test*

Figure 8. *Levels of cell oxidative stress comparing the control and experiment subgroups (colons with and without stream) in the different times of exclusion.*

Table 2. *Variation of β-catenin content values in the apical and basal regions of colonic glands in the segments without fecal stream in relation to the different times of exclusion.*

	Colon without stream		
	Median (crosses)		
	Apical	Basal	p-value
6 weeks	++	+	
12 weeks	++	+++	0.04*
18 weeks	++	+++	0.04*

**Significant; Kruskal-Wallis test.*

cytosol, the ubiquitin system is unable to degrade the β-catenin in the proteasome, and this way the excess protein migrates inside the nucleus and joins the gene transcription factors, thus activating the transcription of genes related to cell proliferation, such as *C-MYC* and *cyclin-1*[7,8,11,13]. Therefore, β-catenin, besides being essential to AJs, actively participates in the Wnt signaling, one of the main mechanisms responsible for the induction of cell division. This double functional domain is important for the renewal of cells in the colonic epithelium, in constant replacement.

It has been shown that, in patients with URC, who suffers from constant apoptosis of the superficial cells of the colonic epithelium, the β-catenin protein presents more expression in the deep regions of colonic glands, where the germinative zone is located, and is the main responsible for the process of cell proliferation[13,45]. On the contrary, the substantial reduction of β-catenin expression in cells of the mucosal surface of subjects with URC was shown, and in this situation there is constant cellular death[13,22,24]. The comparison between normal and sore tissues in subjects with URC showed significant reduction of the tissue content of E-cadherin and β-catenin proteins just in locations in which the disease is active, but not in normal tisses[26]. These results confirm the strong relation between the rupture of AJs and the development of colitis[24].

Many mechanisms are able to damage the AJs in the colonic epithelium. Among them, the oxidative stress stands out[20,21,32,45]. Studies show that the colonic mucosa exposed to high concentrations of OFR oxidizes the Ca^{++} ions, which keep the E-cadherin molecules together in the intercellular space[20]. The rupture of Ca^{++} molecules degrading the intercellular bridges of E-cadherin causes the accumulation of β-catenin cytoplasmic content, thus stimulating cellular division[1,11,20,21,46]. The OFRs can also dissociate the junctions between E-cadherin and β-catenin in the cytosol by a dependent tyrosine-kinase mechanism, which leads to the cytoplasmic accumulation of β-catenin[44]. It was experimentally shown that the migration of β-catenin from its membranous cytoplasmic domain to the inner nuclear cell is considered as the first event in the animal models of chemically induced colitis, which comes even before the neutrophilic infiltration[19,47,48]. However, it is difficult to demonstrate this possibility in the models of chemical colitis, since the rupture of AJs could result from the action of harmful agents, such as TNBS and DSS. With the DV model proposed in this study, it is possible to observe the role of OFR in the rupture of AJs[30]. Recent evidence shows that in experimental models of DC, despite the non existence of intestinal epithelium exposure to any toxic substance, there is increased production of OFR, which determines the appearance of colitis by the rupture of different lines of defense of the colonic mucosal barrier[29-31,37-40]. Even though the relations between the changes in content and pattern of tissue expression of E-cadherin and β-catenin in sick patients and experimental models of URC have been subjectively demonstrated, this possibility had not been assessed in experimental models of DC[19,23].

It is also worth mentioning that the evaluation of tissue content of proteins that compose AJs in models of colitis is usually subjective, which depends on the pathologist's experience. The possibility to use methods of image analysis with the assistance of computers leads to more accuracy, uniformity and reliability in relation to the results, since it quantifies the tissue content of the protein analyzed objectively. The analysis of computer-assisted image, also known as computerized morphometry, presents additional advantages in relation to conventional methods, such as the fast and low cost quantitative evaluation of microscopic structures[37-40]. In this study, with the use of computerized morphometry, it was possible to determine the tissue content of the β-catenin protein objectively, which enabled more precise comparisons between normal and sore tissues[38]. Computerized morphometry had not been previously used to quantify tissue levels of β-catenin in models of colitis.

The evaluation of tissue oxidative stress levels can also be performed by different techniques. Among the most used ones are the malondialdehyde tissue dosimetry, which is a product of the cell membrane phospholipid peroxidation, the plasmatic and urinary dose of 8-hydroxyguanosine levels, and the advanced oxidation protein products[29,49]. However, these are sensitive biochemical techniques, subject to variations, and they need a reasonable amount of tissue to be executed[42]. With the advent of the single cell gel electrophoresis (comet assay), it became possible to quantify the oxidative stress levels in the whole tissue[50]. The technique enables the comparison of oxidative damage levels in cells of the normal colonic mucosa and the sore epithelium[50].

The comet assay is one of the most sensitive methods to assess levels of oxidative stress, presenting greater accuracy when compared to other techniques[50]. Because of its high sensitivity, allied to its low cost, it has been more and more used[30,31,40,42,50]. The comet assay had not been employed to assess the relation between tissue oxidative stress and changes in tissue content and expression of the β-catenin protein in DC models.

At first, with the objective to confirm if animals used for experimentation developed colitis in segments without stream, we evaluated the histological changes in the diverted colon. We observed the presence of colitis in this segment for all rats, regardless of diversion time, when compared to segments with preserved stream[30,31]. In the control group and in colonic segments with fecal stream in the animals from the experiment group, even though we did not find the formation of epithelial ulcers, we identified some degree of inflammatory infiltrate, especially those constituted of neutrophils. Differently, in the colon without stream there was the formation of more epithelial ulcers, sometimes deep, destroying the whole epithelial surface, and larger tissue inflammatory infiltrate, regardless of considered time. It is worth mentioning that in segments without stream, after 12 and 18 weeks of intestinal exclusion, the inflammation grading scale increased in relation to animals submitted to bypass for 6 weeks, thus suggesting that the worsened epithelial aggression could be related to the deficient supply of SCFA, modifying the cellular energetic metabolism for a longer period of time. Confirming this possibility, we found higher levels of oxidative stress in these segments, which increased with the experiment. It is possible that the increased production of OFR be responsible for the larger number of ulcers, and, consequently, for the higher inflammation score observed in segments without stream after 12 and 18 weeks. When we measure the tissue levels of oxidative stress in the segments without stream we observe that they were directly related to the worst inflammation score. The high levels of oxidative stress after six weeks of diversion may be related to the increased production of OFR, both for the presence of neutrophils – cells that produce OFR – and for the changes in cell metabolism resulting from SCFA deficiency.

When we measure the tissue content of β-catenin in animals submitted to bypass for six weeks, we find reduction in the segments without fecal stream in relation to those in the control group and the segments with preserved stream. When we study the β-catenin content separating the apical and basal regions of the colonic glands, we notice the reduction of content in the apical region, and, on the other hand, its increase in the basal region. These findings suggest that the reduction of β-catenin expression in cells of the epithelial surface may be related to higher levels of damage to the cells in this region, probably due to greater local oxidative stress. The worsened inflammation in the segments without stream could lead to the degradation of β-catenin, and consequently to the greater production of OFR. It is possible that cytokines and proteases produced by activated neutrophils, which are present at this stage of the experiment, could also be responsible for the greater degradation of β-catenin, and also that the rupture of E-cadherin/β-catenin bridges in the cells of the epithelial surface could lead to the migration of β-catenin free of cytosol to the inner part of the nucleus in cells of the germinative zone, with the objective to increase the transcription of genes related to cellular division. It is also possible that the greater β-catenin content found in deep cells of the colonic glands can be explained by the greater need for cell proliferation, with the goal to replace the dead cells from the apical surface, destroyed by tissue stress. However, only the analysis of cell division genes, such as *C-myc* or *Ciclyn-1*, and the β-catenin expression, comparing apical and basal cells from the colonic glands, could confirm this possibility[11].

In animals submitted to intestinal bypass for 12 weeks, we found worsened inflammation score in the colonic segments without fecal stream, when compared to those with bypass for six weeks. This is mainly due to the greater presence of epithelial ulcers, once we found lower neutrophilic infiltrate. The levels of oxidative stress in these segments were higher when compared to those found in animals with bypass for six weeks, and were directly related to the worst inflammation score.

The worsened levels of tissue oxidative stress in the colon without stream in animals with bypass for 12 weeks could also be a result of the presence of neutrophils. However, it is likely that for these animals, the greater formation of OFRs because of the SCFA deficiency is more relevant for tissue damage when compared to animals with bypass for six weeks.

The total β-catenin content in segments without stream after 12 weeks was similar to that of animals in the control groups and segments with fecal stream. In the diverted colon, there was no variation in protein content when compared to that found in animals with bypass for six weeks. When analyzing the β-catenin expression separately in the apical and basal regions of colonic glands, we noticed the reduction of content in the apical region, while the basal one presented with increase. We found significant variation in protein content between the apical and basal regions of colonic glands in relation to time of exclusion. These findings reinforce our suspicion that the proportional increase of β-catenin concentration in the proliferative zones of the crypts is related to the greater need for cell division in the proliferative zones of crypts, with the goal to replace the cells from an increasingly damaged epithelial surface.

The inflammation score of the colon without stream in animals with bypass for 18 weeks did not increase in relation to those with bypass for 12 weeks. However, in the diverted segments for 18 weeks, despite the major presence of epithelial ulcers, the neutrophilic infiltrate was insignificant. These findings suggest that the worsened epithelial lesion cannot be related to the higher presence of neutrophils. In these segments, we found higher levels of oxidative stress than for animals with bypass for 12 weeks, suggesting that after 18 weeks, the greater tissue oxidative stress caused by energy deficiency can be the main responsible for the worsened epithelial lesion.

The total β-catenin tissue content in segments without fecal stream, diverted for 18 weeks, was similar to that of animals with bypass for 6 and 12 weeks. The intensity of β-catenin expression in the apical regions of colonic glands was similar to that of animals submitted to bypass for 6 and 12 weeks. However, in the basal region, the intensity of the expression remained high, with values similar to those found in the colon without stream for 12 weeks, thus suggesting the greater need for gene activation related to cell division by β-catenin. As what happened with diverted colons for 12 weeks, the intensity of β-catenin expression in segments without stream presented significant variation when comparing apical and basal regions. The greater β-catenin expression in the proliferative zone of colonic glands after 18 weeks reaffirms the need to maintain the cell division process in the cells by the germinative zone.

Studies have shown the important role of SCFA to maintain the proper tropism of epithelial cells from the colonic mucosa[35-38]. They are important substracts to preserve the integrity of barrier mechanisms, since they induce the expression of genes that form the proteins that are responsible for the selective permeability of AJs and avoid oxidative stress, which cause lesions in these defense systems[35-38]. The inhibition of SCFA metabolization leads to the appearance of colitis[36], while the establishment of fecal stream and the administration of SCFA, mixed nutritional solutions and omega-3 and omega-6 rich polyunsaturated fatty acids, improve histological alterations, probably by diminishing tissue oxidative stress[30].

The results found in this study add new evidence to support the theory of colitis induction by OFR. In this paper, it was possible to demonstrate that cells from the colonic mucosa without regular SCFA supply suffer from more oxidative stress, especially in the late stages of fecal diversion. It was possible to show that colonic segments without stream present histological changes that are indistinguishable from those found in human DC, and similar to those described in experimental models of chemically induced colitis[37-40]. The results also showed that SCFA deficiency, despite practically keeping the total β-catenin tissue content, drastically changes the place of protein expression, reducing its content in the damaged epithelial surface and increasing it in the proliferative region. These findings are in accordance with previous studies, which demonstrated the double functional β-catenin domain,

favoring the mechanisms of cellular adhesion to the epithelial surface and leading to cellular proliferation in germinative zones of colonic glands. Finally, the results in this study show, for the first time in literature, that the content and place of β-catenin expression is changed in DC, as it happened with URC. From the practical point of view, the findings suggest that the reestablishment of SCFA supply to the diverted colon, be it by the reconstitution of fecal stream or by the administration of nutritional solutions rich in SCFA, or even the use of antioxidant substances, can be considered as a valid strategy to prevent and treat DC.

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