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Small Intestine

Protective effect of adipose tissue–derived mesenchymal stromal cells in an experimental model of high-risk colonic anastomosis

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ABSTRACT

Background: Dehiscence of intestinal anastomosis results in high morbidity and mortality. The aim of this study was to investigate the effects of locally administered adipose tissue-derived mesenchymal stromal cells in a model of high-risk colonic anastomosis in rats.

Methods: Seven days after induction of colitis with 2,4,6-trinitrobenzene sulfonic acid, Wistar rats were submitted to a transection of the descending colon followed by end-to-end anastomosis and were then treated with 2×10^6 adipose tissue-derived mesenchymal stromal cells (from the preperitoneal fat) or an acellular culture solution instilled onto the surface of the anastomosis. At day 14, after macroscopic survey of the abdominal cavity, the anastomotic area was submitted to histologic and immunohistochemical analysis, evaluation of myeloperoxidase activity, fibrosis, epithelial integrity, NF-κ B activation, expression of inflammatory cytokines, and extracellular matrix-related genes.

Results: Anastomotic leakage and mortality associated with high-risk anastomosis decreased with treatment with adipose tissue-derived mesenchymal stromal cells ($P < .03$). Application of adipose tissue-derived mesenchymal stromal cells resulted in lower histologic scores ($P = .011$), decreased deposition of collagen fibers ($P = .003$), preservation of goblet cells ($P = .033$), decreased myeloperoxidase activity ($P = .012$), decreased accumulation of CD4+ T-cells ($P = .014$) and macrophages ($P = .011$) in the lamina propria, a decrease in the number of apoptotic cells ($P = .008$), and the activation of NF-κ B ($P = .036$). Overexpression of IL-17, TNF-α, IFN-γ, and metalloproteinases in the acellular culture solution–treated, high-risk anastomosis group decreased ($P < .05$) to near normal values with adipose tissue-derived mesenchymal stromal cells treatment.

Conclusion: Improvements in outcomes of a high-risk colonic anastomosis with adipose tissue-derived mesenchymal stromal cells therapy reflect the immunomodulatory activity and healing effect of these cells, even after just topical administration and reinforces their use in future translational research.

Introduction

Among the postoperative complications in colorectal operations, anastomotic dehiscence represents a serious problem to the patient because of its potential consequences, such as fecal peritonitis and sepsis.[1] It has been estimated that about one third of deaths related to anastomotic dehiscence can be attributed to the

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extravasation of colorectal contents after operative procedures.[2] The morbidity associated with anastomotic dehiscence results in increased durations of hospital stay, costs, and mortality, and a greater risk of cancer recurrence.[3–5]

The incidence of anastomotic dehiscence with leakage has been related to several variables, such as the operative procedure (elective or emergency), the topography (right or left large bowel and upper or lower rectum), the presence of intestinal obstruction, and local factors such as intestinal microbiota, shear forces resulting from peristalsis, fecal transit, and underlying diseases.[2] In particular, in the presence of inflammatory bowel disease (IBD), dehiscence of an intestinal anastomosis represents a severe, postoperative complication with greater morbidity and mortality. Moreover, it has been estimated that more than half of the patients with Crohn's disease (CD) will require an operative procedure within 10 years after diagnosis.[6,7] Currently, notwithstanding all accumulated knowledge in the field of IBD, available therapies are still of limited efficacy and continue to impose important safety concerns. In view of the need for safer and more effective options, cell-based therapies have been investigated as potential novel alternatives.[8,9]

Mesenchymal stromal cells (MSCs) constitute a subset of non-hematopoietic stem cells with multi-lineage potential that can be found in various tissues, including bone marrow, skin, muscle, and fat.[10] In fat tissues, these cells are referred to as adipose tissue-derived mesenchymal stromal cells (AT-MSCs) and represent an abundant source of functional cells, potentially relevant to therapeutic applications using autologous or allogeneic MSCs.[11] The interest in using MSCs for treating chronic inflammatory diseases is because they are home to inflammatory sites where they promote immunomodulatory effects and participate in tissue repair.[12,13] In human IBD, AT-MSCs have been used recently with relatively successful results. For example, complete healing of fistulas was observed in >80% of patients with CD in a modified, intention-to-treat analysis in which AT-MSCs were injected directly into fistula tracts, with no adverse events.[14] In a phase 3, randomized, double-blind controlled trial in patients with CD with perianal fistula, a single intralesional injection of expanded allogeneic AT-MSCs resulted in more effective closure with less treatment-related adverse events than the placebo group.[15] In experimental models of anastomosis, performed either on ischemic colon[16] or after high-dose irradiation,[17] treatment with MSCs promoted accelerated healing. In this study, our aim was to evaluate the potential beneficial effect of locally administered AT-MSCs in a rodent model of a high-risk colonic anastomosis.

Methods

Ethics statement

All procedures reported in this study were in compliance with Brazilian national regulations for animal experiments and were in accordance with the guidelines of the International Care and Use Committee of the National Institutes of Health, and Guide for the Care and Use of Laboratory Animals.[18] The institutional animal care committee of the Federal University of Rio de Janeiro approved the care and use of animals and the procedures carried out in this study (approval number # 004/15).

Animals and experimental groups

The study was performed at the Center of Experimental Surgery of the School of Medicine of the Federal University of Rio de Janeiro. Sixty-six 9-week-old male Wistar rats (*Rattus norvegicus*) (weighing between 300–350 g) were maintained under specific pathogen-free conditions on a 12 h/12 h light and dark cycle in a temperature-controlled room (24°C). Animals were fed with standard rat chow and submitted to fasting for 24 hours before the operative procedure. Animals were fed with standard rat chow, and water was offered ad libitum except for the 12 hours of fasting before and after the operative procedure in order to prevent bronchoaspiration.

After an acclimation period of 1 week, rats were assigned randomly to experimental groups as follows: group 1, 2,4,6-trinitrobenzenesulfonic acid (TNBS)–induced colitis ($n = 11$); group 2, animals submitted to a laparotomy only ($n = 11$); group 3, laparotomy with simple anastomosis ($n = 14$); group 4, TNBS-induced colitis followed by anastomosis and application of adipose tissue-derived mesenchymal stromal cells (AT-MSCs) onto the surface of the anastomosis ($n = 15$); group 5, TNBS-induced colitis followed by anastomosis and application of acellular culture solution (ACS) onto the surface of the anastomosis ($n = 15$).

Adipose tissue-derived mesenchymal stromal cells

AT-MSCs were obtained from cellular culture of preperitoneal adipose tissue. After being harvested, adipose tissue was minced finely and washed extensively with phosphate buffered saline (PBS) containing antibiotics (100 U/mL of sodium-penicillin and 100 mg/mL of Streptomycin, both from Sigma-Aldrich, St. Louis, MO). The adipose tissue was digested enzymatically at 37°C in Dulbecco's modified Eagle medium-low glucose (LGC Biotecnologia, Sao Paulo, SP, Brazil) with 2 mg/mL of collagenase type II (Sigma-Aldrich) for 30 to 90 minutes. Next, after neutralizing the collagenase activity by addition of fetal bovine serum, preparations were passed through a density gradient separation. The pellet was resuspended in Dulbecco's modified Eagle medium-low glucose with 20% fetal bovine serum and antibiotics, and then plated in 25 cm^2 culture flasks and incubated overnight at 37°C with 5% CO_2 at a concentration of 10^5 cells/cm^2. After 2 to 3 days in culture, the non-adherent cells were removed and the adherent cells were maintained in culture until they reached 70% confluence. The monolayer was trypsinized and expanded. After this process, 10 g of adipose tissue is expected to generate approximately $5x10^6$ cells after the fourth passage (30 days), with a cell viability of at least 97%. As quality control for ex vivo expanded cells, AT-MSCs were characterized phenotypically by flow-cytometry (after 4 passages). AT-MSCs were positive for CD90 and CD73, classic markers that specifically define the MSCs population, but negative for CD45 and CD34, indicating that no hematopoietic cells remained in the MSCs suspension used in our experiments. The percentage of CD90 (clone OX-7; BD Biosciences, San Jose, CA) and CD73- (clone 5F/B9; BD Biosciences) positive cells was analyzed, and the absence of hematopoietic cells was verified with CD34 (clone ICO115, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and CD45 (clone OX-1; BD Biosciences) markers. Isotype identical antibodies served as controls.

Induction of colitis

Colitis was induced by instillation of 0.7 mL of a solution containing 20 mg of 2,4,6-trinitrobenzenesulfonic acid (TNBS; Sigma Chemical Co., St. Louis, MO) in 37.5% ethanol (Merck, Damstadt, Germany) using a Nelaton cannula (4 cm long) inserted through the rectum, under light anesthesia. Immediately after the enema application, animals were kept in the Trendelenburg position for one minute and were thereafter allowed access to standard chow and water ad libitum. Two groups of animals not receiving TNBS but submitted to anastomosis (group 3) or to a simple laparotomy (group 2) served as additional controls.

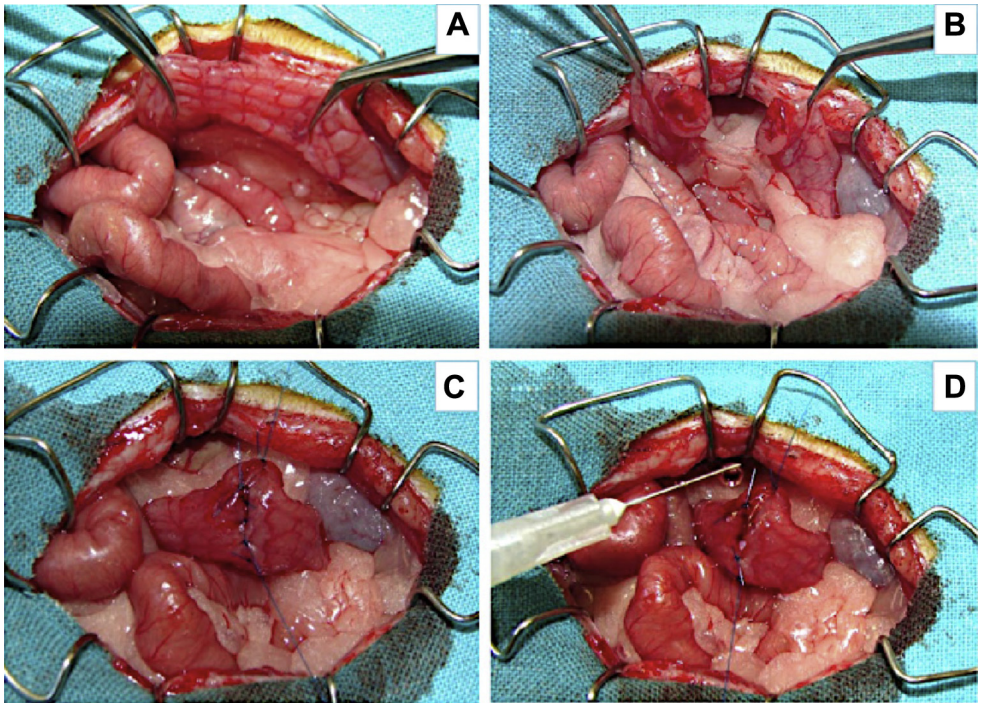

Fig 1. Operative procedure. Isolation of the distal colon preserving the vascular arcade (*A*). Transection of the descending colon (*B*). Anastomosis (*C*). Topical instillation of adipose tissue-derived mesenchymal stromal cells solution (*D*).

Colonoscopic assessment

Colonoscopy was performed under anesthesia 7 days after TNBS application with a flexible bronchoscope device FB120P (Fujinon Corporation, Tokyo, Japan) that had an external diameter of 2.8 mm and was connected to a video camera for recording images. One investigator who was blinded to the treatment protocols and experimental groups performed the endoscopic examinations. Endoscopic analysis was based on the following 4 parameters: hyperemia (absent, mild, moderate, severe localized, severe diffuse); vascularization (normal, increased vascularization with vessels of small diameter, medium diameter, partial large diameter, or large diameter in all segments); bleeding (absent, mild and occasional, several petechial and bleeding spots, intense bleeding during colonoscopic manipulation, or spontaneous at insufflation); and ulceration (absent, erosions, nonconfluent ulcerations, confluent ulcerations).[19,20] Each parameter was attributed 0 to 4 points, and the sum of all parameters was used to generate a final endoscopic score (total score ranging from 0–16). A minimum score of 5 was considered to be satisfactory of the inflammatory process in the experimental groups. Only after confirming the presence of overt colitis by colonoscopy were TNBS-induced rats submitted to the operative procedure.

Anesthetic procedure

The anesthetic procedure consisted of the intraperitoneal administration of ketamine (1 mg/kg) with xylazine (0.1 mg/kg) solution. If necessary, an extra dose was given (usually a quarter of the initial dose) during the procedure to maintain an adequate anesthetic level, which was identified by regular breathing, absence of reflexes, and sagging skeletal muscles. Immediately after anesthetic administration, animals were immobilized in dorsal decubitus, and the skin was cleaned with an antiseptic, surgical scrub solution.

Operative procedure

The operation was performed immediately after colonoscopic examination (to establish the presence of colitis in induced animals; ie, 7 days after TNBS application). We performed a transection of the descending colon preserving the blood supply, 4 cm from the rectum and above the pelvic bone, in all groups except groups 1 and 2. The colon was sutured end-to-end with 6-0 polypropylene (Prolene, Ethicon, Brazil) interrupted stitches. Immediately after anastomosis, instillation of 0.6 mL of a $2X10^6$ AT-MSCs solution in group 4, or 0.6 mL of an acellular culture solution (ACS) in group 5, were administered through direct instillation onto the entire external surface of the anastomosis surface in a single dose. The abdominal wall was closed with 4-0 nylon running suture (Mononylon, Ethicon, Brazil; Fig 1).

One week later, a new laparotomy was performed using the same anesthetic procedure as described previously using sterile technique. After surveying the abdominal cavity for signs of peritonitis, abscess, or dehiscence, a sample of 3 cm containing the anastomotic area was removed (groups 3, 4, and 5). A 3-cm distal intestinal segment was removed in animals that had not undergone anastomosis (groups 1 and 2). After this procedure, animals from each group were killed by anesthetic overdose, without any pain or suffering (4 times the dose applied for usual anesthesia).

Histologic examination

After removal, specimens were washed with sterile saline and fixed in 40 g/L formaldehyde saline, embedded in paraffin, cut into serial sections of 5 μm, and submitted to the various staining procedures. The following histologic parameters involving inflammatory and trophic alterations were studied in hematoxylin-eosin stained slides: mucosal edema, lymphoplasmacytic inflammatory infiltrate, lymphoid follicular hyperplasia, vascular congestion, number and depth of crypts, and the density of cellular elements in

the lamina propria. The following histologic parameters were studied: ulceration, hyperplasia, and inflammatory infiltrate. For both inflammatory infiltrate and hyperplasia, the following grading was considered: 3 = severe; 2 = moderate; 1 = mild; and 0 = absent. For ulcers, grading was as follows: 4 = diffuse glandular disruption or extensive deep ulceration; 3 = glandular disruption or focal deep ulceration; 2 = diffuse superficial ulceration; 1 = focal superficial ulceration; and 0 = absent.[21] To evaluate the anastomosis, the presence of ulcers and mucous juxtaposition between the edges of the anastomosis were considered (0 = absent; 1 = present). The total absence of inflammation corresponded to zero and the maximum score was 12 points.

Assessment of collagen deposition in the colon wall

Phosphomolybdic acid-picrosirius red dye was used to stain collagen fibers in tissue from the serial paraffin sections obtained as described above. At least 10 different areas per tissue section were analyzed under light microscopy at ×100 magnification. The density of collagen fibers was defined by the area positively stained for collagen in relation to the total intestinal tissue using an imaging analysis system.

Assessment of goblet cells in the colonic mucosa

Periodic acid Schiff (PAS) staining was used to stain goblet cells within the intestinal epithelium. The density of goblet cells was defined as the percentage of periodic acid Schiff–positive cells within at least 500 epithelial cells in the crypts and in the surface epithelium of longitudinally sectioned colonic pits.

Immunohistochemical analysis of the colon

Immunologic assessment of the intestinal mucosa was made using an indirect immunoperoxidase technique and the following antibodies: mouse monoclonal anti-rat ED1 (Serotec Ltd., Oxford, UK) to macrophages, mouse monoclonal antibodies anti-rat CD4 (PC4/188) to lymphocytes, and anti-rat p65 (F-6) to NF-κ B (all from Santa Cruz Biotechnology, Inc.). Briefly, serial sections of 5 μm were immersed in 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity. After rinsing in PBS containing 0.5% Tween 20 for 10 minutes, tissue sections were incubated with nonimmune horse serum for 30 minutes and, subsequently, with the respective monoclonal antibody in a humidified chamber overnight at room temperature. Two sections from each sample were incubated with either PBS alone or mouse monoclonal IgG1 (concentration-matched; Dako A/S, Glostrup, Denmark) as negative controls. After being rinsed in PBS for 10 min, all tissue sections were incubated for 30 minutes with the LSAB+ system HRP kit (Dako A/S). Additional rinsing of sections with PBS was followed by development with a solution containing hydrogen peroxide and diaminobenzidine (Dako A/S). Preparations were lightly counterstained in Harris's hematoxylin, dehydrated, and mounted in Permount (Fisher Scientific, Pittsburgh, PA).

Assessment of apoptosis in the colon

Apoptosis was investigated in tissue sections of the colon by a terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick-end labeling (TUNEL) assay. Samples from all experimental groups were analyzed using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore Corporation, Billerica, MA). After deparaffinization, sections were hydrated and incubated with a proteinase K solution. Next, the endogenous peroxidase activity was blocked, and slides were covered with the equilibration buffer and then incubated with a solution containing TdT enzyme. A second section from each sample, without TdT enzyme, served as negative controls. After termination of the reaction, the specimens were incubated with nonimmune horse serum and then incubated with an antidigoxigenin antibody peroxidase conjugate. As described above for the indirect immunoperoxidase, the sections were developed with diaminobenzidine, counterstained in Harris's hematoxylin, dehydrated, and mounted in mounting medium. Morphologically preserved TUNEL-positive cells and apoptotic bodies were defined as apoptotic cells.

Quantitative assessment of colon sections

All histomorphologic analyses of tissue sections under light microscopy were performed using a computer-assisted image analyzer (Leica QWin Plus V 3.5.1, Leica Microsystems Ltd, Switzerland) by an experienced observer who was blinded to the experimental data. Any epithelial and lamina propria cells exhibiting identifiable reactivity distinct from background were regarded as positive. In the immunoperoxidase studies, the percentages of the different cell subsets or apoptotic cells were defined by the number of immunoreactive cells in relation to total cells (immunoreactive and nonimmunoreactive cells) in the lamina propria (counted in at least 10 different areas) or in at least 500 epithelial cells.

RNA isolation and cDNA synthesis

Immediately after death, a tissue fragment approximately 0.5 cm from the region of the anastomosis was separated for the gene expression studies. Briefly, total RNA was obtained from fresh rat colonic tissue, and isolation was performed using the Z3100 SV Total RNA Isolation System kit (Promega, Madison, WI). Equal amounts were then reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Wilmington, DE). The Nanodrop 2000 UV-vis Spectrophotometer (Thermo Scientific) was used for quantifying and determining the RNA purity of samples. Experimental protocols were performed according to the manufacturer's instructions. To complete the cDNA preparation, the samples were incubated in the thermocycler (Gene Amp PCR9700, Applied Biosystems, Foster City, CA) at 25°C for 10 minutes, followed by 120 minutes at 37°C and for 5 minutes at 85°C. The samples were then stored in the freezer at −20°C.

Quantitative real-time polymerase chain reaction

To quantify the changes in mRNA levels, quantitative reverse transcription polymerase chain reaction (PCR) was performed on the ABI Prism 7500 (Applied Biosystems) using RT2 Real Time SYBR Green/Rox PCR Master Mix (SABiosciences, Frederick, MD). For this purpose, levels of mRNA were normalized to the expression of glyceraldehyde phosphate dehydrogenase and constitutive 18S genes. For data analysis, the DDCt method was used by determining the fold change for the target gene in each sample with 95% confidence. Real-time PCR was performed with 2 μg of cDNA in triplicate, and the following genes were analyzed: IL-10, IL-17, IFN-γ, TGF-β, TNF-α, MMP2, and MMP-9. PCR cycles were performed according to the manufacturer's instructions. A complete list of the primers used can be assessed in Supplementary Table S1.

Statistical analysis

Statistical analyses were performed using the statistical software SPSS for Windows (version 10.0.1, SPSS Inc.). Statistical differences among the experimental groups were evaluated via

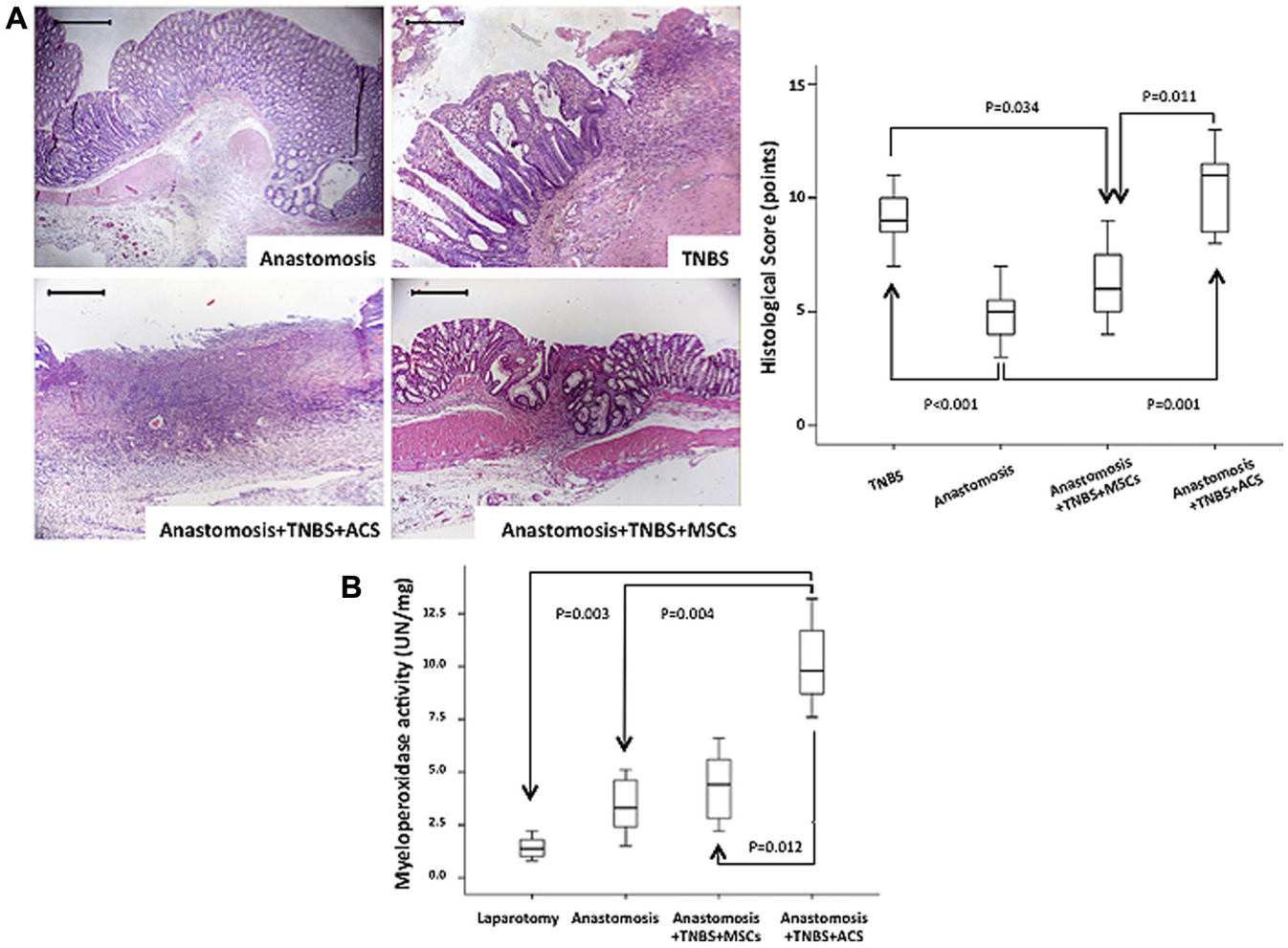

Fig 2. Treatment with adipose tissue-derived mesenchymal stromal cells (AT-MSCs) attenuates inflammation and improves wound healing in a high-risk colonic anastomosis. Histopathologic analysis by hematoxylin and eosin staining of colon samples shows that AT-MSCs prevent the severe inflammatory changes and tissue damage of intestinal anastomosis in the presence of 2,4,6-trinitrobenzenesulfonic acid (TNBS)—induced colitis (*A*). Quantitative analysis of the samples shows that AT-MSCs decrease of myeloperoxidase activity of the colonic anastomosis in the presence of TNBS-induced colitis (*B*). The analysis was performed by Kruskal-Wallis analysis of variance on ranks test, in which multiple comparisons were carried out using the Dunnett's test. The horizontal bars represent the medians, the boxes represent the 25th and 75th percentiles, and the vertical lines below and above the boxes represent the minimum and maximum values, respectively. Length bars represent 50 μm. The data are representative of 3 independent experiments, with 4 to 5 animals per group.

Kruskal-Wallis ANOVA on ranks test in which multiple comparisons were carried out using the Dunnett's test. The values are expressed as the medians with interquartile ranges. All tests were 2-tailed.

Availability of materials and data

The raw data supporting the conclusions of this article, including protocols, analytic methods, and study material will be made available by the authors to any qualified researcher.

Results

Treatment with AT-MSCs decreased postanastomotic morbidity and mortality

During the follow-up period after the operative procedure, no changes in weight were detected among the animals regardless of the treatment. Macroscopic analysis of the abdominal cavity, however, showed greater colonic distension, wall thickening, and more exuberant perianastomotic adhesions in groups 1 and 5 (submitted to TNBS-induced colitis). Local complications, including fistula, abscess, and peritonitis, were observed in 8 animals submitted to the high-risk anastomosis and treated with ACS (group 5, 53%), more than complications occurring in animals submitted to simple anastomosis (group 3, 14%) and in all other groups (0%; $P = .012$). In group 4, no relevant postoperative complications were observed with only occasional slight intraperitoneal adhesions. The simple anastomosis (group 3) resulted in one death (7%), which occurred in the first animal operated in the study and was attributed to anesthetic complications. TNBS-induced colitis followed by anastomosis and treatment with ACS (group 5) resulted in 4 deaths (27%). The analysis of cumulative survival showed a decrease in mortality in group 4 compared to all other groups ($P = .028$). No postoperative deaths occurred among AT-MSCs-treated animals, group 4 (0%).

Treatment with AT-MSCs attenuates intestinal damage owing to the operative procedure

Animals that had undergone the operative procedure without previous exposure to TNBS (group 3) showed an inflammatory process restricted to the anastomosis site. In group 1, animals

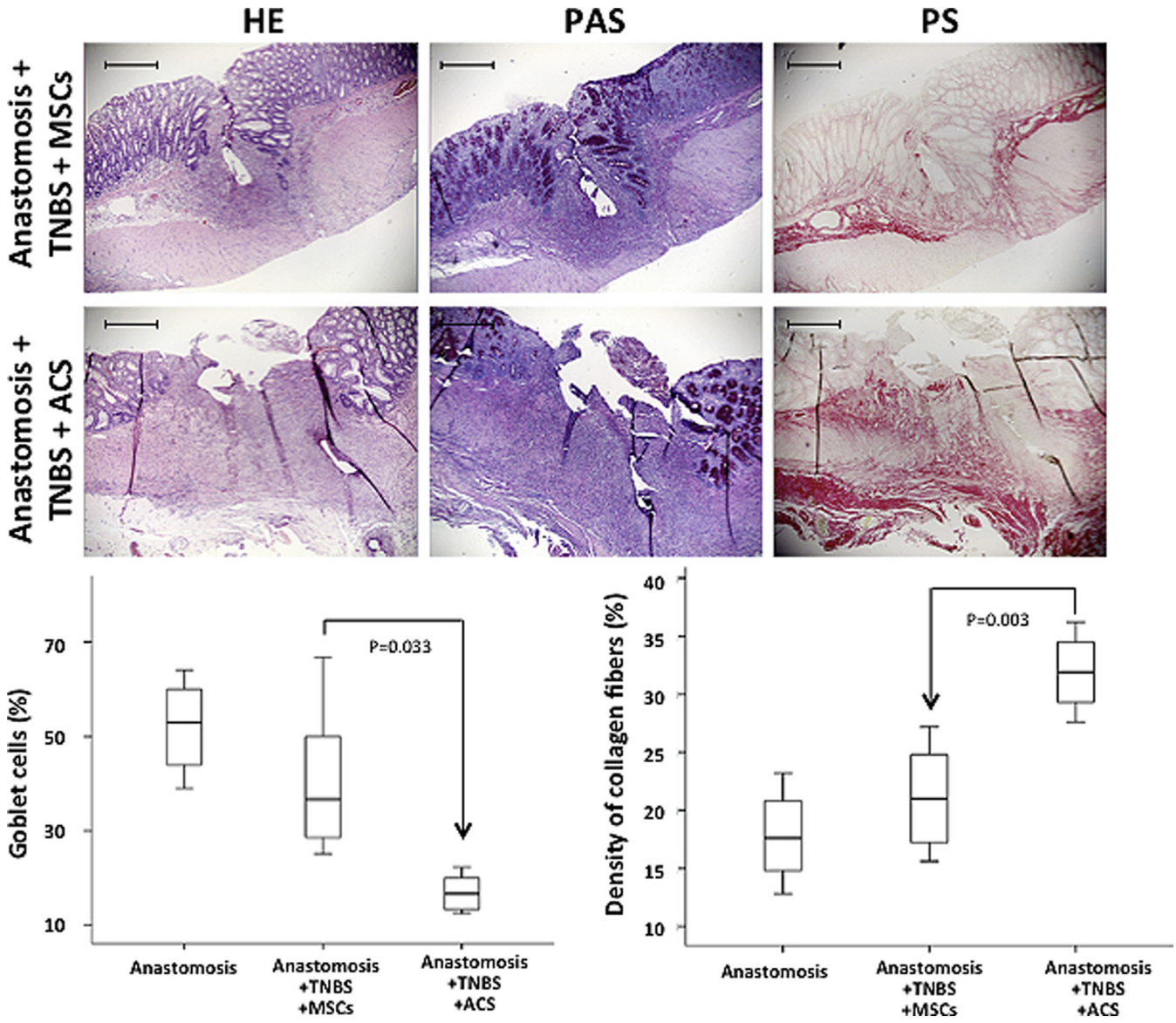

Fig 3. Treatment with adipose tissue-derived mesenchymal stromal cells (AT-MSCs) preserves the specialized epithelial cells and decreases the densities of collagen fibers in the high-risk colonic anastomosis. Quantitative analysis based on staining with periodic acid-Schiff (PAS) and phosphomolybdic acid-picrosirius red dye (PS) shows that AT-MSCs prevent the loss of goblet cells and the deposition of collagen fibers in intestinal anastomosis in the presence of TNBS-induced colitis. The analysis was performed by Kruskal-Wallis ANOVA on ranks test, in which multiple comparisons were carried out using the Dunnett's test. The horizontal bars represent the medians, the boxes represent the 25th and 75th percentiles, and the vertical lines below and above the boxes represent the minimum and maximum values, respectively. Length bars represent 50 μm. The data are representative of 3 independent experiments, with 4 to 5 animals per group.

with TNBS-induced colitis showed mucosal edema, inflammatory infiltrate in the lamina propria, lymphoid follicular hyperplasia, vascular congestion, decreased number of crypts, and transmural inflammation. In group 5 (TNBS-colitis plus anastomosis followed by ACS application), in addition to all changes related to TNBS-induced inflammation described in group 1, larger ulcers and apparent noncoaptation of the edges of the anastomosis were observed. In group 4, the local application of AT-MSCs resulted in an overall decrease in the histologic score, including improvement in the inflammatory process and less tissue damage, with clear signs of epithelization in the anastomosis area, compared to group 5 ($P = .011$; Fig 2, *A*). To investigate the role of neutrophils in the inflammatory process of this experimental model, myeloperoxidase activity was measured in colon explants. Myeloperoxidase activity was increased in samples from the TNBS-induced animals submitted to anastomosis treated with ACS (group 5) but was decreased after AT-MSCs ($P = .012$) to levels comparable to those observed in animals submitted to just a simple anastomosis (Fig 2, *B*).

In the epithelial compartment, the density of mucous-secreting goblet cells was inversely proportional to the inflammatory process observed in the colon. The densities of goblet cells were greater in samples from animals treated with AT-MSCs compared with TNBS-induced animals ($P = .033$; Fig 3).

Analysis of the density of collagen fibers in the colon tissue one week after performing the anastomosis revealed enhancement of fibrosis in TNBS-induced animals involving the mucosa, submucosa, and the anastomosis area. Separation and disorganization of mucosal muscle fibers was also observed, suggesting a direct effect of the enzymatic activity and tissue remodeling caused by the

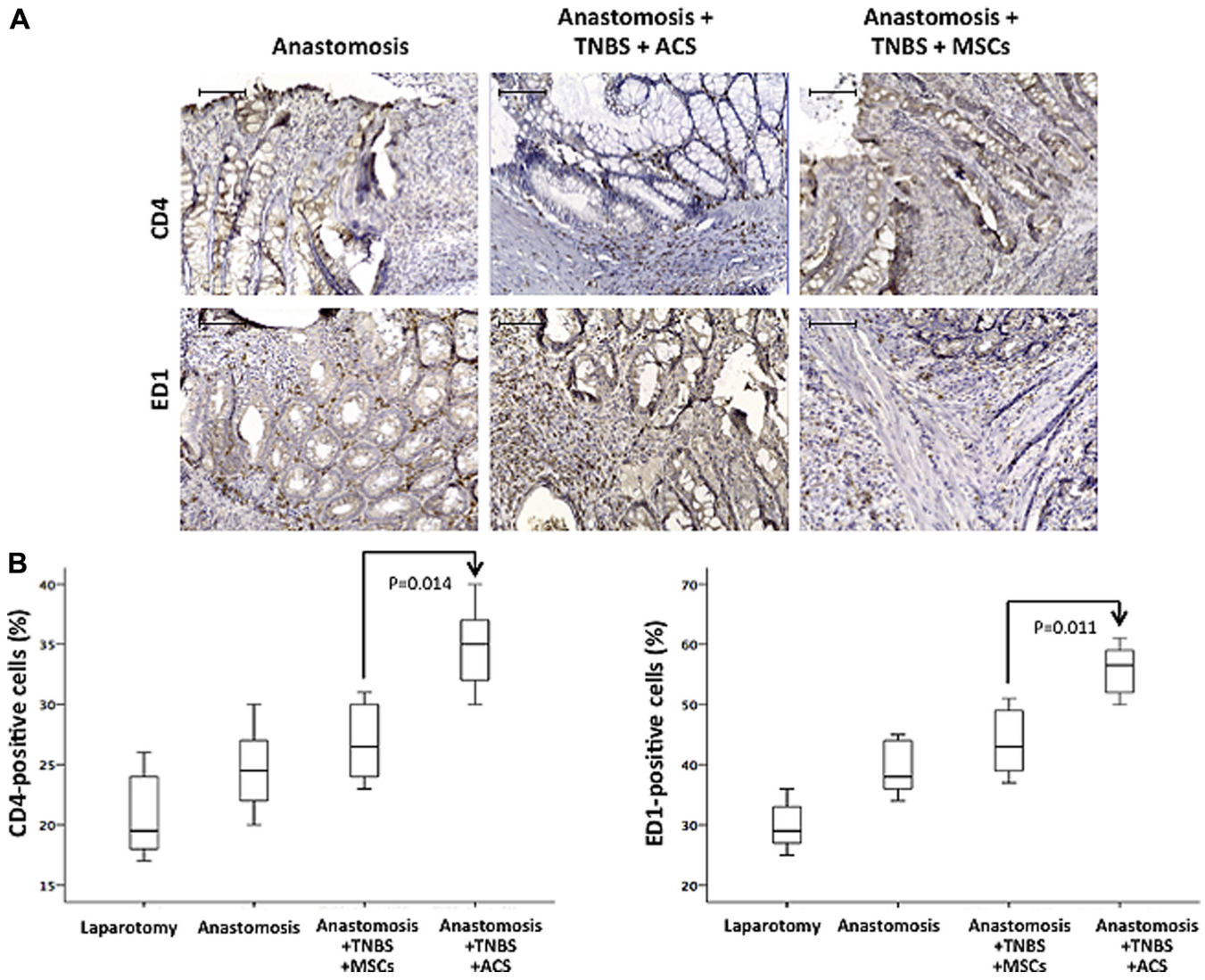

Fig 4. This high-risk colonic anastomosis is characterized by an intense inflammatory cell infiltration in the lamina propria, including CD4-positive T-lymphocytes and ED1-positive macrophages. Treatment with adipose tissue-derived mesenchymal stromal cells (AT-MSCs) attenuates accumulation of CD4- (*A*) and ED1-positive cells in the high-risk anastomosis (*B*). Colonic samples were submitted to immunoperoxidase analysis using mouse monoclonal anti-rat CD4 or mouse monoclonal anti-rat ED1. The analysis was performed by Kruskal-Wallis analysis of variance on ranks test, in which multiple comparisons were carried out using the Dunnett's test. The horizontal bars represent the medians, the boxes represent the 25th and 75th percentiles, and the vertical lines below and above the boxes represent the minimum and maximum values, respectively. Length bars represent 50 μm. The data are representative of 3 independent experiments, with 4 to 5 animals per group.

transmural inflammatory process. Deposition of collagen fibers was attenuated after treatment with AT-MSCs ($P = .003$; Fig 3).

To characterize the different cell populations present in the colon, immunoperoxidase experiments were performed to label CD4- and ED1-positive cells. The inflammatory cell accumulation observed in the lamina propria of TNBS-induced animals submitted to anastomosis treated with ACS (group 5) comprised an increased concentration of both CD4- and ED1-positive cells, however, both CD4- ($P = .014$) and ED1-positive cells ($P = .011$) decreased significantly after treatment with AT-MSCs (Fig 4).

To analyze the role of cell turnover in this model, we used a TUNEL assay to label apoptotic cells and anti-Ki67 to label proliferating cells. Samples from TNBS-induced animals submitted to anastomosis treated with ACS (group 5) showed markedly greater rates of apoptosis, but with a decrease after treatment with AT-MSCs ($P = .008$; Fig 5, *A*). No differences in cell proliferation rates analyzed by Ki67 immunohistochemical labeling were detected among the experimental groups (Fig 5, *B*).

Effect of AT-MSCs on gene expression and intracellular signaling in the colonic tissue

To investigate the molecular mechanisms by which AT-MSCs might control the local inflammatory response, we examined the mRNA expression of several genes potentially involved in the inflammatory process and tissue remodeling. The overall mRNA expression was increased for most target genes studied in samples from animals submitted to TNBS-induced colitis followed by anastomosis. In colon samples from TNBS-induced animals submitted to anastomosis and treated with ACS (group 5), mRNA levels of TGF-β, MMP-2, MMP-9, TNF-α, IFN-γ, and IL-17 were increased ($P < .05$) compared to animals submitted to a simple laparotomy (group 2), while MMP-2 and IL-17 were also increased compared to animals submitted to anastomosis only ($P < .05$). Among the animals in the high-risk anastomosis group, the only change observed after treatment with AT-MSCs compared to ACS was with IFN-γ mRNA expression ($P = .020$). Nevertheless, it is important to

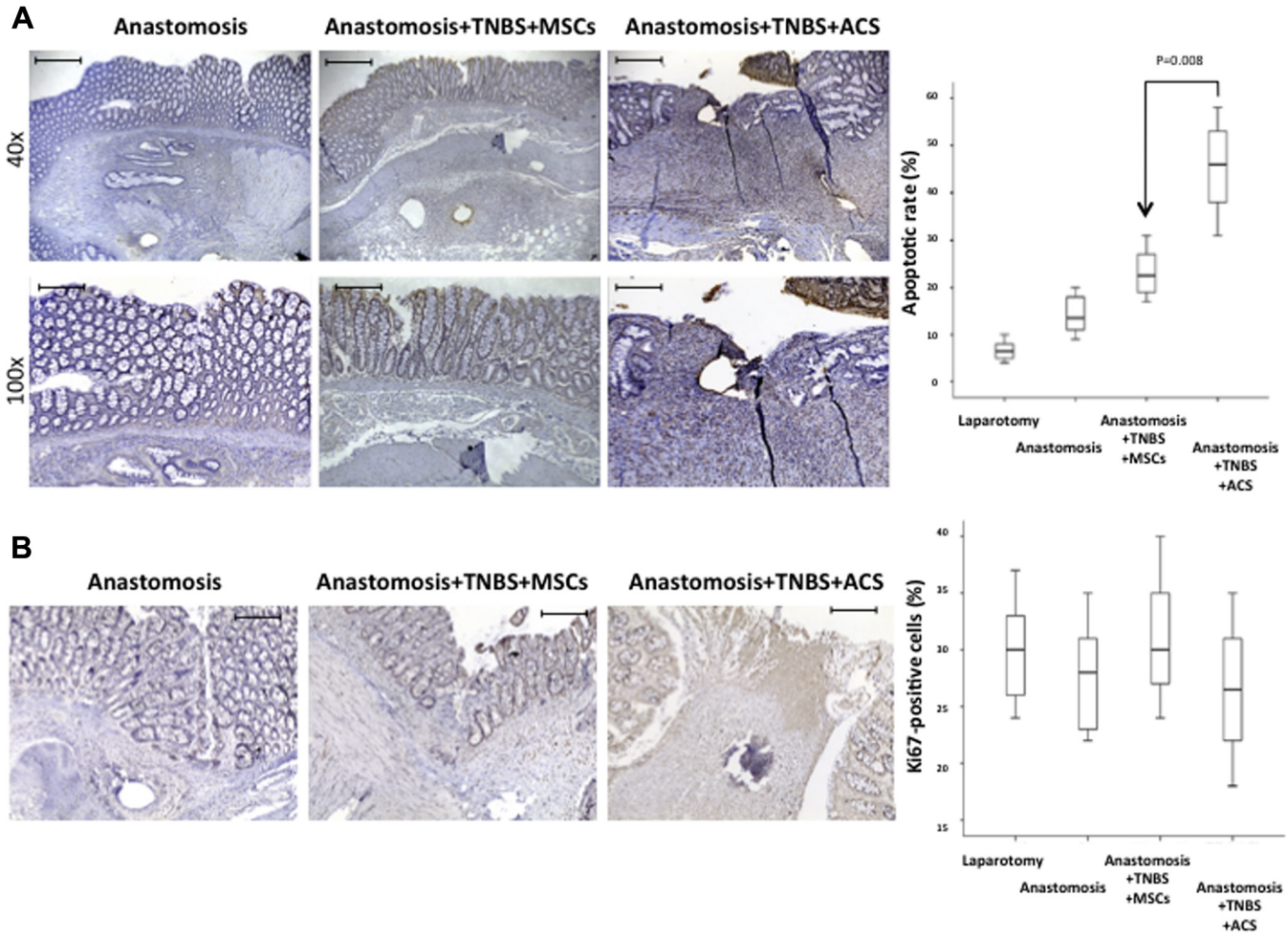

Fig 5. Treatment with adipose tissue-derived mesenchymal stromal cells (AT-MSCs) protects the colonic mucosa against apoptotic cell loss induced by the high-risk colonic anastomosis. Apoptotic cells in the colon were detected using a terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick-end labeling assay as shown by the representative photomicrographs (*A*). Quantitative analysis of Ki67-positive cells of intestinal anastomosis shows that the densities of Ki-67 proliferating cells are not different among the groups (*B*). The analysis was performed by Kruskal-Wallis analysis of variance on ranks test, in which multiple comparisons were carried out using the Dunnett's test. The horizontal bars represent the medians, the boxes represent the 25th and 75th percentiles, and the vertical lines below and above the boxes represent the minimum and maximum values, respectively. Length bars represent 50 μm. The data are representative of 3 independent experiments, with 4 to 5 animals per group.

highlight that treatment with AT-MSCs tended to decrease the expression of TGF-β, MMP-2, MMP-9, TNF-α, and IL-17 and to increase the expression of IL-10 (Fig 6).

With respect to signaling pathways, NF-κ B activation as measured by the nuclear translocation of the p65 subunit was drastically decreased in samples from TNBS-induced animals submitted to anastomosis after AT-MSCs treatment compared with ACS-treated animals ($P = .036$; Fig 7).

Discussion

In this study, the beneficial effects of the topical application of AT-MSCs were demonstrated in a high-risk colonic anastomosis in a rat model of colitis. Regarding efficacy, AT-MSCs prevented the mortality and complications associated with this high-risk colonic anastomosis, and consistently stabilized the intestinal architecture after 1 week of treatment. In particular, we demonstrated that topical application of AT-MSCs contributed to the preservation of epithelial integrity, stabilizing the number of mucous-producing goblet cells and the rate of epithelial apoptosis, while attenuating collagen deposition, inflammatory cell infiltration, and the local production of proinflammatory mediators. Moreover, as proposed previously by our group,[22] we present not only an experimental model of high-risk anastomosis but also a consistent novel model of transmural inflammation of the colon, with frequent formation of fistula and abscess, similar to what is expected in penetrating human CD.

Currently, a potential limitation to the successful implementation of MSC-based therapy is the inability to drive these cells to specific locations in the body. In a previous study from our group investigating cell trafficking and tissue localization of exogenously applied MSCs, we demonstrated that intravenously administered MSCs do not reach the colon and do not improve TNBS-induced inflammation. In contrast, intraperitoneal administration rapidly directed cells toward the inflamed colon with relevant beneficial therapeutic effects.[13] Our previous experience with MSCs and in particular AT-MSCs homing to the inflamed colon after intraperitoneal injection, prompted us to investigate whether direct administration on the anastomosis would facilitate the possible anti-inflammatory actions of AT-MSCs.

Appropriate operative technique, including gentle handling of the bowel, approximation of well-vascularized tissues, and avoidance of tension at anastomosis are regarded as critical principles for a successful intestinal anastomosis.[23] In this study, the greater

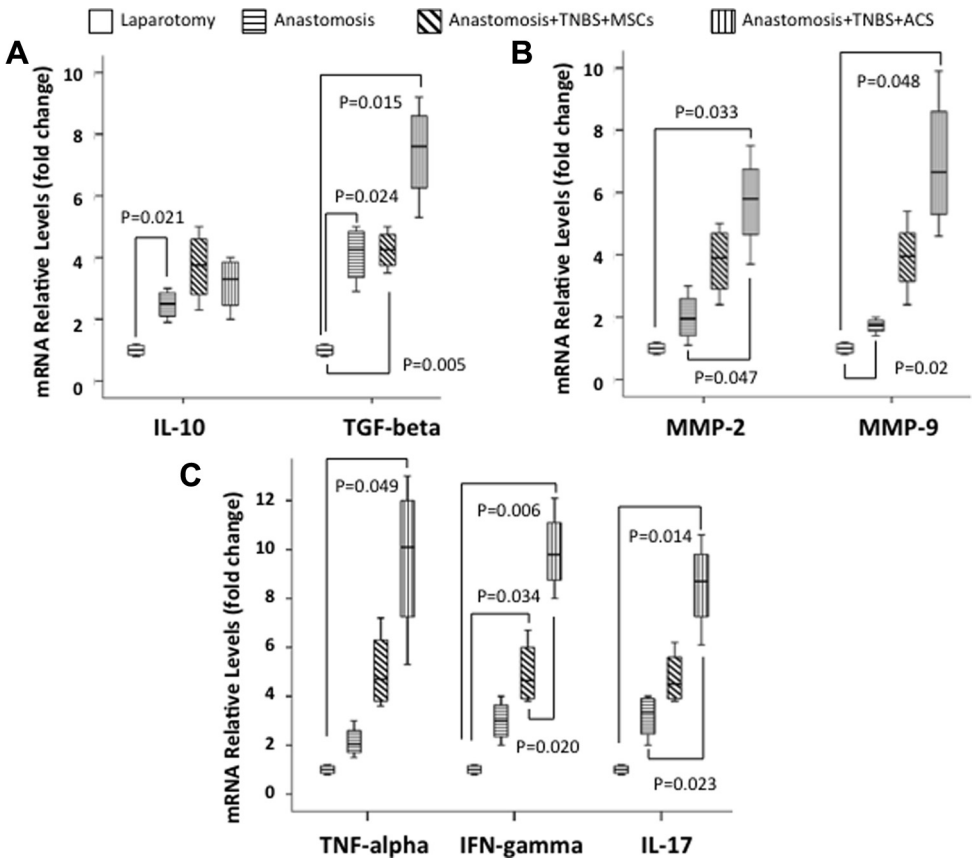

Fig 6. Treatment with adipose tissue-derived mesenchymal stromal cells (AT-MSCs) modulated the expression of genes involved in the immune response and tissue remodeling affected by the high-risk colonic anastomosis. Quantitative real-time PCR was used to measure the levels of IL-10 and TGF-β (*A*), the metalloproteinases MMP2 and MMP9 (*B*), and TNF-α, IFN-γ, and IL-17 (*C*). The horizontal bars represent the medians, the boxes represent the 25th and 75th percentiles, and the vertical lines below and above the boxes represent the minimum and maximum values, respectively. The analysis was performed by Kruskal-Wallis analysis of variance on ranks test, in which multiple comparisons were carried out using the Dunnett's test. The data are representative of 3 independent experiments, with 4 to 5 animals per group.

mortality and development of complications among animals submitted to anastomosis seem to indicate the inherent risks of the procedure and corroborate well-known anastomosis drawbacks reported in human studies, especially in an inflamed bowel.[1,24,25] It is notable that no deaths and fewer postoperative complications were observed among the animals treated with AT-MSCs, clearly indicating a protective effect in the model of high-risk anastomosis. Similar to previous studies using models of bowel anastomosis in rats,[26–28] perianastomotic adhesions were detected in practically all animals subjected to anastomosis. Nevertheless, in this study, adhesions were dramatically more abundant among the animals with induced colitis, but they were consistently prevented by the treatment with AT-MSCs.

Histologic analysis of samples from animals submitted to the anastomotic procedure showed several abnormalities within the bowel wall, including inflammatory cellular infiltration with follicular lymphoid hyperplasia, edema, vascular congestion, and a decrease in the number of crypts similar to previous observations.[29] In this study, histopathologic findings were greatly accentuated in the animals with the colitis, with large areas of deep ulcerations and fistula formation, whereas treatment with AT-MSCs markedly improved the morphologic aspects of the bowel, with clear decreases in inflammatory changes secondary to high-risk anastomosis. In regard to the epithelial layer, the tissue damage consisting of ulcerations and the depletion of terminally differentiated mucous-secreting goblet cells was greatly reversed after treatment with AT-MSCs. With respect to epithelial homeostasis, although cell proliferation does not appear to have had an important role, mucosal apoptosis was dramatically decreased in the colitic areas of anastomosis after treatment with AT-MSCs.

Comparable to human CD, the TNBS-induced colitis murine model has been known to result in deep inflammatory cell infiltrates composed of CD4-positive T cells, macrophages, and neutrophils in the intestinal lamina propria.[30,31] The accumulation of CD4-positive T cells and macrophages in the lamina propria, in addition to activated neutrophils as reflected by the increased myeloperoxidase activity, showed that this model of high-risk anastomosis is compatible with the triggering of both the adaptive and innate immune mechanisms and is markedly mitigated by treatment with AT-MSCs.

The beneficial effects of AT-MSCs have also been demonstrated in other models of high-risk anastomosis. For example, in a model of anastomosis performed in a previously irradiated colon, AT-MSCs were shown to promote healing via a mechanism attributed to enhanced vessel formation alongside decreased inflammation.[17] In a model of ischemic colonic anastomosis in rats, the favorable effects of AT-MSCs on healing acceleration were similarly attributed to increased angiogenesis, but also to augmented bursting pressure and collagen deposition.[32]

Considering the underlying mechanisms fueling the local inflammatory process investigated in this study, the results support a predominant Th1 and Th17 response, with increased expression of IFN-γ, TNF-α, and IL-17 in samples from high-risk anastomosis. Previous studies have shown that some models of experimental

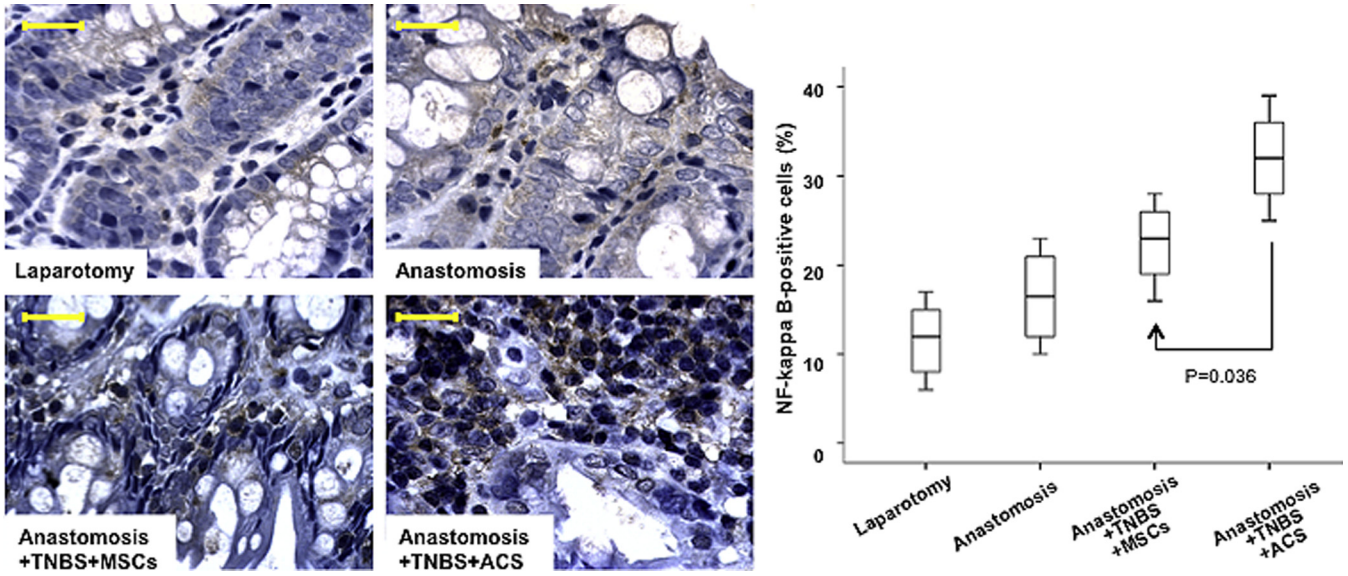

Fig 7. Treatment with adipose tissue-derived mesenchymal stromal cells (AT-MSCs) attenuated the expression of the NF-κ B intracellular signaling pathway involved in cytokine production and cell survival triggered by the high-risk colonic anastomosis. Colonic samples were submitted to immunoperoxidase analysis using mouse monoclonal anti-rat NF- κ B. The analysis was performed by Kruskal-Wallis analysis of variance on ranks test, in which multiple comparisons were carried out using the Dunnett's test. The horizontal bars represent the medians, the boxes represent the 25th and 75th percentiles, and the vertical lines below and above the boxes represent the minimum and maximum values, respectively. Length bars represent 20 μm. The data are representative of 3 independent experiments, with 4 to 5 animals per group.

colitis[33,34] and human CD[35] are based on both Th1 and Th17 responses. Therefore, in this study, we have interpreted the nature of the immune response as being basically resultant from TNBS-induced inflammation, with a marked enhancement caused by the operative procedure. In fact, although only IFN-γ was decreased in the high-risk anastomosis after AT-MSCs, both TNF-α and IL-17 also showed a clear tendency to be decreased. It is possible that the number of animals in the experiment might have influenced the statistical analysis. It is also likely that the number of AT-MSCs injected might not have been sufficient to sustain the anti-inflammatory response during the study period. Nevertheless, analyzing gene expression only may not reflect the actual production of cytokines and other molecules, while the timing of tissue harvesting may also affect the levels of mRNA in the present study.

In the present model of high-risk anastomosis, the increased activation of NF-κ B suggested the probable engagement of this intracellular signaling pathway in the underlying inflammatory process. Proinflammatory cytokines in turn can induce NF-κ B activation, thereby leading to the overexpression of adhesion molecules in endothelial vessels and the consequent increase in leukocyte recruitment.[36] Therefore, NF-κ B activation can lead to a positive feedback loop controlling the immune response in experimental models[37] and in human IBD[38] that is also activated by proinflammatory cytokines.[39]

Of the anti-inflammatory genes analyzed in this study, the expression of IL-10 showed an opposing pattern compared to the proinflammatory genes; however, no difference in the expression of IL-10 could be demonstrated in samples from high-risk anastomosis after treatment with AT-MSCs. In contrast, the greatest expression of TGF-β, another key anti-inflammatory cytokine, detected in samples from animals submitted to high-risk anastomosis displayed a similar pattern as IFN-γ, TNF-α, and IL-17. Although the expression of TGF-β did not decrease after AT-MSC treatment, we noticed a tendency toward reduction, reaching levels similar to the samples from animals submitted to a simple anastomosis. These findings seem to be in accordance with the notion that TGF-β is also regulated by inflammatory cytokines.[40] Moreover, these observations support the idea that the immunomodulatory action of MSCs is pleiotropic and, similar to other studies,[41,42] it might affect predominantly the production of proinflammatory cytokines.

In samples from animals with the high-risk anastomosis, the increased densities of collagen fibers throughout the intestinal wall were markedly decreased after treatment with AT-MSCs. This effect also seems to involve TGF-β, which plays a critical role in extracellular remodeling in inflammatory diseases,[43] whereas excessive collagen deposition has been implicated in tissue fibrosis.[44,45] In fact, in addition to TGF-β, proinflammatory cytokines have been shown to stimulate the production of matrix metalloproteinases (MMPs) and collagen fibers by myofibroblasts and fibroblasts in the intestinal mucosa, further contributing to matrix remodeling and fibrosis.[46] In IBD and especially in CD, remodeling of the extracellular matrix resulting from chronic inflammation has been implicated frequently in tissue fibrosis.[47] With regard to MMPs, greater expression has been associated with the greater recurrence of fibrotic strictures in samples from patients with CD.[48] In agreement with these reports, we also found an overexpression of MMPs, particularly MMP2 and MMP9, in samples from animals with high-risk anastomosis, mimicking transmural penetrating CD. Between the third and fifth day after the operative anastomosis, weakening of mechanical resistance is expected to occur, and dehiscence during this period has been associated with increased enzymatic expression.[49,50] Although this may seem conflicting with our results showing that the healing effect of AT-MSCs is accompanied by a tendency toward a decrease in the expression of MMPs, this may be explained owing to the later time-point analyzed in this study. In fact, the dynamics of gene expression and extracellular matrix homeostasis during the inflammatory process would require serial analyses for a precise understanding of the healing process.

Our work has limitations intrinsic to experimental models and also regarding the study design. Although obviously not completely representative of human disease, experimental models have provided valuable insights into the complex mechanisms underlying the development and pathogenesis of various diseases. Whereas

translation of results obtained with animal models of noncolitic colonic anastomosis have been quite successful, experimental data in high-risk anastomosis mimicking human IBD are currently lacking. In this study, we used the model of TNBS-induced colitis first described in rats,[51] but given its limitations regarding correspondence to humans, we understand that additional studies with different species will be required to assure a reliable translational outcome. In contrast, it is important to be aware of discrepancies in the biologic responses between animal and human,[52] and results of experimental systems should be translated into clinical practice with caution. [51,53,54] Regarding the route of administration of mesenchymal stem cells, currently there is still no consensus about the best mode of delivery and targeting to the site of need. In a previous study conducted by our research group, we showed that intravenous administration of MSCs not only failed to reach the colon but also did not attenuate the inflammation induced by TNBS. In contrast, intraperitoneal administration had favorable therapeutic effects.[13] Owing to divergent results in the scientific literature regarding the best route of administration, in the present study, we proposed the topical administration onto the surface of the anastomosis, considering the easy applicability of this method and similarity with our previous successful result (intraperitoneal). Instilling cells directly onto the anastomosis is easy to perform during the operative procedure and, in theory tissue biodistribution may be faster than a nonspecific intraperitoneal administration. Moreover, it has the advantage of being directly in contact with the surface of the anastomosis unlike the intraperitoneal administration, which may suffer an unpredictable distribution due to the formation of abdominal adhesions. Additional studies will be necessary to establish the best route for achieving the best outcome.

In conclusion, the successful treatment of a high-risk colonic anastomosis in a colon with colitis with local administration of AT-MSCs supports the idea of using these cells for the prevention of anastomotic dehiscence in the context of intestinal inflammation. Improvements in operative outcomes of high-risk anastomoses in response to AT-MSCs therapy reflect the immunomodulatory activity and healing effect of these cells, even after topical administration. In light of the ease and safety of isolation from abundant adipose tissue and the rapid ex vivo expansion, AT-MSCs have emerged as an alternative to nonmyeloablative cell-based therapy for the prevention of dehiscence of a high-risk colonic anastomosis and reinforces their use in future translational research. In addition, we present herein a rat model of high-risk colonic anastomosis that can be regarded as a novel and effective model for penetrating CD.

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Conflict of interest/Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.surg.2019.07.023.

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