Effects of Ghrelin on the Oxidative Stress and Healing of the Colonic Anastomosis in Rats

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Abstract

Background: Anastomotic leakage is the deadliest complication of colonic procedures. Ghrelin is an orexigenic hormone with potent actions on growth hormone release and functions in the processes of growth, tissue inflammation, repair, and oxidative stress. We evaluated the hypothesis that the exogenous administration of ghrelin causes beneficial effects on the healing of colonic anastomosis.

Materials and methods: Sixty-four male Wistar rats were randomly assigned to eight subgroups receiving postoperative intraperitoneal administration of ghrelin (23 µg/kg/d) or saline after a colonic anastomosis. The anastomotic tissue was evaluated on the third, seventh, and 14th postoperative days. Anastomotic bursting pressure, histological parameters, hydroxyproline content, and tissue oxidative stress markers were compared.

Results: There was a significant increase in the mean anastomotic bursting pressure in the ghrelin subgroup on the seventh postoperative day (P = 0.035). Histological evaluation demonstrated a significant difference in the neutrophilic infiltrate (P = 0.035) on the third and 14th day and in apoptosis (P = 0.004), granulation tissue (P = 0.011) and peritoneal inflammation (P = 0.014) on the 14th postoperative day. There was a statistically significant increase in the hydroxyproline content in the ghrelin subgroup on the 14th postoperative day (P = 0.043). There were significant differences in the nitrite tissue levels (P = 0.021) on day 3 and in reactive oxygen species (P = 0.012) on day 14.

Conclusions: The administration of ghrelin had beneficial anti-inflammatory and antioxidant effects, increasing the resistance of the anastomosis and the hydroxyproline tissue content in the postoperative period.

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Introduction

Colorectal surgery has incorporated important technical advances and scientific knowledge in the last several decades, including staplers, antibiotics, bowel preparation, and laparoscopic access, all of which have improved the safety and efficacy of this procedure. Nevertheless, colorectal procedures are still associated with important postoperative complications. Anastomotic leakage is the most severe complication and has an incidence between 2% and 16%. It is associated with higher rates of morbidity and mortality, higher hospital costs, and worse oncological results.

Ghrelin was originally identified, purified, and characterized by Kojima et al. in 1999 from rat stomach. It subsequently received notoriety as the first known endogenous ligand of the GH-secretagogue receptor (GHS-R). Ghrelin is a potent GH-releasing and appetite-stimulating peptide consisting of 28 amino acids, in which serine 3 is modified by a fatty acid (n-octanoic acid) in the activated form.

It has been demonstrated in experimental studies that the intracerebral, intraperitoneal, or subcutaneous administration of ghrelin avoids damage to the gastric mucosa and accelerates healing in ethanol ischemia/reperfusion injury and in injuries due to alendronate or hydrochloric acid. Ghrelin increases the release of local nitric oxide (NO) and the serum levels of tumor necrosis factor-α (TNF-α) and nuclear factor kappa B (NF-κB), which stimulate proinflammatory tissue cytokines. In addition, ghrelin has decreased myeloperoxidase and malondialdehyde activity and decreased cell apoptosis, cytochrome P, and caspase 3 release, which are associated with oxidative stress and healing delays.

In the bowel, it has been proven that ghrelin administered intravenously or intracerebroventricularly has important postoperative protective effect against the damage induced by ischemia and reperfusion in rats. Ghrelin induces a reduction in the release of inflammatory cytokines (TNF-α and IL-6) and neutrophilic infiltrate by decreasing myeloperoxidase activity. Sen et al. found a significant decrease in NF-κB levels, recently described these findings and showed the inhibition of glutathione oxidase depletion in the ileal segments after ischemia/reperfusion injury, suggesting an antioxidant protective effect.

It has also been recently demonstrated that the administration of ghrelin in rats subjected to acetic acid–induced colitis increases blood flow and local DNA synthesis in the inflamed colonic mucosa, exhibiting potent anti-inflammatory actions, promoting a significant reduction in the levels of TNF-α, IL-1β and myeloperoxidase activity, and accelerating the process of cell regeneration.

These data demonstrated that ghrelin influences and improves repair and intestinal healing, acting as an anti-inflammatory or antioxidant agent. In the present study, we demonstrated that the administration of ghrelin in the postoperative period after colonic anastomosis could attenuate inflammation, apoptosis, and oxidative stress and improve the anastomotic bursting pressure in rats.

Animals and surgical procedures

Sixty-four male Wistar rats (Rattus norvegicus) from the Central Animal Facility at the Federal University of Santa Catarina (UFSC) were used in this research, which was previously approved by Ethics Committee on Animal Use of UFSC, under case PP0915/146–2014. The animals weighed between 300 and 350 g and were between 160 and 180 d old. They were acclimated for 7 d at the Laboratory of Experimental Surgery and Operative Technique of the UFSC while receiving food and water ad libitum in standardized conditions. The room was maintained at a constant temperature of 23 ± 2°C, with light and dark cycles alternating every 12 h and humidity from 50% to 60%. All procedures were performed in accordance with the Brazilian guidelines for animals in research.

The procedures were performed with the animals under general anesthesia by using an intramuscular solution of ketamine (9 mg/100 g body weight) and thiaze (1.25 mg/100 g body weight). A 2-cm middle laparotomy was performed under aseptic conditions. The sigmoid colon was identified and sectioned with straight scissors 2 cm above the peritoneal reflection. Then, an end-to-end anastomosis was performed with eight separate stitches of 6-0 polypropylene (Ethicon, Somerville, NJ) in the extramucosal layer. Abdominal wall closure was performed in two planes with continuous 4-0 nylon sutures (Ethicon, Somerville, NJ).

Reoperations were performed under the same conditions. The prior anastomosis was identified, and the sigmoid colon containing the anastomosis segment was extracted for analysis.

Groups

The animals were randomized into two groups based on the postoperative administration of ghrelin (G) or saline (S). Each group was distributed into four subgroups of eight rats (n = 8), which were named according to the time of the reoperation and euthanasia in the postoperative period: sham saline (SS), sham ghrelin (SG), saline and 3 d (S3), ghrelin and 3 d (G3), saline and 7 d (S7), ghrelin and 7 d (G7), saline and 14 d (S14), and ghrelin and 14 d (G14) (Fig. 1).

In the saline group, the animals received 1 mL of saline solution; in the ghrelin group, the animals received 23 μg/kg/d of rat ghrelin (Tocris, Bioscience, Bristol, UK) dissolved in 1 mL of saline solution. In both groups, the administration was made by daily intraperitoneal (IP) injection during the postoperative period with randomization.

Anastomotic bursting pressure measurements

A 2-cm sigmoid segment containing the anastomosis centrally was identified and maintained in the abdominal cavity. One end was closed with 2-0 silk suture, and the other end was introduced into a 6-Fr polyvinyl catheter occluded with 2-0 silk thread. The catheter was connected in a “Y” configuration system to a continuous infusion pump (Samtronic, São Paulo, Brazil).
Paulo, Brazil) on one side and to a mercury column manometer (Unitec, São Paulo, Brazil) on the other side. The abdominal cavity was full of saline solution, and the colon segment was submerged and inflated with air at 2 mL/min. The anastomotic bursting pressure value was recorded in millimeter of Hg at the moment of rupture of the anastomotic line and exhaust air.

Immediately after the pressure measurement, a 2-cm segment containing the anastomosis was resected and sectioned longitudinally into three identical strips, each containing a portion of the anastomosis centrally. One of the fragments was stored in a 10% formalin solution for histopathological study, and the other two were frozen with liquid nitrogen and stored in cryotubes separately and placed in a freezer at −80°C for the analysis of oxidative stress enzymes and markers.

**Histopathological evaluation**

The fragments were preserved in 10% formalin solution and were dehydrated and embedded in paraffin. The paraffin blocks were sectioned on a microtome (Leica 2040 Autocut, Wetzlar, Germany) with 2-micron thick cuts, and the slices were stained with hematoxylin and eosin and with Masson trichrome stain.

The tissues with anastomosis were examined under an optical microscope (Nikon Eclipse Ni, Sendai, Japan) at 400 magnification by a single pathologist in a blinded fashion using the semiquantitative method. The parameters evaluated were neutrophil infiltrate, fibrin deposition, fibroblast infiltration, collagen deposition, granulation tissue, edema, and apoptosis. These parameters were classified by assigning scores: 0 = absent, 1 = mild, 2 = moderate, and 3 = severe.

**Measurement of antioxidant enzymes, nitrite levels, reactive oxygen species, glutathione levels, and nuclear factor erythroid 2–related factor 2**

The frozen fragments were homogenized in ice-cold PBS. After centrifugation, the supernatant was used in a blinded fashion for the measurement of the glutathione (GSH), nitrite (NO₂⁻), and reactive oxygen species (ROS) levels.

**Protein content analysis by western blot analysis**

Glutathione peroxidase, NADPH quinone dehydrogenase 1, heme oxygenase 1, Nrf2, and p-Nrf2

The colon was homogenized in 5 volumes of 50 mM Tris (pH 7.0) containing 1 mM EDTA, 100 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM Na₂VO₄, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail (Roche, Mannheim, Germany). Then, the samples were centrifuged at 10,000 x g for 10 min at 4°C, and the supernatant was boiled at 100°C for 5 min. The protein content was quantified by Lowry's method. Samples were diluted in 25% 100 mM Tris buffer (pH 6.8, with 40% glycerol and bromophenol blue) and 8% β-mercaptoethanol. An aliquot of 50 μg of total protein was size-separated by electrophoresis in a 14%-16% SDS-polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane. After washing and blocking, the membranes were incubated overnight with the primary antibodies antiguathione peroxidase 1 (GPx-1, 22 kDa, 1:5,000, Abcam, Cambridge), NAD(P) H:quinone oxidoreductase 1 (NQO1, 29 kDa, 1:1,000, Cell Signaling, Danvers), heme oxygenase 1 (HO-1, 33 kDa, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA), nuclear factor (erythroid-derived 2)–like (Nrf2, 97-100 kDa, 1:1,000, Cell Signaling, Danvers) and phospho-Nrf2 S40 (p-Nrf2, 100 kDa, 1:1,000, Abcam, Cambridge). Loading evenness was verified by incubating the membranes with the primary antibody β-actin (β-actin, 43 kDa, 1:2000, Santa Cruz Biotechnology, Santa Cruz, CA). Afterward, the membranes were exposed to the anti-rabbit (1:2000; Cell Signaling; Danvers) or anti-mouse (1:2000; Cell Signaling; Danvers) secondary antibodies. The immunocomplexes were visualized using an luminol-based enhanced chemiluminescence detection system (GE Healthcare, São Paulo, SP, Brazil) and the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

**Protein determination**

The sample protein content was determined by Lowry’s method using bovine serum albumin as the standard.

**Reduced glutathione levels**

GSH levels were quantified by a fluorimetric assay according to Hissin et al. using o-phthalaldehyde (Sigma Aldrich, St. Louis, MO) as a derivative. The GSH content was calculated based on a GSH standard curve and expressed in μmol GSH/mg protein.

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*Fig. 1 — Distribution of the study subgroups. S = saline subgroup; G = ghrelin subgroup; PO = postoperative.*
Nitrite levels
The concentration of NO (indirectly determined by nitrite dosages) was measured by the Griess method. Equal volumes of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl) ethylene diamine, St. Louis, MO) and colon homogenate were mixed and incubated for 10 min at room temperature. The absorbance was read at 500 nm. The concentration of NO was determined by comparison with a standard curve of sodium nitrite, and the results are expressed as μmol nitrite/mg protein.20

Reactive oxygen species
This assay is based on the reaction of nitroblue tetrazolium with ROS. The reagent nitroblue tetrazolium is reduced by superoxide (O2•−) to formazan, a purple-blue compound with absorbance at 630 nm. ROS generation was expressed as the percentage of saline.21

Statistical analysis
The results are expressed as the mean ± standard deviation. The Shapiro–Wilk normality test showed that the data had a normal distribution (P < 0.05). The means between the subgroups on the same postoperative day were compared using an unpaired two-tailed t test. All statistical analyses were performed using the software SPSS 22 for Windows (Chicago, IL).

Results
No signs of anastomotic leakage were identified in the evaluated colonic segments, and no deaths occurred during the study. There were differences in weight loss and weight gain between the subgroups, but these differences were not statistically significant when comparing the subgroups in the same postoperative period.

Bursting pressure
The anastomotic bursting pressure showed no statistically significant differences between subgroups SS and GS, between subgroups S3 and G3, and between subgroups S14 and G14. However, on the seventh day, the means (±standard deviations) of subgroups S7 and G7 were 100.25 (±48.94) and 147.00 (±22.90) mm Hg (P = 0.035), respectively (Fig. 2).

Histopathological assessment
There were no statistically significant differences in the histopathological parameters between the SS and GS subgroups. The means of neutrophil infiltrate were higher in saline subgroups S3 and S7 than in ghrelin subgroups G3 and G7 (2.75 ± 0.46 versus 2.00 ± 0.75, P = 0.035) (Table 1) and (2.00 ± 0.75 versus 1.25 ± 0.46, P = 0.035) (Table 2), respectively.

Similarly, on the 14th d, there were statistically significant differences between subgroups S14 and G14 in terms of granulation tissue (2.38 ± 0.51 versus 1.25 ± 0.46, P = 0.011) and apoptosis (2.00 ± 0.75 versus 1.75 ± 0.70, P = 0.011), respectively (Table 2).

The mean differences of the scores were not statistically significant for any of the parameters evaluated in subgroups S7 and G7 on the seventh day (Table 3).

Table 1 – Means, standard deviations (SDs), and P values of histopathological scores in the third postoperative day.

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>Histological parameters</th>
<th>Saline Mean (±SD)</th>
<th>Ghrelin Mean (±SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil infiltrate</td>
<td>2.75 (±0.46)</td>
<td>2.00 (±0.75)</td>
<td>0.035</td>
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<tr>
<td>Fibrin deposition</td>
<td>2.63 (±0.51)</td>
<td>2.38 (±0.51)</td>
<td>0.350</td>
<td></td>
</tr>
<tr>
<td>Fibroblast infiltration</td>
<td>1.25 (±0.46)</td>
<td>1.25 (±0.46)</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Collagen deposition</td>
<td>1.13 (±0.35)</td>
<td>1.13 (±0.35)</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Granulation tissue</td>
<td>1.75 (±0.48)</td>
<td>1.88 (±0.83)</td>
<td>0.717</td>
<td></td>
</tr>
<tr>
<td>Edema</td>
<td>2.25 (±0.46)</td>
<td>2.00 (±0.75)</td>
<td>0.438</td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>2.38 (±0.51)</td>
<td>1.88 (±0.64)</td>
<td>0.108</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05/unpaired two-tailed t test.
Means, standard deviations (SDs), and \( P \) values of histopathological scores in the 14th postoperative day.

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>Saline</th>
<th>Ghrelin</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological parameters</td>
<td>Mean (±SD)</td>
<td>Mean (±SD)</td>
<td></td>
</tr>
<tr>
<td>Neutrophil infiltrate</td>
<td>2.00 (±0.75)</td>
<td>1.25 (±0.46)</td>
<td>0.035</td>
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<tr>
<td>Fibrin deposition</td>
<td>1.50 (±0.92)</td>
<td>1.00 (±0.53)</td>
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<tr>
<td>Fibroblast infiltration</td>
<td>2.25 (±0.70)</td>
<td>1.75 (±0.46)</td>
<td>0.120</td>
</tr>
<tr>
<td>Collagen deposition</td>
<td>2.38 (±0.51)</td>
<td>2.38 (±0.74)</td>
<td>1.000</td>
</tr>
<tr>
<td>Granulation tissue</td>
<td>2.38 (±0.91)</td>
<td>1.25 (±0.46)</td>
<td>0.011*</td>
</tr>
<tr>
<td>Edema</td>
<td>0.00 (±0.00)</td>
<td>0.25 (±0.46)</td>
<td>1.000</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>2.00 (±0.75)</td>
<td>0.75 (±0.70)</td>
<td>0.004*</td>
</tr>
</tbody>
</table>

\* \( P < 0.05/\text{unpaired two-tailed } t \text{ test.} \)

Reactive oxygen species and nitrite levels

The tissue concentrations of ROS increased after trauma due to an elevation in oxidative status. These levels returned to basal levels by the action of antioxidant defenses. In this study, the ROS levels increased immediately after the surgical trauma in the surgery subgroups compared with sham subgroups. In the comparison between subgroups S3 and G3 (150.18 ± 39.02 versus 133.31 ± 45.97, \( P = 0.761 \)) and subgroups S7 and G7 (110.50 ± 15.74 versus 113.50 ± 29.91, \( P = 0.820 \)), there were no statistically significant differences. However, the decrease in ROS levels was greater in G14 than in S14 on the 14th d (98.93 ± 19.46 versus 163.19 ± 48.61, \( P = 0.012 \)), and this difference was statistically significant (Fig. 3).

The NO\textsubscript{2} levels decreased in all surgery subgroups compared with the sham subgroups. There were statistically significant differences between subgroups S3 and G3 (5.04 ± 0.88 versus 3.91 ± 0.85, \( P = 0.021 \)). The comparison between subgroups S7 and G7 and between S14 and G14 did not identify statistically significant differences (Fig. 4).

Antioxidant enzymes: HO-1, GPx, and NQO1

Antioxidant enzymes are released after surgical trauma in response to ROS elevation. In this study, HO-1 and GPx increased in all surgery subgroups compared with the sham group. The HO-1 levels were much higher in the ghrelin subgroup on the third day (33.04 ± 23.37 versus 19.48 ± 18.09, \( P = 0.289 \)) and were higher on the seventh day (14.91 ± 13.15 versus 10.99 ± 10.87, \( P = 0.587 \)) than in the sham group; however, these results were not statistically significant (Fig. 5).

The GPx levels were constant and high in the ghrelin subgroups, as in the saline subgroups, these changes did not present statistical significance (Fig. 5).

The level of NQO1 decreased after trauma in all surgery subgroups compared with the sham subgroups. The return to basal levels on the 14th d was faster in the ghrelin subgroup than in the saline subgroup (0.94 ± 0.48 versus 0.72 ± 0.31, \( P = 0.368 \)); however, this difference was not statistically significant (Fig. 5).

Glutathione levels

The amounts of GSH in the scar tissue in the surgery subgroups were lower than those in the sham subgroups. Between subgroups S3 and G3 and between S7 and G7, there were no statistically significant differences; however, between S14 and G14 (560.34 ± 121.06 versus 661.43 ± 75.58, \( P = 0.073 \)), the differences were almost significant (Fig. 6).

Nuclear Factor Erythroid 2–related factor 2

The relative intensities of Nrf2 and p-Nrf2 were much higher in the surgery subgroups than in the sham subgroups. In all surgery subgroups, the means in the saline subgroups were higher than those in the ghrelin subgroups; however these results were not statistically significant, as shown in Figure 7.

Discussion

The reduction in the incidence of dehiscence and anastomotic fistulas in the postoperative period of colorectal procedures and the identification of their risk factors have been the subject of several studies. Different strategies have been tested with the objective of reducing their serious clinical repercussions, which often involve multiple surgical interventions, ostomies, longer hospital stays, and worse cancer prognoses.

Ghrelin is an orexigenic hormone with a potent central action, inducing the release of GH, regulating several growth factors and acting on mechanisms of cellular migration, angiogenesis, and proliferation that are indispensable for healing. Ghrelin also has potent anti-inflammatory and antioxidant effects, with an impact on redox homeostasis that accelerates the healing process in several tissues.

Ceran et al. observed an increase in the bursting pressure and tissue hydroxyproline levels on the seventh postoperative day in colonic anastomoses in rats with IP administration of 10 ng/kg/d of ghrelin. Warzecha et al. showed effects on the healing of oral mucosal ulcers at does of 4, 8, and 16 nmol/kg/d. Konturek et al. using 20 μg/kg/d via IP concluded that there was improvement in colonic healing with TNBS-induced colitis in rats. Similarly, Sen et al. demonstrated the protective effects against ileal ischemia/reperfusion in rats receiving a...
10 ng/kg ghrelin dose intravenously. Recently, Matuszyk et al. also showed protective effects of the administration of ghrelin IP at 8 nmol/kg/dose on the colonic mucosa of rats that underwent acetic acid–induced colitis.

Analysis of the mean values of bursting pressure of the anastomoses showed significantly higher values in the group receiving ghrelin on the seventh postoperative day ($P = 0.035$). This time corresponds to the re-epithelialization phase. The values were also higher in the animals that received ghrelin on the third and fourteenth postoperative days; however, in these periods, statistically significant differences were not observed. This result seems to demonstrate a greater resistance in the anastomosis line to intraluminally induced pressure factors that simulate intestinal physiology (secretion and feces) and peristalsis, especially on the seventh postoperative day in the animals that received ghrelin.

These findings are similar to those of experimental studies that evaluated the influence of ghrelin administration on the bursting pressures of colonic and gastric anastomoses on the seventh postoperative day and showed a statistically significant increase in the bursting pressure means in those subgroups receiving ghrelin at doses of 10 ng/kg/d and 10 nmol/kg/d.

In this study, histopathological evaluation was completed by two distinct methods of staining: hematoxylin-eosin staining for the determination of cellular components and Masson trichrome staining for the assessment of connective and muscular tissues. Differences were observed in the comparison of the means of histological parameter scores between the ghrelin and saline subgroups in all postoperative periods evaluated, but the differences were statistically significant on the third and fourteenth days. These differences indirectly suggest possible anti-inflammatory effects in the subgroups receiving ghrelin, associated with a decrease in neutrophil recruitment in the inflammatory phase of the healing process (third day, $P = 0.035$). From the fourth postoperative day, which is considered the critical period of anastomotic healing, this production of collagen is essential, since the anastomosis is the site of most of the postoperative dehiscence and intestinal fistulas. Sen
et al. have demonstrated similar protective effects with the administration of ghrelin in the ileal mucosa of rats after the induction of mesenteric ischemia/reperfusion injury, suggesting that this anti-inflammatory action would decrease the time required for the recovery of the damaged tissue. The anti-inflammatory effects of ghrelin administration have also been demonstrated in human endothelial cells and in rats with sepsis induced by suppressing the release of proinflammatory cytokines such as IL-1, IL-6, and TNF-α \((P = 0.035)\), granulation tissue \((P = 0.011)\), apoptosis \((P = 0.004)\), and peritoneal inflammation \((P = 0.011)\). On the fourteenth postoperative day, significant differences were detected in the means of neutrophil infiltrate. All of these parameters had decreased mean scores in the ghrelin subgroups compared with the saline group. Again, these results suggest that ghrelin acts as a regulator of inflammation and a promoter of final tissue healing. In this last period of the healing process, which corresponds to the wound maturation phase, there was a reduction in the granulation tissue; abundant granulation tissue has a detrimental effect and a negative impact on the tensile strength of the anastomotic line, thereby preventing collagen deposition.

In this study, the reduction in apoptosis that was also observed on the fourteenth day also suggests decreased injury and better tissue regeneration in this phase, directly affecting the results of the anastomotic healing. Recently, Taati et al. demonstrated a reduction in apoptosis and TNF-α and IL-6 levels in testicular germinative tissue after the induction of ischemia/reperfusion with a consequent improvement in the tissue repair process in the group receiving ghrelin IP. This result corroborates the findings of this study, suggesting similar effects of ghrelin on the same phases of the healing process and a prevention of the exacerbation of the inflammatory phase.

Regulated redox processes influence healing, and the maintenance of redox hemostasis is important to avoid the deleterious effects caused by the overproduction of ROS resulting from aerobic cellular metabolism. In wounds, there is a temporary increase in ROS levels in the inflammatory phase (respiratory burst) that act as a line of defense against invading pathogens and as mediators of cell signaling for angiogenesis. However, the maintenance of high levels of ROS at the healing site is responsible for the activation of proapoptotic proteins, death, and cell necrosis.

In this study, the tissue levels of ROS were determined by measuring \(\text{O}_2^-\), which is the main byproduct of the oxidation of molecular oxygen and which originates from all other reactive radicals involved in oxidative reactions. On the third postoperative day, an increase in the tissue concentrations of ROS was observed in both subgroups, characterizing the initial respiratory burst. The concentrations in this period were higher in the saline subgroup, but this difference was not statistically significant \((P = 0.458)\). This effect may have influenced neutrophil chemotaxis during this period because a significant increase in this cellular type was demonstrated in the saline subgroup. This would in turn lead to a greater level of inflammation as previously discussed. The concentrations

Fig. 5 – Relative intensity/β-actin of antioxidant enzymes in colonic anastomosis tissue in rats. The rats underwent surgery received saline or ghrelin in the postoperative period. The relative intensity of HO-1 (A), GPx (B), and NQO1 (C), was measured in the intestinal tissues at 3, 7, and 14 d after surgery.
of ROS in the subgroups that received ghrelin gradually decreased on the seventh day and returned to basal levels in the fourteenth day, demonstrating the effective performance of the antioxidant systems and the maintenance of redox homeostasis, which are essential for complete wound healing.

This behavior was observed in the saline subgroups on the seventh day, whereas on the fourteenth day, there was a marked and statistically significant increase in the ROS levels relative to those in the ghrelin subgroups ($P = 0.012$).

These findings suggest possible beneficial effects of ghrelin on the recovery and maintenance of redox homeostasis and an optimization of the healing process. These results also corroborate the histopathological findings of this study. There was a significant decrease in the neutrophils, granulation tissue, and apoptosis scores on the fourteenth day in the ghrelin subgroups, suggesting a protective effect against the deleterious effects of ROS in the final phase of wound maturation.

In the nitro-oxidative stress state, the excessive production of NO$_2$ activates the latent form of the human neutrophilic protease, causing greater degradation of the components of the extracellular matrix, mainly collagen, and negatively affecting the final healing results.

In this study, we also measured the tissue concentrations of NO$_2$ on the third, seventh, and fourteenth postoperative days, these concentrations decreased in all subgroups compared with the sham subgroups. This finding suggests a decrease in the tissue concentration of NO in tissues that underwent resections and sutures due to the local ischemia produced by the surgical procedure. On the third day, there was a statistically significant difference in the means of the tissue concentrations of NO$_2$ in the ghrelin subgroups, the concentrations were lower. This result may indicate a decrease in the production of reactive radicals resulting from the oxidation of NO induced by ghrelin, with a consequent reduction in nitro-oxidative stress in this period. These effects would result in decreased activation of the neutrophilic proteases and improvement in the healing of the anastomosis line, as demonstrated by the increase in the anastomotic bursting pressure levels on the seventh day in the ghrelin subgroup.

In addition, there was less mobilization of neutrophils to the wound on the third day, as was also demonstrated in this study in the ghrelin group at a statistically significant level. In the other periods, the differences were not statistically significant, with the NO$_2$ concentrations returning to the basal levels on the fourteenth day in both subgroups, suggesting that there are no effects of ghrelin on nitro-oxidative stress in the late phases of colonic healing.

The Nrf2/Keap 1 pathway has been recognized as one of the main lines of oxidative defense because this pathway activates genes that encode the main antioxidant enzymes, such as SOD, CAT, NQO1, thioredoxin, HO-1, and GPx. In this study, we measured the relative tissue intensity levels of total Nrf2 and p-Nrf2. p-Nrf2 is the active phosphorylated form that migrates to the nucleus and acts on genes to induce enzymatic expression. In both subgroups and in all postoperative periods evaluated, there was activation of the Nrf2/Keap 1 system, as evidenced by an increase in the relative intensity of p-Nfr2, likely induced by the previously demonstrated increase in the tissue ROS levels.

The total Nrf2 and p-Nrf2 levels were higher in the saline subgroups; however, these differences were not significant, suggesting that ghrelin does not directly stimulate this route of antioxidant defense, despite having proven effects that decrease intestinal oxidative stress.

Protective effects with increased activity of HO-1 and NQO1 by the Nrf2/Keap 1 system against antioxidant injury have already been demonstrated in endothelial cells and in neurological cells. In this study, increases were observed in the postoperative relative expression levels of GPx and HO-1. These changes were consistent with the observed increases in p-Nfr2 and decreases in NQO1 expression. Although different, these results were not statistically significant, suggesting that the beneficial antioxidant effects of ghrelin are
not associated with the activation of these enzymes specifically. Future studies expanding the range of antioxidant enzymes analyzed are needed to determine the level of action of ghrelin in the antioxidant defense cascade.

GSH has antioxidant and antiapoptotic effects, and its tissue and plasma levels are inversely proportional to the state of oxidative stress, indicating the redox balance. In this study, the GSH tissue levels fell in both subgroups during the postoperative periods evaluated, indicating the consumption of this substance by the enzymatic defenses to maintain redox hemostasis and coinciding with ROS elevations demonstrated previously.

On the fourteenth day, there was a greater increase in the GSH concentration in the ghrelin subgroup, which showed a trend toward a significant difference compared with that in the saline group in this period. This result coincides with the decrease in ROS levels and the lowest histopathological score of apoptosis found in the same subgroup in the same period. Taken together, these data strongly suggest that the administration of ghrelin may be beneficial in the remodeling phase of the healing process in colonic anastomoses, avoiding cellular lesions and decreasing apoptosis by optimizing the redox hemostasis in that period.

Although our study suggests a reduction in the intensity of inflammatory activity in the wound healing process by the reduction of the neutrophilic infiltrate, more specific tissue parameters, such as proinflammatory cytokine (IL-1, IL-6), anti-inflammatory cytokine (IL-10), and growth factors (vascular endothelial growth factor and insulin-like growth factor 1) levels, should be assessed in further studies to strengthen these findings. Likewise, additional measurements of other oxidative stress biomarkers such as catalase, superoxide dismutase, and thioluric acid reactive substances are required to complement and confirm the beneficial influence and the reduction in the anastomotic tissue oxidative stress state.

Nevertheless, we believe that this pioneering research has provided results that offer a starting point for new studies related to oxidative stress, intestinal anastomosis healing and perioperative ghrelin administration.

Conclusions

The administration of ghrelin in the postoperative period was associated with an increase in the bursting pressure of the anastomosis on the seventh day, a reduction in the neutrophilic inflammatory infiltrate on the third and fourteenth days, and a reduction in the granulation tissue and apoptosis on the fourteenth day. This treatment also resulted in lower ROS tissue levels and higher GSH levels on the fourteenth day and lower NO2 production on the third day. There was no influence on the tissue expression of NOQ1, OH-1, GPx, Nrf2, and p-Nrf2.

These results suggest that the administration of ghrelin may have beneficial effects on the healing of colonic anastomoses and therefore represents a promising strategy for the preventing surgical complications, such as dehiscence and anastomotic fistulas. Further studies with larger sample sizes are required to prove these effects.

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