

Evaluation of the effects of S-methylisothiourea hemisulfate, an inducible nitric oxide synthase inhibitor, on the healing of colonic anastomosis in rats¹

Avaliação dos efeitos da S-metilisotiouréia, um inibidor da enzima óxido nítrico sintase induzível, na cicatrização de anastomoses colônicas em ratos

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ABSTRACT

PURPOSE: To evaluate the effects of S-methylisothiourea hemisulfate (SMT) on the healing of colonic anastomosis in rats.

METHODS: Sixty rats Wistar were distributed into two groups of 30 animals: experimental (E) and control (C). The animals of experimental group received intraperitoneal SMT at 50mg/kg/dose every 12 hours for 72 hours. The control group received intraperitoneal saline at the same volume of SMT. The rats were subdivided into subgroups groups of 10 for euthanasia on the third, seventh, and 14th postoperative days (POD). We evaluated clinical and weight evolution, breaking strength and histopathology; also, a blood sample was collected for serum dosage of nitrite/nitrate.

RESULTS: There was more vascular neoformation (p=0.006) and granulation (p=0.002) in the E3 group, and more mononuclear infiltrates in the C3 group (p=0.041). There was also more edema in the C14 group (p=0.008). There was no statistically significant difference in breaking strength, nitrite/nitrate dosage, and the remaining histopathological parameters.

CONCLUSION: The use of S-methylisothiourea hemisulfate improved the healing of colonic anastomosis in rats on the third postoperative day by accelerating the proliferative stage of healing, but without interfering with the breaking strength of the anastomosis.

Key words: Nitric Oxide. Nitric Oxide Synthase. Wound Healing. Anastomosis. Surgical. Colon. Rats.

RESUMO

OBJETIVO: Avaliar os efeitos do hemissulfato de S-metilisotiouréia (SMT) na cicatrização de anastomoses colônicas em ratos no terceiro, sétimo e 14^o dia de pós-operatório (DPO).

MÉTODOS: Sessenta ratos Wistar foram distribuídos em dois grupos: experimental (E) e controle (C), com 30 animais cada. No grupo experimental foi administrado SMT 50mg/kg/dose, intraperitoneal a cada 12 horas por 72 horas. O grupo controle recebeu NaCl a 0,9%. Os ratos foram subdivididos em grupos de 10 para eutanásia no terceiro, sétimo e 14^o DPO. Avaliou-se a evolução clínica e o peso dos animais, a resistência tênsil e histopatologia da anastomose, e a dosagem de nitrito/nitrato no soro.

RESULTADOS: Houve mais neoformação vascular (p=0,006) e de granulação (p=0,002) no grupo E3, e maior infiltração de mononucleares no grupo C3 (p=0,041). Houve também mais edema no grupo C14 (p=0,008). Não houve diferença estatisticamente significativa na resistência tênsil, a dosagem de nitrito / nitrato, e os restantes parâmetros histopatológicos.

CONCLUSÃO: A utilização do hemissulfato de S-metilisotiouréia acelerou a cicatrização das anastomoses colônicas, a melhoria ocorreu no terceiro DPO: verificou-se que a fase proliferativa da cicatrização foi acelerada. Não houve interferência na resistência tênsil das anastomoses.

Descritores: Óxido Nítrico. Oxido Nítrico Sintase. Cicatrização. Anastomose Cirúrgica. Colo. Ratos.

Introduction

The metabolism of nitric oxide (NO) has been widely investigated in wound healing, especially in intestinal anastomosis. NO is synthesized by macrophages and is an important cell mediator of tissue repair. The release of NO is found to increase during trauma, surgical stress, and sepsis. Macrophage oxidation of L-arginine to NO is catalyzed by nitric oxide synthases (NOS). This process also generates citrulline and the intermediate hydroxy-L-arginine. There are three enzyme isoforms: two are constitutive (neuronal and endothelial) and one is inducible (inducible nitric oxide synthase, iNOS)¹.

During healing of wounds and burns there is an increase in iNOS activity². The resulting increase in NO formation may have a negative influence on fibroblast collagen synthesis; it may also deteriorate cases of inflammation and sepsis. The increase has also been implicated in the pathophysiology of a variety of diseases, including circulatory shock. Studies show that nitrite and nitrate are the stable metabolites for the consumption of L-arginine and production of NO, and their plasma or urine dosage represent this production^{3,4}.

Several drugs may reduce or inhibit the effects of NOS. One such drug is S-methylisothiourea hemisulfate (SMT). Several studies have shown the specificity of SMT for iNOS. SMT *in vitro* is 10 to 30 times more potent than any other iNOS inhibitor⁴.

Shaffer *et al.*⁵ showed that wound fibroblasts are phenotypically altered during the healing process to synthesize NO. The authors also showed that SMT *in vitro* not only reduces NO synthesis, but also does not affect the proliferation of fibroblasts and increases collagen contractility.

The aim of the present study was to evaluate the effects of S-methylisothiourea hemisulfate on colonic anastomosis healing in rats.

Methods

The present study was approved by the Ethics Committee for Animal Use of the Biological Sciences Institute at Brasilia University. All experiments and procedures were performed in accordance with the Brazilian guidelines involving animals in research.

The study used 60 male, healthy Wistar rats (*Rattus norvegicus*); rat body weight ranged between 248g and 364g; age ranged between 90 to 120 days. Rats were randomized into two groups, experimental (E) and control (C), with 30 rats in each group. The rats were subdivided into groups of 10 rats for

euthanasia on the third (subgroups E3 and C3), seventh (subgroups E7 and C7), and 14th (E14 and C14) POD.

Animals in the experimental group were given intraperitoneal SMT (S-Methylisothiourea hemisulfate salt, Sigma-Aldrich Co, São Paulo, SP, Brazil) at 50mg/kg/dose every 12 hours. The administration started at the end of surgery and was maintained for 72 hours. Animals in the control group were administered saline at 0.9% following the same procedures established for the experimental group⁶.

General anesthesia was induced using 5mg/kg of weight xylazine hydrochloride (RompumTM) and 25mg/kg of weight ketamine hydrochloride (KetaminTM) via intramuscular injection. Next, we performed a midline incision and identification of the descending colon, resection of 1cm of colon 3cm above the peritoneal reflection, and termino-terminal anastomosis (6-10 polypropylene suture)⁷.

Reoperation was carried out on the previously set date for each group. Nitrite/nitrate (NO_x) concentrations were measured using the Greiss reaction on serum obtained after centrifuging 5ml of arterial blood (drawn by cardiac puncture). We carried out resection of a central part of the intestine submitted to anastomosis; the segment was then longitudinally divided. One segment was used for analysis of the tensile strength. Analysis was performed using a digital test apparatus named Versa Test (MecmesinTM Versa Test, United Kingdom) coupled to a digital dynamometer AGF (MecmesinTM Versa Test, United Kingdom). Breaking strength was expressed in Newtons (N). Histopathological evaluation was carried out on the second segment by an experienced pathologist blind to animal groups. The evaluation was carried out with hematoxylin and eosin staining. The following indicators were evaluated: congestion, edema, focal hemorrhage, ulceration, necrosis, mononuclear infiltrates, polymorphonuclear infiltrates, vascular neof ormation, granulation, and fibrosis. Each indicator was classified as absent (-), mild (+), moderate (++) , strong (+++) , or severe (++++), as described by Sousa *et al.*⁸. All animals killed in the PO period were submitted to necropsy.

Statistical analysis was carried out using SPSS (version 12.0) and Sigma Stat (version 32). To compare initial and final weights, and NO_x serum dosage was used one-way ANOVA and t-test. To evaluate the histopathological criteria was used Fisher's exact test. Statistical analyses were considered significant for p<0.05.

Results

Six animals died during the experiment: one from

subgroup E3, two from subgroup E7, two from subgroup E14, and one from subgroup C3. All deaths occurred in the immediate PO period. Necropsy did not show signs of intraabdominal or intrathoracic bleeding, abscess, anastomosis dehiscence, or peritonitis.

The initial weight of animals was similar in both groups ($p=0.454$); the average weight was 313.21g (95.0% CI=304.28-322.14) for the experimental group, and 311.54g (95.0% CI=303.55-319.54) for the control group. There was an initial weight loss in both groups, which was also not different between the groups. By the end of the study, animals regressed to their initial weight. The comparison of animal weight in each subgroup, carried out at the time of euthanasia on the third, seventh, and 14th POD, did not show a statistically significant difference (Figure 1).

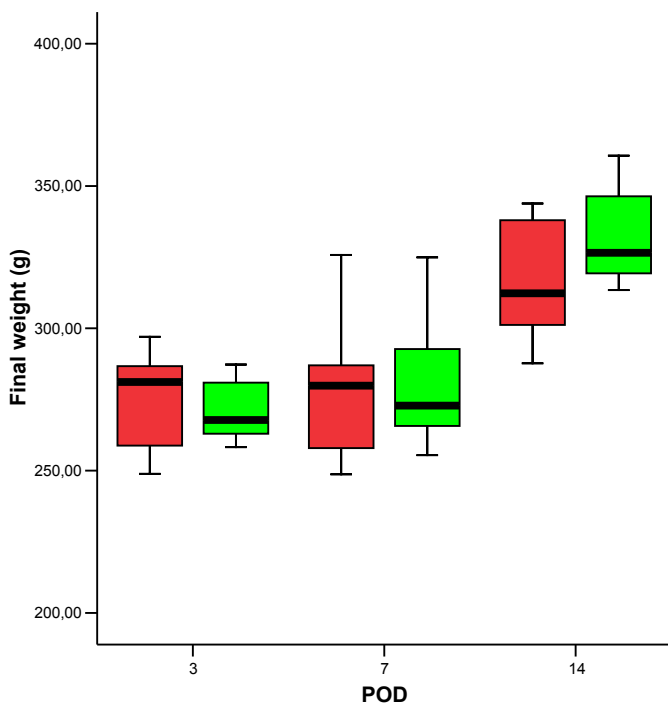


FIGURE 1 - Median and interval for animal weight in both groups and according to postoperative day of euthanasia (weight in grams). Legend: third (3), seventh (7), 14th (14) POD, SMT = S-methylisothiurea hemisulfate

Intracavitary adhesions were shown in 88.9% of animals of subgroup E3, and in 100.0% of animals in the other subgroups. Adhesions showed lower resistance to breaking on the third postop day and became more resistant on the seventh and 14th days. Incidence of anastomosis dehiscence was one on control group and four on the SMT group but there was no difference between the subgroups ($p=0.05$). Anastomosis dehiscence was blocked by the omentum and adjacent organs. There were no cases of diffuse peritonitis, abscess, or anastomosis leakage.

Breaking strength increased with time in all subgroups with no statistically significant differences between them (Figure 2).

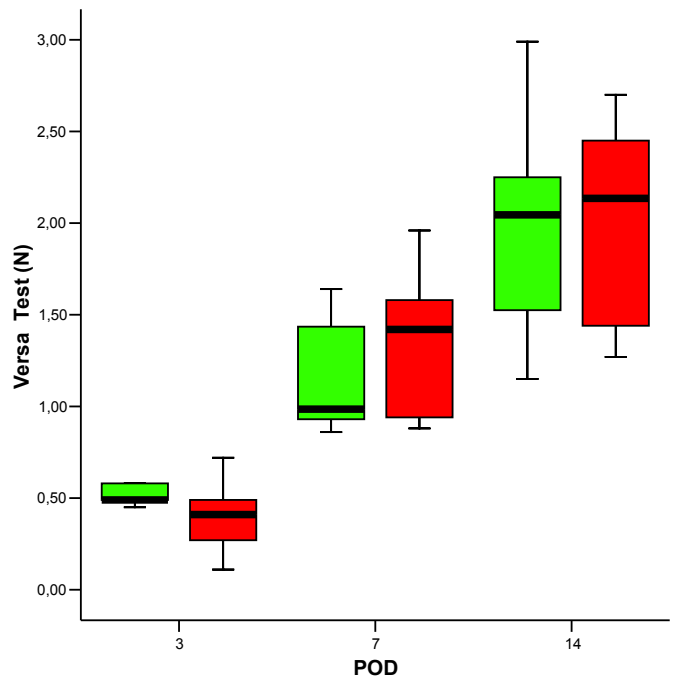


FIGURE 2 - Median and intervals for breaking strength of anastomoses in the different groups and subgroups (values in Newtons). Legend: third (3), seventh (7); 14th (14) POD; SMT = S-methylisothiurea hemisulfate

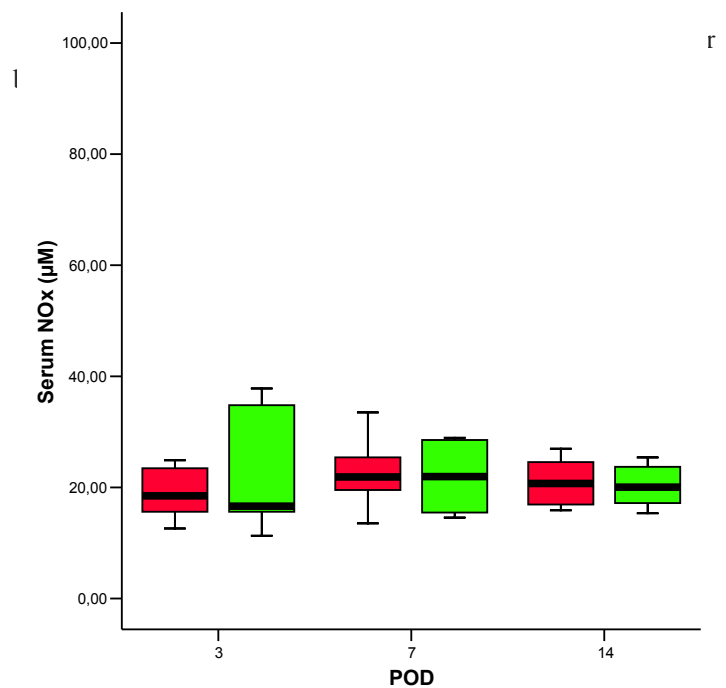


FIGURE 3 - Median and intervals for nitrite dosage in serum collected at euthanasia (values in μM). Legend: third (3), seventh (7); 14th (14) POD; SMT = S-methylisothiurea hemisulfate

Group E3 showed significantly more vascular neoformation ($p=0.002$) and more granulation ($p=0.0001$). Subgroup E14 had a statistically significant greater presence of edema in comparison to C14 ($p=0.002$); this difference was not observed in the third and seventh POD. There were no differences between the groups in presence of congestion, focal hemorrhage, ulceration, necrosis, mononuclear infiltrates, or fibrosis.

Discussion

SMT is one of the most selective drugs among iNOS inhibitors. Other studies have been carried out on the effect of SMT on colon anastomoses healing with intraperitoneal injections that ranged from 3.0mg/kg/day to 200mg/kg/day (every 12 hours or by continuous infusion)^{4,6,9}. In the present study, SMT was administered at 72 h. The rationale behind this choice is that iNOS is more active during the first days after surgical trauma. iNOS peaks during the first 24 hours and remains active for up to 10 days¹⁰. Other authors have corroborated the finding that the most active period of NOS is during the early stages of wound healing¹¹.

The mortality rate in the study was 10%. Deaths were most likely caused by anesthetic complications: there were no signs of bleeding, anastomosis dehiscence, or peritonitis. Previous studies on healing of intestinal anastomoses in rats have reported mortality rates that ranged from zero to 11.4%^{7,12,13}.

The weight loss was followed by weight recovery up until the 14th POD. The losses may have been associated with surgical stress¹⁴. Weight loss has been described in previous studies on wound healing^{15,16}. Others reported weight recovery as early as the seventh POD¹⁷.

The increase in breaking strength found with the passing of the PO period in both groups corroborates other studies in the literature¹⁸. The lack of a statistically significant difference on the third POD might be because the operated area is kept together by the suture during this period¹⁹.

The histopathological evaluation by an experienced examiner blind to experimental groups is an important criterion for the evaluation of the evolution of the healing of colon anastomoses^{7,8}. Among the indicators evaluated, there was a statistically significant higher presence of vascular neoformation and granulation in group E3; of necrosis in subgroup C3; and of edema in subgroup E14. These findings indicate that iNOS inhibition by SMT favored an improvement in the process of healing.

The study did not show any difference between the subgroups for plasma dosage of nitrite/nitrate on the third, seventh,

and 14th POD. This result was not expected; our expectation was that there would be a higher dosage of nitrite/nitrate on the third POD. This dosage would decrease with time and, in the experimental group (i.e. administered SMT), the dosage of NO would be smaller than in the control group.

Conclusion

The use of S-methylisothiourea hemisulfate improved the healing of colonic anastomosis in rats on the third postoperative day by accelerating the proliferative stage of healing, but without interfering with the breaking strength of the anastomosis.

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