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Effects of arginine on abdominal wall wound healing in *Wistar* rats.

Efeitos da arginina na reparação cicatricial da parede abdominal em ratos Wistar.

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ABSTRACT

Objective: to evaluate the effects of arginine on abdominal wall healing in rats. **Methods:** we submitted 20 *Wistar* rats to laparotomy and divided them into two groups, arginine and control, which then received, respectively, daily intraperitoneal treatment with arginine (300mg/kg/day) and weight-equivalent phosphate buffered solution, during five days. On the seventh postoperative day, we collected blood and scar wall samples from both groups. We evaluated serum nitrate and nitrite levels, wound evolution by tissue hydroxyproline dosages, granulation tissue formation, percentage of mature and immature collagen, myofibroblast density and angiogenesis. We used the ANOVA and the Student's t tests with p=0.05 for comparisons between groups. **Results:** there were no significant differences between the groups studied for nitrate and nitrite (p=0.9903), tissue hydroxyproline (p=0.1315) and myofibroblast density (p=0.0511). The arginine group presented higher microvascular density (p=0.0008), higher percentage of type I collagen (p=0.0064) and improved granulation tissue formation, with better angiofibroblastic proliferation rates (p=0.0007) and wound edge reepithelization (p=0.0074). **Conclusion:** in the abdominal wall healing evaluation of *Wistar* rats under arginine treatment, there was no change in serum nitrate and nitrite levels, total collagen deposition and myofibroblast density. There was an increase in type I collagen maturation, microvascular density and improvement in scar granulation tissue formation by better edge reepithelization and angiofibroblastic proliferation.

Keywords: Arginine. Wound Healing. Hydroxyproline. Collagen Type I. Abdominal Wall. Rats, Wistar.

INTRODUCTION

Wound healing is a dynamic process involving the phenomena of homeostasis, inflammation, cell proliferation and tissue remodeling[1]. Significant volumes of resources have been directed to the treatment of postoperative complications caused by delayed healing or non-healing, which has led to major investments in research aimed at better understanding the pathophysiology of these complications. In addition, thanks to advances in surgical metabolism, nutrition, and biochemistry, other research has sought to investigate the synthesis of substances capable of improving or shortening the healing process, such as an experimental study that showed that arginine supplementation in rat diets increased tensile strength and collagen deposition in the injured tissue[2].

Arginine, a semi-essential amino acid, plays a fundamental role in the metabolism of various body components, displaying numerous and peculiar pharmacological effects[3]. It is recruited in the acute and chronic phases of healing and it is a precursor, among other molecules, of nitric oxide (NO), which favors angiogenesis and the healing process as a whole. Angiogenesis, on its turn, is a healing phenomenon of the proliferative phase that occurs so that cells and growth factors can continue the remodeling phase, with collagen maturation and increased tensile strength[4].

The aim of this study is to investigate the effects of intraperitoneal administration of arginine on abdominal wall healing of *Wistar* rats, considering as indicators the synthesis and maturation of collagen, the formation of granulation tissue and the microvascular and myofibroblast tissue densities.

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METHODS

We maintained 20 *Wistar* rats (*Rattus norvegicus albinus, Rodentia mammalia)* aged 180 to 210 days and weighing 408.4±40.1g at a controlled temperature of 19ºC to 23ºC and light cycles automatically adjusted every 12 hours. We divided them into two groups with statistically similar weights (p=0.2452), and placed them in polypropylene boxes with wood shavings (replaced every 24 hours), with a maximum of four rats in each box. All animals received species-specific chow and water *ad libitum*. They remained in these conditions for seven days prior to and also during the experiments, which were performed in a single cycle of activities.

We observed the Ethical Principles on Animal Experimentation of the Brazilian College of Animal Experimentation (COBEA-2000) and the project was approved by the Animal Research Ethics Committee (CEPA) of the Angelina Caron Maternity Hospital, Campina Grande do Sul, PR, according to protocol number 012/11-CEPA/HAC.

We performed the surgical procedures in a properly sanitized and air cooled environment. For anesthetic induction, we used isoflurane (Isothane-Baxter®) sedation through inhalation in a closed circuit, followed by Ketamine hydrochloride (Ketamin-Cristalia®) at a dose of 100mg/kg body weight, measured by an analytical balance (Coleman® SK280251), combined with 10mg/kg xylazine hydrochloride (Xylazin-Syntec®) using two 1ml disposable syringes (BD-Plastiplak®) and hypodermic needles (BD-Plastiplak®) for each rat.

After confirming the anesthetic induction due to the loss of the interdigital reflex, we performed a wide abdomen trichotomy, put the animal in supine position with duct tape on the surgical board, applied iodinated alcohol to the trichotomized area, and placed sterile fenestrated surgical drapes. By means of a 3cm median laparotomy, a single surgeon performed the opening of the skin and muscle tissue. Then, the surgical wound was sutured in two planes (muscle-aponeurotic and skin), with continuous stitches using a 3.0 Mononylon thread (Figure 1).

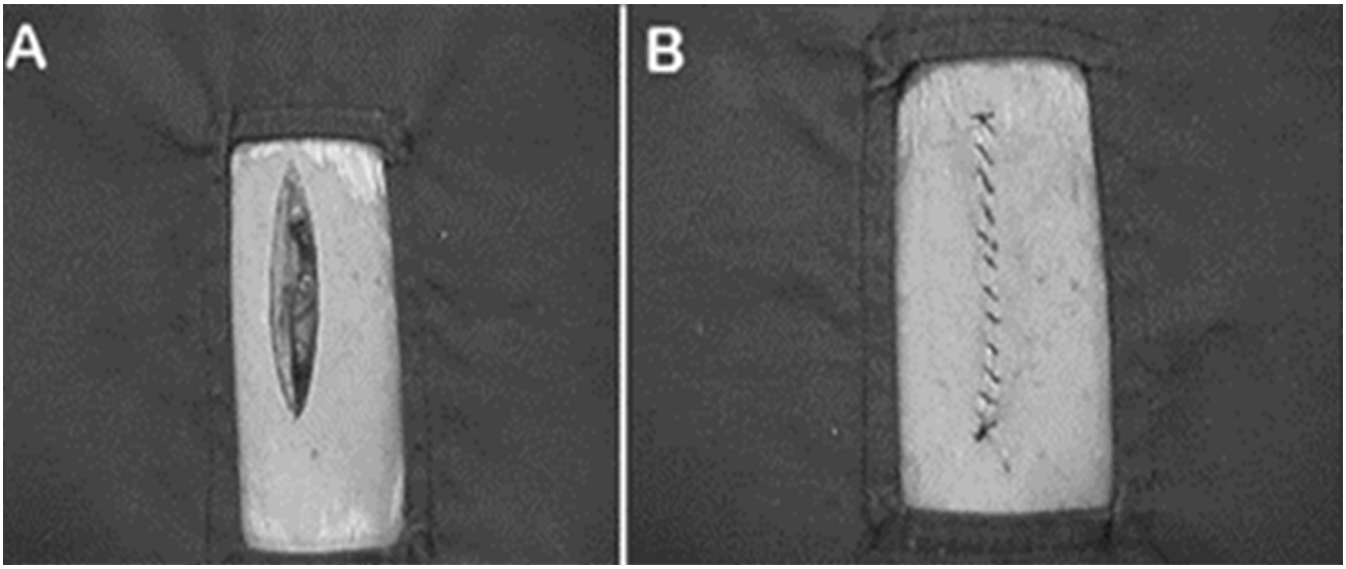

Figure 1. *Steps of the surgical procedure: median laparotomy with opening of the skin and muscle tissue and access to the peritoneal cavity (A); surgical wound sutured in two planes: muscle-aponeurotic and cutaneous (B).*

After closure, the surgical wound was cleaned with saline and iodinated alcohol was reapplied. Dipyrone was administered orally at a dose of 20mg/kg and 10ml of saline subcutaneously in the dorsal region and the rats were kept warm until anesthetic recovery. They were then placed in properly identified boxes and kept for 24 hours with food and water *ad libitum* until the seventh postoperative day.

Treatments consisted of intraperitoneal injections of arginine at a dose 300mg/kg/day for the arginine group and equivalent dose of phosphate buffered solution (PBS) for the control group, which were administered to rats sedated by isoflurane inhalation once daily. for five days. Initiation of treatment (first dose) was in the immediate postoperative period with rats still sedated.

For the arginine group, we used a 20% solution of L-Arginine (Merck® article K21051142) in PBS pH 7.4, sterilized by 20m membrane filtration (Millipore-SCWP304F0®). For the control group, we used PBS pH 7.4 solution, sterilized by MF membrane filtration (SCWP304F0 Millipore®). In both cases, the PBS solution was aliquoted in sterile vials and kept at 4±2°C.

On the seventh postoperative day, the rats underwent halothane (Tanohalo-Cristália®) inhalation sedation in a closed circuit, anesthetized by intramuscular injection with ketamine hydrochloride at a dose of 100mg/kg and fixed in surgical pads. We then performed a cardiac puncture and collected 8ml to 10ml of blood, corresponding to an exanguinative puncture and inducing cardiopulmonary arrest. Still under anesthetic plan and with evidence of death, we resected the surgical wound containing the aponeurotic layer and skin. The collected samples were spread on filter paper, washed in PBS pH 7.4 solution and separated into two fragments.

One of the fragments for hydroxyproline dosing was immersed in PBS pH 7.4 solution and kept in a freezer at -80°C until laboratory processing. The other fragment, intended for histopathological evaluation, was immersed in an identified vial containing buffered formalin.

We analyzed tissue hydroxyproline concentration by the spectrophotocolorimetric method[5,6], which evaluates the total hydroxyproline content in tissue samples, providing a measure of total collagen deposition in microgram (µg) per milligram (mg) of wet tissue. Samples were evaluated in duplicates and, for validation of results, we tabulated and statistically validated 560 nm spectrophotometric readings.

For microscopic evaluations, the samples were fixed in buffered formalin and, after suture removal, subjected to automated histological processing consisting of dehydration, diaphanization and blocking phases. Subsequently, 4mm-thick sections were made perpendicular to the largest suture axis, in triplicate, which were fixed on slides to be stained according to the evaluation to be performed, following the items below.

To evaluate the percentage of collagen types I and III, the histological sections were stained with Picrosirius-Red-F3BA[7] and analyzed by optical microscope at 400 times magnification using polarized light source. The images were taken by camera, transmitted to a color monitor and digitized.

We performed a histometric analysis of the images using the Image Pro-Plus version 4.5 software for Windows to identify the color-based collagen type. The colors red, yellow and orange corresponded to type I collagen, while the shades of green corresponded to type III collagen. The result was expressed as percentage area of type I and type III collagen at each histological section site.

We carried out three different field readings on each slide, which were tabulated and statistically validated.

For assessment of granulation tissue formation, three independent pathologists evaluated Harris's hematoxylin-eosin-stained (HE) histological sections, considering, in each reading, the intensity of the cicatricial inflammatory reaction by evaluating the following phenomena: fibrinoleukocyte crust formation by observing fibrin deposits and blood cells on the wound surface; reepithelialization of the edges, paying special attention to the advancement of the epithelium around the wound; and angiofibroblastic proliferation, by morphological evaluation of neovascularization (angiogenesis). These findings were attributed grades 0, 1 and 2 for the absence of reaction, mild and moderate reaction, respectively[8].

From the paraffin-embedded material, we obtained 4mm-thick histological sections, extended on glass slides and immuno-labeled with anti-CD34 antibodies, which allowed the quantification of scar microvascular density. The CD34 antigen is a transmembrane protein found on the surface of endothelial cells, being diffusely expressed in the scarring microvessels, and which acts in binding to specific adhesion receptors. In addition, its expression level may correlate with the quality of the healing process[9].

We employed the immunoperoxidase method using the streptavidin-biotin technique, with positive and negative controls, using the Biotin anti-CD34 antibody clone QBEnd/10® reagent (Dako Corporation, Glostrup, Denmark) at 1:350 dilution, following the Manufacturer's specification[10]. We considered the test positive when a brown reaction was detected, excluding likely areas of nuclear reaction pattern background, and any brown-colored endothelial cell (or group of endothelial cells) clearly separated from other immunostained elements, regardless of size, was considered a microvessel. The analysis was performed by computerized histometry and the results were expressed in tissue density of microvessels over an area of 7,578.94mm^2.

From the paraffin-embedded material, we obtained 3mm-thick sections, which were mounted on glass slides and immuno-labeled with anti-smooth muscle actin monoclonal antibodies for the identification of myofibroblasts. We employed the immunoperoxidase method[11,12], using the streptavidin-biotin technique, with positive and negative controls, using Biotin anti-aSMA antibody clone reagent PJC10® (Dako Corporation, Glostrup Denmark) at 1:400 dilution, following the manufacturer's specification. We deemed the test positive when a brown reaction was detected, excluding the probable background staining areas with nuclear reaction pattern. The analysis was performed by computerized histometry and the results were expressed in myofibroblast tissue density over an area of 7,812.26mm^2.

We measured Nitrate on plasma samples obtained from blood collected by the cardiac puncture. We evaluated four aliquots of the same sample (tetraplicates) and used the Griess reagent (2% sulfanylamide, 0.2% N-1-naphthyl ethylenediamine and orthophosphoric acid) for the dosages. The sample/Griess reagent mixture was incubated for 5min at 37ºC and then the optical density was read by a spectrophotometer at 520nm[13]. Simultaneously to the ANOVA test for validation of results, we prepared a calibration curve with a nitrite standard at concentrations of 40, 80, 120, 160 and 200 mmol/l. After method validation, we interpolated the average optical densities by linear regression on the calibration curve and thus obtained the results in mmol/l.

For statistical validation of the analyzes, we used Student's t-test (hydroxyproline) or the ANOVA test (other variables) for comparison of the results of each of the measurements of the same sample. We found no significant differences (p>0.05) between the readings in any of the variables analyzed in this study, which allowed validating these results and making comparisons between group means by calculating the arithmetic mean of each sample and the group mean with standard deviation. We expressed results as mean±standard deviation and student's, with significance level at 0.05 for comparisons between groups, using the GraphPad Instat software.

RESULTS

When compared by the Student's t-test, the average hydroxyproline dosages of the arginine and control groups showed no significant difference (0.0304±0.0073 *versus* 0.0250±0.0081; p=0.1355). Figure 2 depicts the comparison between the means of the control and arginine groups for the percentages of collagen I (p=0.0064) and collagen III (p=0.0274), with higher values for the arginine and control groups, respectively.

Table 1 shows the comparisons between the control and arginine means for edge reepithelialization (p=0.0074) and angiofibroblastic proliferation (p=0.0007).

Regarding the histometric evaluation of the wound microvascular density (Figure 3), we observed significantly higher values for the arginine group (282.06±99.12 in the control group *versus* 446.33±81.59 in the arginine group; p=0.0008). Similarly, the tissue density of myofibroblasts was higher in the arginine group, but without statistical significance (367.49±68.34 in the control group *versus* 429.28±63.78 in the arginine group; p=0.0511). The mean serum nitrate and nitrite dosage were 1.35±0.26mmol/l and 1.35±0.23 mmol/l for the control and arginine groups, respectively, with no statistically significant difference (p=0.9903) (Figure 4).

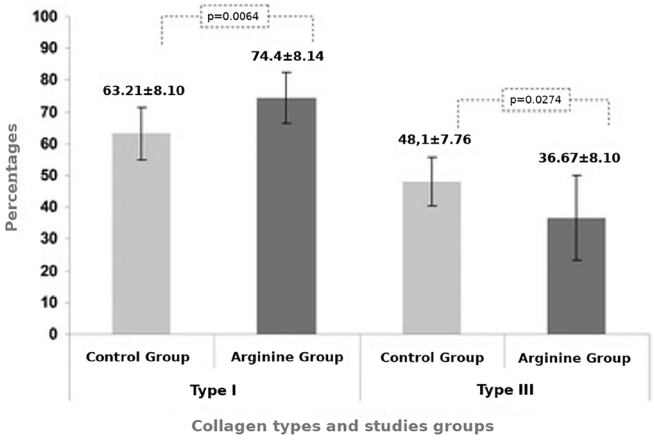

Figure 2. *Comparison between the mean percentages of collagen types I and III between the evaluated groups.*

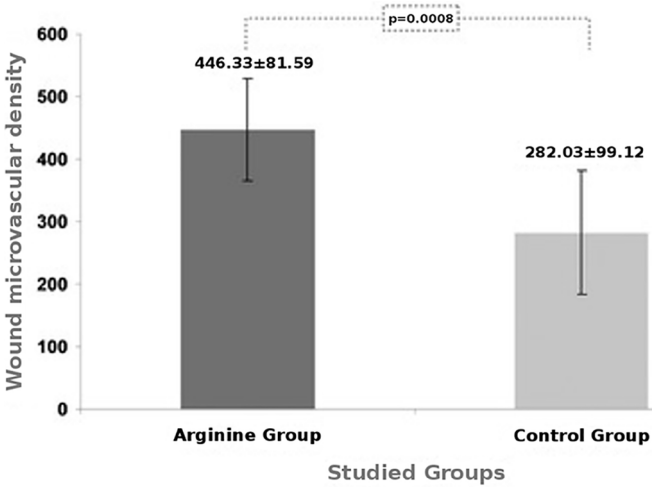

Figure 3. *Comparison between the mean microvascular densities between the evaluated groups.*

Table 1. *Comparison between the means of the control and arginine groups in granulation tissue formation according to the criteria of fibinoleukocyte crust formation, edge reepithelization and angiofibroblastic proliferation.*

Groups	Fibrinoleukocyte crust	Edge reepithelization	Angiofibroblastic proliferation
Control	1	1.4±0.52	1.37±0.46
Arginine	1	1.93±0.21	1.97±0.11
p	n/s*	0.0074	0.0007

** n/a: not suitable for statistical calculation; values expressed as mean±standard deviation.*

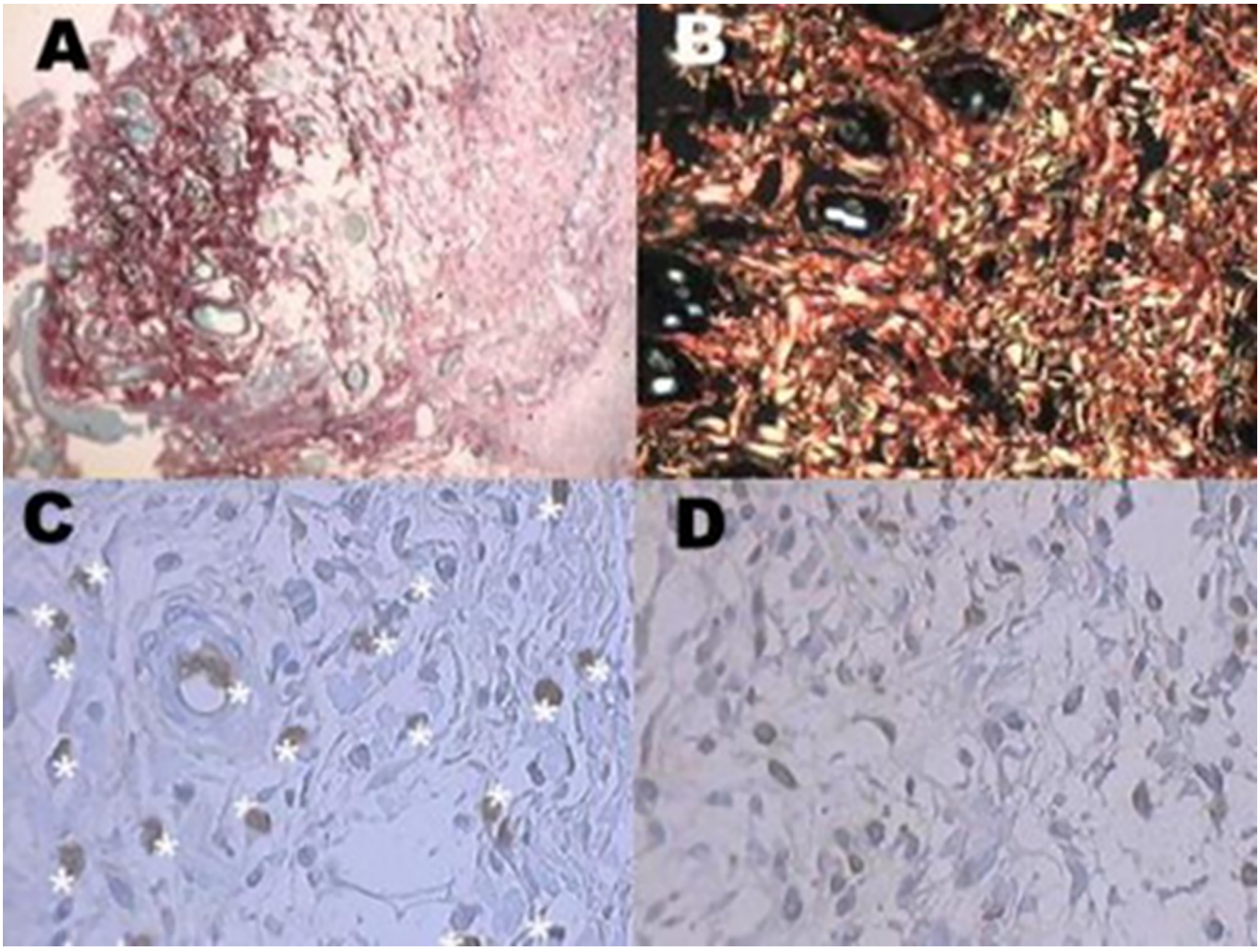

Figure 4. *Histological evaluations: A and B) quantification of type I and III collagen by computerized histometry by picro-sirius staining. In detail "A" light microscopy and in detail "B" after polarization, the colors red, yellow and orange correspond to type I collagen, while the green tones correspond to type III collagen; C) evaluation of wound microvascular density by the immunoperoxidase method with anti-CD34 monoclonal antibody, and the brown reaction on any endothelial cell or group of endothelial cells was considered positive, clearly separated from other immunostained elements; D) evaluation of myofibroblast tissue density by the immunoperoxidase method with anti-smooth muscle actin monoclonal antibodies for the identification of myofibroblasts. The brown reaction in any cell was considered positive as a myofibroblast.*

DISCUSSION

Previous studies have shown the importance of Nitric Oxide (NO) in wound healing and angiogenesis[14]. In addition, a challenging study model of wound healing in *Zucker* rats (diabetic and obese) showed improvement of the healing process with the use of arginine-enriched hyperproteic formula, besides proline[15]. Considering these findings, the present study aimed to compare the healing processes in the abdominal wall of rats, according to biochemical and histopathological parameters, after treatment with arginine+PBS solution (arginine group) or PBS solution (control group), both injected intraperitoneally.

Experimental studies in rats were performed to verify the effects of arginine on postoperative healing of intestines and skin, burns, incorporation of synthetic material in the abdominal wall, with different doses and routes of administration, such as oral, subcutaneous and intraperitoneal[16-20].

One of them concluded that, in trauma, arginine supplementation is essential, as its use in humans is related to increased wound healing, a positive effect on the immune system, and reduced days of hospitalization. This occurs through the promotion of lymphocyte and fibroblast proliferation, which serves as an intracellular substrate for nitric oxide production in macrophages to improve bactericidal activity[21].

Treatment with arginine, at the same dose used in the present study, but administered subcutaneously, induced angiogenesis and increased total collagen deposition in the skin flap healing process in rats[17].

In the present study, we evaluated the effects of arginine treatment on the surgical wound, both biochemically, by determining the tissue hydroxyproline content, which represents total collagen deposition, and histologically, by quantifying collagen I and III and by evaluating granulation tissue, angiogenesis and tissue density of myofibroblasts.

The evaluation of the amount of total collagen, indicated by tissue hydroxyproline, allows a valuable but partial analysis of the healing process, since one should also consider the different types of collagen, which differ in their chemical composition and in the form of association between their molecules, interfering with scar resistance. Concomitant with collagen deposition and maturation in a scar, there is a progressive increase in tensile strength, which may increase for up to two years after the beginning of the healing process[6].

Hydroxyproline can be synthesized from L-arginine by two metabolic pathways: the arginase pathway and the ornithine aminotransferase pathway, which produce the largest amount of tissue proline. In the present study, in contrast to the results of Shukla *et al.*[18], there was no significant difference between tissue hydroxyproline averages in the arginine and control groups, despite a numerical increase.

In addition to the absolute collagen concentration, represented by its hydroxyproline content, the arrangement of its fibers is also responsible for tissue resistance. Among the 19 collagen isoforms identified to date, type I collagen is the most frequent and predominant in bones and tendons, being considered mature collagen. It is also the most important, as it is responsible not only for maintaining the integrity of most tissues due to its mechanical properties[22], but also for its active participation in tissue functionality, through its interaction with cells present in the extracellular matrix. Type III collagen, in turn, is most commonly found in soft tissues such as blood vessels, dermis and fascia. Granulation tissue expresses 30% to 40% of type III collagen, being considered immature collagen.

In the present study, there was a higher mean type I collagen percentage in the arginine group when compared to the control group ($p=0.0064$), which can be explained when evaluating the results of the quantification of wound microvascular density, which also demonstrated a significant increase in the arginine treated group ($p=0.0008$).

The healing mechanisms occur in a sequence of three phases: inflammatory, proliferative or granulation tissue formation on day seven, with greater angiogenic stimulation and collagen synthesis, and remodeling, by collagen deposition and maturation, and consequently, of scar maturation.

Granulation tissue formation begins with infiltration of macrophages, which produce and release chemical mediators that intensify fibroblast migration and activation, thus explaining the most intense angiofibroblastic proliferation observed in the arginine-treated group in this study.

Fibroblasts are the major components of granulation tissue and the main cells of the proliferative phase. However, they need to be activated out of their quiescence state. The release of tumor growth factor beta (TGF-ß) stimulates fibroblasts to produce collagen and to become myofibroblasts, promoting wound contraction. In the present study, the tissue densities of myofibroblasts did not differ between the arginine and control groups (p=0.0511), although there was a numerical difference. An experimental study in rats that investigated the density of myofibroblasts in wound healing by the same method employed here concluded that metronidazole, applied topically to wounds with secondary intention healing, does not interfere with wound contraction and delays the appearance of myofibroblasts[23]. On the other hand, a recent experimental study concluded that the topical administration of metronidazole solution to second intention healing skin wounds was able to improve fibroblast differentiation, which can be used in favor of the wound healing process[24].

The importance of myofibroblasts in the healing process also stems from the fact that the wound contraction process reaches its maximum efficiency during fibroblast fixation and its phenotypic maturation for collagen-producing cells. This is due to the phenotype change of fibroblasts from the wound margins to myofibroblasts, which are intermediate cells between smooth muscle and fibroblasts. Although their contractile mechanism remains unclear, these cells are found aligned around deposits of the new extracellular matrix, producing cell-to-cell unions and generating tensile strength.

After the influence of growth factors, fibroblasts are activated and migrate from the wound margins to their center, which allows to infer the effects of arginine on better edge reepithelization in the present study. This process is called fibroplasia and, for its efficiency, parallel the formation of new blood vessels is necessary, that is, the neovascularization of the region[25,26].

In the present study, the arginine group showed significantly higher tissue microvascular density than the control one (p= 0.0008). This result can be explained by the two recognized pathways of direct arginine degradation. One is mediated by the enzyme arginase, which transforms it into ornithine and urea, while in the other arginine is catalyzed by the enzyme nitric oxide synthase and its product is NO, a potent activator of the vasodilator factor associated with vascular endothelium, which is responsible for angiogenesis[27].

An experimental study showed that L-arginine improves cardiac function and reduces infarct size in rats with acute myocardial infarction. The possible mechanism is related to the dual function of promoting angiogenesis and arteriogenesis, in addition to regulating collagen expression[28].

NO is a metabolite that crosses the cell membranes very easily, which makes it an intra- and inter-cellular messenger involved in different physiological and pathological responses during wound healing[29,30]. Due to NO's short lifetime (about 3 to 9 seconds), direct dosing is impractical. Considering that its metabolism generates nitrates and nitrites, which are stable and allow for dosing in biological fluids, such as cerebrospinal fluid, urine, wound fluids and plasma, these determinations are considered indicators of arginine metabolism.

However, such measures do not represent the equimolar formation of NO, as non-enzymatic formation also occurs[31].

In the present study, serum nitrate and nitrite dosages in the arginine and control groups showed no significant difference (p=0.9903). Regardless of the species, the nitrite toxicological mechanism of action is similar. Nitrite oxidizes Fe^{2+} from hemoglobin, transforming it into methemoglobin, which has no ability to carry O_2, causing tissue anoxia. Animal deaths have been reported with 80% to 90% of oxidized hemoglobin[32]. Nitrate and nitrite dosing was used to assess whether the administered dose of arginine could alter the serum nitrate and nitrite level, thus being an indicator of a possible toxic effect if higher than the control group.

The present study contributes some important and promising questions regarding healing and use of arginine. The starting point was the possibility that NO, an arginine catabolite with angiogenic effect, could improve collagen synthesis, deposition and maturation, with consequent improvement in healing. Promising results were observed regarding the improvement of tissue microvascular density, collagen maturation and granulation tissue, with prospects for future studies in organisms with healing difficulties, such as those with chronic corticotherapy or vascular impairment, transplanted and elderly, among others.

R E S U M O

Objetivo: *avaliar os efeitos da arginina na cicatrização da parede abdominal de ratos Wistar.* **Métodos:** *vinte ratos Wistar foram submetidos à laparotomia e separados em dois grupos (arginina e controle), que receberam tratamento diário por via intraperitoneal com arginina (300mg/kg/dia) e solução tampão fosfato em dose equivalente ao peso, respectivamente, durante cinco dias. No sétimo dia pós-operatório, coletaram-se amostras de sangue e da cicatriz da parede abdominal de ambos os grupos. Avaliaram-se o nível sérico de nitratos e nitritos, a evolução cicatricial pelas dosagens de hidroxiprolina tecidual, formação de tecido de granulação, determinação da porcentagem de colágeno maduro e imaturo, densidade de miofibroblastos e angiogênese. Empregaram-se os testes de ANOVA e t de Student com p=0,05 para as comparações entre os grupos.* **Resultados:** *não ocorreram diferenças significantes entre os grupos estudados para dosagens de nitratos e nitritos (p=0,9903), hidroxiprolina tecidual (p=0,1315) e densidade de miofibroblastos (p=0,0511). O grupo arginina apresentou maior densidade microvascular (p=0,0008), maior porcentagem de colágeno tipo I (p=0,0064) e melhora na formação do tecido de granulação, com melhores índices de proliferação angiofibroblástica (p=0,0007) e re-epitelização das bordas (p=0,0074).* **Conclusão:** *na avaliação cicatricial da parede abdominal de ratos Wistar sob tratamento com arginina, não houve alteração do nível sérico de nitratos e nitritos, da deposição de colágeno total e da densidade de miofibroblastos. Verificaram-se aumento da maturação de colágeno do tipo I, da densidade microvascular e melhora na formação do tecido de granulação cicatricial pelas melhores re-epitelização de bordas e proliferação angiofibroblástica.*

Descritores: *Arginina. Cicatrização. Hidroxiprolina. Colágeno Tipo I. Parede Abdominal. Ratos Wistar..*

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