

Evaluation of topical metronidazole in the healing wounds process: an experimental study

Avaliação do uso tópico do metronidazol no processo de cicatrização de feridas: um estudo experimental

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A B S T R A C T

Objective: To assess the efficacy of topical a 4% metronidazole solution in wounds healing by secondary intention in rats. **Methods:** We made circular wounds two inches in diameter at the back of rats and studied healing at 3, 7, 14 and 21 days. The wound contraction and epithelialization were assessed by peripheral digital planimetry and myofibroblasts by immunohistochemistry with α -SMA. **Results:** There was no difference between groups regarding wound contraction. In wounds treated with metronidazole peripheral epithelialization was evident early on day 3 ($p < 0.001$) and there were no differences in other periods. In the control group, the number of myofibroblasts was higher on day 7 ($p = 0.003$) and day 14 ($p = 0.001$) and in the experimental group it was suggestively higher on day 3 ($p = 0.06$). **Conclusion:** Metronidazole 4% solution at a dose of 50 mg/kg applied topically to wounds healing by secondary intention facilitates early peripheral epithelialization, does not interfere with wound contraction and delays the appearance of myofibroblasts.

Key words: Metronidazole. Wound Healing. Fibroblasts. Rats.

INTRODUCTION

Authors reported that older adults have slower process of wound healing and increased risk of skin breakdown with formation of ulcerated lesions, and chronic ulcers of the lower limbs, generating early retirements¹ and being the second cause of absence from work².

In most public institutions there are scarce resources to meet the demands of low-income population, which consequently develops diseases with greater severity. To control costs, private institutions and insurance companies are setting increasingly strict criteria to allow the use of resources and sophisticated technologies³.

Ashford et al. reported, for the first time in 1984, the use of oral metronidazole to control the stench of neoplastic ulcerated lesions⁴. Currently, topical metronidazole is recommended in oncology wounds with the same purpose, because it acts against the anaerobic bacteria responsible for the production of volatile acids that cause the odor, without the side effects of its oral useage⁵⁻⁷.

Trindade Neto et al.⁸ described the use of topic 0.5% metronidazole gel in a case of granulomatous rosacea

with good results and referred that metronidazole gel has been successfully used in cases of mild to moderate rosacea, maintaining patients in remission.

There is little research on the use of topical metronidazole in benign wounds healing by secondary intention, although it is a low cost medication, simple to handle and of easy access to the general population, being able to give back the patient's independence in his/her self care.

This study aimed to evaluate the action of a topical 4% metronidazole solution in wounds healing by secondary intention in mice, analyzing the epithelialization, wound contraction and the correlation with myofibroblasts.

METHODS

The study project was submitted to the Ethics Committee on Animal Use of the Pontifical Catholic University of Paraná and was approved under number 232, having followed the guidelines of the Federal Law No. 6638 and the recommendations of the Brazilian College of Animal Experimentation (COBEA).

Work done at the Post-Graduation in Surgical Clinic Program, Pontifical Catholic University of Paraná (PUCPR)-Curitiba-PR-BR.

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We used 80 male Wistar rats (*Rattus norvegicus* albinos, Rodentia mammalia), young adults aged 90 days and weighing between 184.28 and 255.56 g. They were kept in cages suitable for the species, under controlled environmental conditions with light/dark cycles of 12 hours and temperature $20 \pm 2^\circ$ C. They had free access to water and standard chow for the species.

The animals were randomly divided into two groups, control (CG) and experimental (GE), and evaluated 3, 7, 14 and 21 days after injury in the skin. They were anesthetized with 0.1ml/100g bodyweight of a mixture of 1ml of ketamine (50 mg) with 1ml of 2% xylazine (20 mg) intramuscularly in the posterior portion of the right thigh. We shaved the dorsal region of each animal, creating a hairless area of approximately 24 cm². We proceeded to antisepsis made with PVPI and delimitation of the operative area with a fenestrated sterile field, marking the skin by rotation of the cutting edge of a metallic punch with two centimeters in diameter and the resecting the circular skin demarcated by the punch until exposure of the dorsal muscle fascia was achieved⁹.

The rats in the control group had their wounds cleaned with 0.9% saline, and then occluded with dry gauze postoperatively. The rats in the experimental group also had clean wounds like the control group, but soon after cleaning gauze dressings soaked in metronidazole solution (benzoilmetronidazol) 40mg/ml (4%) in qsp vehicle were applied, and the wound remained occluded for 24 hours. In the remaining days of the study, all animals had their wounds cleaned with 0.9% NaCl solution and the experimental group, after cleaning the wound, received the application of 0.3 ml of metronidazole 4% solution on the wound, corresponding to 12, 5mg/day, once a day.

The animals were kept in separate cages after recovering from anesthesia, and put on shelves with the same distance from the light source, receiving water and balanced chow *ad libitum*.

After completion of three days of treatment 10 animals from each treatment group were euthanized with a lethal dose of intraperitoneal sodium pentobarbital (120mg/kg). The same process occurred on days 7, 14 and 21.

Each animal was placed on a surgical board and photographed by a digital camera, model P71 Cyber-Shot, Sony®, 3.2M pixel resolution, tripod kept at a constant distance of 34 cm, soon after infliction of the wound and after euthanasia in 3, 7, 14 and 21 days. The images obtained were imported into the computer program VeVn MD-measurement Documentation® to assess wound contraction by digital planimetry. To calculate the actual area a reference square of 2x2 cm (material provided by the manufacturer of the program) was put at the right side of the wound at the time of photography, assisting in converting the electronic image to the measure in centimeters.

After evaluation of the contraction, the wounds were excised with a margin of one cm of healthy skin around the lesion, with depth to the dorsal muscles of the rat. The resected segment was stretched on filter paper, fixed in 10% formalin for 24 hours and subjected to conventional histological preparation. To assess the presence of myofibroblasts in the wounds the histologic sections were submitted to immunohistochemistry by tissue array method with the anti- α -SMA antibody, with readings of 10 fields per histological section.

Collection of material was performed by swab culture of the wounds in the rats of control and experimental groups that were sacrificed on the 14th day. This occurred on two occasions, after cleaning the wound with 0.9% NaCl solution on day zero and on the 14th day before euthanasia. The results were evaluated by Fisher's exact test.

For comparison of control and experimental groups, assessed at each time of sacrifice, we used the Student t test for independent samples. To compare the times of euthanasia restricted to each of the control and experimental groups we used analysis of variance (ANOVA) with one factor. The condition of normality of variables was assessed by Shapiro-Wilks. Variables that did not have this condition were subjected to transformation, in this case the square root. P values <0.05 indicated statistical significance. Calculations were performed using the software Statistica® v.7.

RESULTS

One animal died due to an anesthetic accident.

Macroscopic evaluation of the wounds did not reveal bleeding or purulent secretion at all, in both groups, throughout the evaluation process. On the third day it became evident the greater peripheral epithelialization in the experimental group, while mild in the control group. On day 21 one animal from the experimental group had a completely epithelialized wound, whilst in the control group all animals had wounds with incomplete epithelialization.

The peripheral area of reepithelialization as measured by digital planimetry at 7, 14 and 21 days showed no significant differences, but there was difference on day 3 ($p < 0.001$), demonstrating early reepithelialization in the experimental group (Figure 1).

By analyzing wound contraction we observed that the wound areas decreased significantly over time in the control ($p < 0.001$) and experimental ($p < 0.001$) groups. However, when both groups were compared, the averages were similar in the four evaluation periods (Figure 2).

Immunohistochemistry demonstrated a greater number of myofibroblasts in the wounds of the control group in the 7th and 14th day readings, with significant difference ($p < 0.003$ and $p < 0.001$). At day 3 there was no difference, and on day 21 the difference was not significant

but was suggestive ($p=0.06$) to the presence of a greater number of myofibroblasts in the experimental group (Figure 3).

Cultures performed at days 0 and 14 showed no significant difference ($p=1$) as for the presence of bacteria in the wounds in both moments analyzed (Table 1).

DISCUSSION

Authors who did experimental work in rats with wound healing by secondary intention, Prasad et al.¹⁰ and Rao et al.¹¹ with the use of oral metronidazole, and Rao et al.¹² using it topically, reported a significant increase of epithelialization in the wounds of the experimental groups, but this parameter was evaluated in all three studies as the number of days required for complete wound epithelialization.

In our study epithelialization proved to be earlier in the experimental group ($p<0.001$). However, the later evaluations found no difference between the wounds of both groups. Metronidazole probably facilitates the initial epithelialization when used topically at a dose of 50 mg/kg/day

As for the contraction of the wound in the back of the animal as measured by planimetry, Prasad et al.¹⁰ and Rao et al.¹² reported increased contraction with the use of metronidazole oral dose of 160 mg/kg/day and 180 mg/kg/day, respectively. Borden et al.¹³, who used metronidazole intraperitoneally at a dose of 20 mg/kg/day, found no significant difference in wound contraction.

In this study, we observed that the wounds of both groups significantly decreased their area as time passed. But when the groups were compared, there was no difference in any of the moments, demonstrating that metronidazole at a dose of 50mg/kg/day topical use does not interfere with the contraction of the wound that is healing by secondary intention, the same result of Borden et al., despite different routes of administration of the medication.

With regard to myofibroblasts, Grinnell¹⁴, by analysis of markers of the cytoskeleton, and Darby, Skalli and Gabbiani¹⁵, with immunofluorescence labeled with antibodies to detect all isoforms of actin, indicated that these cells are derived from fibroblasts that migrated into the wound.

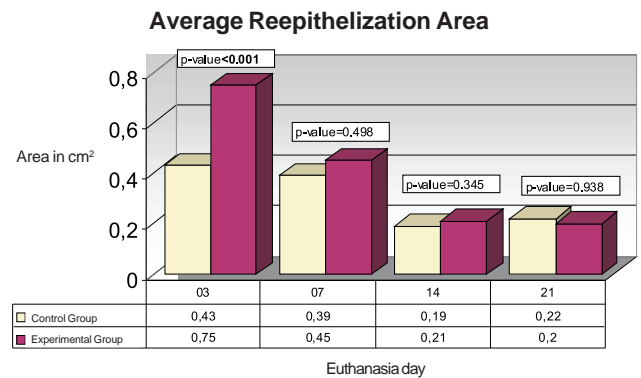


Figure 1 - Reepithelization Area (cm²).

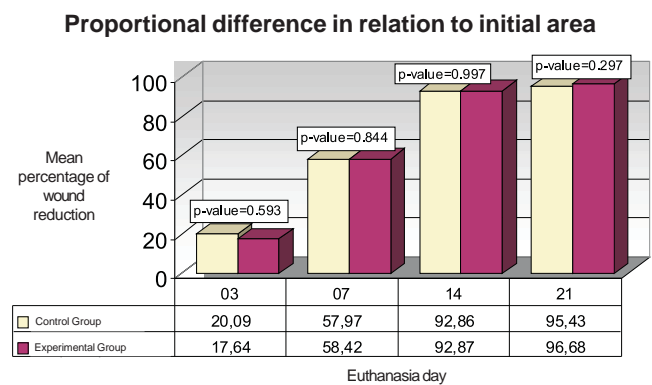


Figure 2 - Percentage of contraction of wound areas in the four periods studied.

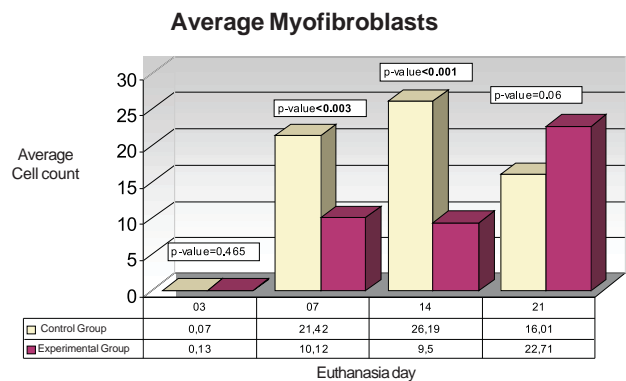


Figure 3 - Average number of myofibroblasts in the wounds from Control and Experimental Groups.

Table 1 - Presence of bacteria in the wounds of rats in days zero and fourteen.

Bacteria	Day 0		Day 14	
	Control Group	Experimental Group	Control Group	Control Group
Absent	9 90,00%	9 90,00%	1 10,00%	9 90,00%
Present	1 10,00%	1 10,00%	9 90,00%	1 10,00%
Total	10	10	10	10

$p\text{-value} = 1$ (Fisher's exact test, $p<0,05$).

Masur et al.¹⁶ and Grinnell¹⁴ reported that the transforming growth factor-beta (TGF- β) acts as a fibroblasts differentiation promoter into myofibroblasts.

Hinz et al.¹⁷ were able to increase the expression of α -SMA in rat subcutaneous fibroblasts cultured on a silicon substrate and the collagen matrix, treating them with TGF- β , demonstrating the effective action of this factor on the contractile activity of fibroblasts.

Besides TGF- β action on the differentiation of fibroblasts into myofibroblasts, it acts on keratinocytes, on the matrix deposition, on fibroblast proliferation and on angiogenesis^{3,18}.

In the current study there was a lower amount of myofibroblasts in the wounds of the experimental animals at 3, 7 and 14 days, raising a possible deleterious effect of metronidazole on the TGF- β . But this factor acts on keratinocytes responsible for epithelialization, and metronidazole in this study had a beneficial effect with regard to initial epithelialization evaluated at day 3, suggesting that this medication does not interfere with the action of that growth factor.

Darby, Skalli and Gabbiani¹⁵ demonstrated that myofibroblasts appeared only on the 6th day of wounds evaluation, and they were positively present in wounds from the 12th to the 15th day. From the 15th to the 20th day there was an intense decline in the presence of myofibroblasts, and on day 30 there were none of these cells in the wounds. These authors emphasized that the first phase of wound contraction is independent of myofibroblasts.

Berry et al.¹⁹ reported that there was actual contraction of wounds in the absence of a high density of myofibroblasts. They suggested that the contractile unit may be the organization of thin collagen fibrils into the thick collagen fiber, operation held by fibroblasts, and the compression of the connective tissue within the granulation tissue, retracting the dermis and fatty tissue around the wound.

Authors report that the mechanical stress existing in the wound's extracellular matrix would be responsible for maintaining the myofibroblasts present^{20,21}.

In this experiment, there was no correlation between the effective presence of myofibroblasts in the lesion and the macroscopic contraction of the wound, because even with significant difference of myofibroblasts at 7, and 14 days for the control group ($p=0.003$ and $p=0.001$), and on day 21 suggestively higher in the experimental group ($p=0.06$), in these same moments there were no significant differences in wound contraction of the groups compared ($p=0.844$, $p=0.997$ and $p=0.297$).

There was minimal presence of myofibroblasts in the wounds of control and experimental groups on day 3, consistent with the findings reported by Darby, Skalli and Gabbiani¹⁴. In the wounds of our experimental groups there were fewer myofibroblasts when compared to control groups on the 7th and 14th day, diverging from those authors. However, metronidazole did not inhibit the appearance of myofibroblasts in the wound. This was demonstrated on

day 21, in which the number of these cells was suggestively higher in the experimental group ($p=0.06$). A larger sample of wounds could confirm this result or rule it out definitely.

Darby, Skalli and Gabbiani¹⁵ reported that the factors that influence the expression of α -SMA in fibroblasts are unknown. In smooth muscle cells α -SMA can be modulated by extracellular components such as proteoglycans and heparin, and cytokines such as α -interferon. Heparin causes increased expression of α -SMA and α -interferon causes it to decrease. The authors said they did not know whether these comments applied to fibroblasts.

The same authors investigated the mechanism of disappearance of fibroblastic cells when the granulation tissue progressed to a scar. Apoptotic figures were observed in fibroblasts and endothelial cells between the 20th and 25th day after injury, suggesting that there was a programmed death of these cells in cases of wound healing.

Moulin et al.²² investigated the influence of myofibroblasts on keratinocyte growth and differentiation using a human skin model in vitro. They demonstrated that the presence of myofibroblasts and/or absence of fibroblasts delayed reepithelialization, but when the fibroblasts were present complete epithelialization occurred from 7 to 10 days.

In the current research topical 4% metronidazole solution may have had a direct action on fibroblasts by preventing the early expression of α -SMA, stimulating the growth of keratinocytes and encouraging the greater reepithelialization that occurred in the experimental group on the third day. It has probably delayed the phenomenon of fibroblasts apoptosis that occurs around the 20th day. This apoptotic phenomenon was observed by Darby, Skalli and Gabbiani¹⁵ between the 20th and 25th day of their research. This assumption would justify the events of the 7th and 14th day of the current work with metronidazole, when the wounds of the experimental groups showed a lower amount of myofibroblasts when compared with control groups.

The mechanism by which metronidazole delays the change of fibroblasts into myofibroblasts remains to be clarified.

The results of the cultures performed in this study suggest that perhaps topical metronidazole does not interfere with the normal colonization of open wounds, thus not being this the mechanism of action on wounds to healing by secondary intention.

CONCLUSION

Metronidazole 4% solution at a dose of 50 mg/kg applied topically to wounds healing by secondary intention in rats facilitates the early peripheral epithelialization, does not interfere with wound contraction and delays the appearance of myofibroblasts.

RESUMO

Objetivo: Avaliar a ação do metronidazol em solução a 4%, tópico, em feridas com cicatrização por segunda intenção em ratos. **Métodos:** Fez-se feridas circulares com dois centímetros de diâmetro no dorso de ratos e estudou-se a cicatrização em 3, 7, 14 e 21 dias. A contração da ferida e a epitelização periférica foram avaliadas por planimetria digital e os miofibroblastos pela imunistoquímica com α -SMA. **Resultados:** Não houve diferença entre os grupos em relação à contração ferida. Nas feridas tratadas com metronidazol a epitelização periférica precoce foi evidente no 3º dia ($p < 0,001$) e não houve diferenças nos demais períodos. No grupo controle, o número de miofibroblastos foi maior no 7º dia ($p = 0,003$) e no 14º dia ($p = 0,001$), e no grupo experimento, foi sugestivamente maior no 3º dia ($p = 0,06$). **Conclusão:** O metronidazol, solução a 4%, na dose de 50mg/kg/dia, aplicado de forma tópica nas feridas com cicatrização por segunda intenção, facilita a epitelização periférica precoce, não interfere na contração da ferida e atrasa o aparecimento dos miofibroblastos.

Descritores: Metronidazol. Cicatrização de feridas. Fibroblastos. Ratos.

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