

Comparative histological study on wound healing on rat's skin treated with Mitomycin C or Clobetasol propionate¹

Maria de Fátima Pereira de Carvalho^I, Celina Siqueira Barbosa Pereira^{II}, José Humberto Fregnani^{III}, Fernando de Andrade Quintanilha Ribeiro^{IV}

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^ILecturer, Department of Morphology, Santa Casa School of Medical Sciences of Sao Paulo, Universidade de Sao Paulo (USP), Brazil. Design of the study, care of animals, technical procedures, manuscript preparation.

^{II}Associate Professor, Department of Otorhinolaryngology, Santa Casa School of Medical Sciences of Sao Paulo, USP, Sao Paulo-SP, Brazil. Intellectual and scientific content of the study, manuscript writing, critical revision, supervised all phases of the study.

^{III}Associate Professor, Research and Teaching Institute, Hospital de Câncer de Barretos, Brazil. Statistical analysis, histopathological examination.

^{IV}Associate Professor, Department of Morphology, Santa Casa School of Medical Sciences of Sao Paulo, USP, Sao Paulo-SP, Brazil. Macroscopic and histopathological examinations, statistical analysis.

ABSTRACT

PURPOSE: To compare histologically the action of Mitomycin C and that of Clobetasol propionate for surgical wound healing in rats.

METHODS: A circular skin fragment was surgically removed from 57 Wistar rats. Twenty-two animals were treated with Mitomycin C with topical medication in a single dose, 22 with Clobetasol propionate with a cream medication once a day for 15 days and 13 did not receive any medication. The animals were euthanized 30 and 60 days, and the scars subjected to histological examination.

RESULTS: The histological analysis on the samples did not show statistically significant differences regarding the quantities of fibroblasts, fibrocytes and vascular proliferation in the three groups, in the evaluations after 30 and 60 days. In the treated groups with Mitomycin C and Clobetasol there was a decrease in collagen concentration over the 30-day period and an increase in collagen concentration over the 60-day period, in comparison with the control group.

CONCLUSIONS: The actions of Mitomycin C and Clobetasol were equivalent and not interfere in fibroplasias and in angiogenesis. Both drugs initially cause a decrease in collagen over a 30-day period and an increase over a 60-day period, demonstrating a delay in the wound healing.

Key words: Wound Healing. Mitomycin. Propionates. Clobetasol. Collagen. Models, Animal. Rats, Wistar.

Introduction

Healing is the natural restorative response of a tissue lesion. The healing process has three distinct phases: inflammation, proliferation and maturation¹. If one of these phases fails or is prolonged, there may be a delay in or failure of wound occlusion. For surgeons, the healing of a surgical wound must follow the process of *restitutio ad integrum*, so as to make the scar as inconspicuous as possible and avoid atrophic or hypertrophic reactions such as keloid.

In otorhinolaryngology, surgical treatment of congenital or acquired stenosis of the external acoustic meatus, choanae and larynx is a challenge because of the risk of postoperative relapse, and the use of a scar-inhibiting agent would be a useful and efficient alternative. Two topical medications that act on the healing of wounds are Mitomycin C (MMC) and Clobetasol propionate (CP). MMC, produced by the fungus *Streptomyces caespitosus*, not only is a potent antibiotic and chemotherapeutic agent, but also is an alkylating agent that blocks RNA transcription for formation of extracellular matrix (ECM) components and induces apoptosis of fibroblasts, with a marked reduction in collagen and ECM protein levels^{2,3}. CP is a glucocorticoid with high anti-inflammatory power and acts on keratinocytes, thereby causing reductions in ECM volume and cell proliferation. This results in epidermal thinning and, through inhibition of mitoses in fibroblasts, reduction of the amount of collagen and ECM volume⁴ in the dermis.

Experimental studies on animals have enabled observations on the topical effect of these medications towards inhibition of the scar response, in relation to MMC^{2,5-12} and CP¹³. A variety of methodological criteria has been used, especially regarding the times for evaluating and reevaluating the wounds, which have often generated conflicting or unenlightening results.

The present study had the objective of using histological techniques to study the effects of the topical action of MMC and CP on the healing process of surgical wounds on the backs of rats, on the 30th and 60th days after the injury.

Methods

The present study was fully approved by the ethics committee for animal experimentation of the Institute of Advanced Otorhinolaryngological Sciences (ICAO number 2/11) and was carried out under veterinarian supervision and follow-up.

A controlled experimental design was selected for this study, which was conducted on 57 Wistar rats: all male adults weighing over 200g. The animals were kept in individual cages, with food and water ad libitum, in an environment with a constant temperature of 21°C.

The rats were anesthetized by means of an intraperitoneal injection of ketamine (40 mg/kg) and xylazine hydrochloride (10 mg/kg). Afterwards, the rats were depilated on their backs so that a circular surgical wound, of 1 cm in diameter, could be made in that region, with removal of the skin and subcutaneous tissue. Once the surgical procedure had been concluded, all the wounds were washed with 10 ml of 0.9% physiological solution.

In the control group (CG), composed of 13 rats, no medication was applied to the surgical wound. Six rats were euthanized after 30 days and the remaining seven, after 60 days.

The group treated with MMC (MMCG), with 22 rats, received a single topical application of 2 ml of MMC at a concentration of 0.5 mg/ml (Mitocin, Bristol-Myers Squibb, São Paulo, Brazil), which was dripped onto a gauze over the wound, for a period of five minutes. After this period, the wound was washed again with 10 ml of 0.9% physiological serum. Ten animals were euthanized after 30 days and 12, after 60 days.

The group treated with CP (CPG), composed by 22 rats, received applications of clobetasol propionate in the form of a 0.05% cream (Psorex, Glaxo Smith Kline, São Paulo, Brazil), at a dosage of 0.25 g/kg, once a day, for 15 days. This dosage was used in accordance with the literature^{14,15}, since CP has a high degree of toxicity for rats. Eleven rats were euthanized after 30 days and 11 after 60 days.

The reason for creating a control group of animals that were not treated instead of using a region where there was no application of the medication on the backs of the treated animals themselves was to avoid possible systemic interferences from topical administration, in relation both to MMC¹⁶ and to CP^{14,15}.

After anesthetization using ketamine and xylazine hydrochloride intraperitoneally, the animals were euthanized by means of an intracardiac injection of sodium thiopental (24 mg/kg) and potassium chloride (0.5 ml). The skin of the dorsal region of the animals, adjacent to the scar, was depilated. The whole area, including the scar, was photographed and then removed surgically. The fragments were fixed in 10% formol and processed using standard histological techniques, with embedding in paraffin and preparation of slides with sections of thickness 3 µm, for histological examination by means of HE and picosirius red stainings.

The following parameters were taken into consideration for the histological evaluation of the slides stained with HE: presence of fibroblasts and fibrocytes and vascular proliferation in terms of the quantity of blood vessels in the dermis. The evaluations were carried out qualitatively (presence or absence) and semi-quantitatively (weak, moderate or high intensity) by two experienced histologists, and the variables were classified according to their intensity, as degree

1 (absent or weak intensity) and degree 2 (moderate or high intensity). We observed all field In the microscopic evaluation with x10, x20, x40 magnification lenses.

The slides stained with picosirius red were used to evaluate the concentration of collagen fibers present in the connective tissue underlying the epithelium. On these slides, with the aid of an optical microscope coupled to an Axiocam MRc 5 camera (Zeiss), the area of the scar was located and photographed between two and eight times, depending on its extent when magnified x200. The Image Pro Plus 4.5® software was used to obtain a quantitative reading of the collagen deposits in selected areas of the skin, by means of digital recognition of the regions stained in red (areas of collagen deposition) and the intensity of the staining (the stronger the staining was, the greater the deposition of collagen fibers was), thus calculating the collagen concentration.

After gathering the data, a computerized database was set up, which was subjected to statistical analysis with the aid of the Statistical Package for the Social Sciences (SPSS) version 13.0 software.

The study population was characterized by means of descriptive statistics. Fisher's exact test was used to compare proportions. Mean values were compared using the Kruskal-Wallis test. Post-hoc analysis was performed using Mann-Whitney test (two by two comparisons), with Bonferroni correction for the significance level. In that case, statistical tests were considered to be significant when the P value was less than 0.017. In the other tests, the significance level was set at 5%.

Results

The wounds on the backs of the rats healed by second intention. In all the rats, a crust was seen to form, and this fell off spontaneously. In the control group, the scar crusts fell off, on average, on the 15th day; in MMCG they fell off around the 25th day; and in CPG, on the 26th day. In the latter group, some rats still presented crusts on the wound until the 30th day.

The histological results from analyses carried out on the fragments from the rats euthanized after 30 days are presented in Table 1, and the results of those after 60 days are in Table 2.

TABLE 1 – Comparison of the histopathological variables according to the study group: observation after 30 days.

Variable		Control	Mitomycin	Clobetasol	P (*1)
Fibroblasts	Degree 1	2 (33.3%)	1 (10.0%)	4 (36.4%)	0.451
	Degree 2	4 (66.7%)	9 (90.0%)	7 (63.7%)	
Fibrocytes	Degree 1	0 (0.0%)	4 (40.0%)	2 (18.2%)	0.209
	Degree 2	6 (100.0%)	6 (60.0%)	9 (81.8%)	
Vascular proliferation	Degree 1	0 (0.0%)	0 (0.0%)	0 (0.0%)	NE
	Degree 2	6 (100.0%)	10 (100.0%)	11 (100.0%)	
Collagen concentration	Mean (with standard deviation)	80.8 (9.3)	63.2 (11.7)	58.8 (7.7)	0.002 (*2)

Degree 1: Absent/weak intensity; Degree 2: Moderate/high intensity; NE: Not evaluable

(*1) Fisher's exact test (fibroblasts, fibrocytes and vascular proliferation) or Kruskal-Wallis test (collagen concentration).

(*2) *Post hoc* analysis: Mann-Whitney test with Bonferroni correction. Comparisons: control vs mitomycin (P=0.009); control vs clobetasol (P=0.001).

TABLE 2 – Comparison of the histopathological variables according to the study group: observation after 60 days.

Variable		Control	Mitomycin	Clobetasol	P (*1)
Fibroblasts	Degree 1	0 (0.0%)	6 (50.0%)	3 (27.3%)	0.069
	Degree 2	7 (100.0%)	6 (50.0%)	8 (72.7%)	
Fibrocytes	Degree 1	5 (71.4%)	3 (25.0%)	4 (36.4%)	0.163
	Degree 2	2 (28.6%)	9 (75.0%)	7 (63.6%)	
Vascular proliferation	Degree 1	0 (0.0%)	2 (16.7%)	2 (18.2%)	0.646
	Degree 2	7 (100.0%)	10 (83.3%)	9 (81.8%)	
Concentration of collagen	Mean (with standard deviation)	66.7 (7.0)	84.3 (9.2)	79.1 (9.2)	0.005 (*2)

Degree 1: Absent/Weak intensity; Degree 2: Moderate/high intensity.

(*1) Fisher's exact test (fibroblasts, fibrocytes and vascular proliferation) or Kruskal-Wallis test (collagen concentration).

(*2) *Post hoc* analysis: Mann-Whitney test with Bonferroni correction. Comparisons: control vs mitomycin (P=0.005); control vs clobetasol (P=0.010).

Discussion

The macroscopic evaluation on the wounds of the animals in the three groups showed that there were differences in the time that the crusts took to fall off. This demonstrated that the healing was slower among the animals that received topical medication, especially those of the CP group. This difference may have been due to the ways in which the two medications were applied: MMC in a single dose and CP, daily for 15 days.

Regarding the quantities of fibroblasts and fibrocytes, there was no statistically significant difference among the three groups in the 30 and 60-day evaluations, thus demonstrating that MMC and CP did not interfere in fibroplasia. This result differed from what was found by Ribeiro *et al.*⁷: over an observation period of 90 days after using topical MMC at a concentration of 0.5 mg/ml on wounds on the backs of six rats for five minutes, these authors reported that there was a significantly greater quantity of fibroblasts and lower quantity of fibrocytes in the fragments removed from these animals, in relation to the control group, which was also composed of six rats.

Vascular proliferation (angiogenesis) is part of the proliferative phase of healing and, in the present study, presented moderate degree in the three groups, in the evaluation carried out after 30 days. No statistical analysis was possible at this time. In the analysis after 60 days, on the other hand, there was no statistically significant difference among the three groups. The findings from the present study therefore indicate that administration of MMC and CP at the specified dosages and posology did not alter the angiogenesis at the times of 30 and 60 days. On the other hand, Ribeiro *et al.*⁷ demonstrated that there was a significant increase in vascular proliferation in scar fragments on the backs of rats, in a group treated with topical MMC at a concentration of 0.5 mg/ml for 5 minutes, after a period of 90 days.

A decrease in the collagen concentration in scar fragments from the vocal folds of pigs stained with picosirius red was observed by Camargo *et al.*¹⁷ and Baptistella *et al.*⁸. Both of these authors applied MMC soaked in cotton to the wound for three minutes and analyzed the effect of the medication on healing after 30 days. However, Camargo *et al.*¹⁷ used MMC at a concentration of 0.4 mg/ml and Baptistella *et al.*⁸, 0.04%.

No other studies demonstrating inversion of the concentration of collagen fibers in scars between 30 and 60 days, as reported in the present study, were found in the literature. This was probably because observations tend to be made preferentially up to 30 days after the injury. A decrease in collagen concentration over a 30-day period has also been observed in the injuries treated

with both medications. The increase in the collagen concentration of collagen in the scars of the treated groups in relation to the control group seen after 60 days demonstrates that these drugs retard healing during the initial period. Nevertheless, an increase in collagen deposition was observed on the 60th day after the date of the procedure. The remodeling phase of the healing process begins 21 days after the injury and can continue indefinitely.

Conclusions

In the rats treated with Mitomycin C and Clobetasol there was a late fall in the scar crusts (delay in the initial healing). The medications did not interfere in fibroplasias and in angiogenesis. In the rats treated with Mitomycin C and Clobetasol there was a decrease in collagen over a 30-day period and an increase over a 60-day period, demonstrating a delay in the wound healing.

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Correspondence:

Prof. Dr. Fernando de Andrade Quintanilha Ribeiro
Rua Itapeva, 366/cj 74
01332-000 São Paulo - SP Brasil
Tel.: (55 11)99235-4363
quintanilha.f@uol.com.br

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