ORIGINAL RESEARCH PAPER

Animal Science

USE OF FIBRINOLISIN / DEOXYRIBONUCLEASE AND BISMUTAL SUBGALATE IN WOUND PALATINE MUCOSA REPAIR. HISTOLOGICAL STUDY IN RATS.

KEY WORDS: Fibrinolisina-deoxyribonuclease, Bismuth Subgalate, Palatal Mucosa, Repair.

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ABSTRACT

This study evaluated histological repair process in rats palatine mucosa with fibrinolysin-deoxyribonuclease application associated or not with bismuth subgalate. 75 Wistar rats were divided into 3 groups. Wounds were made in the central region of the third palatal fold. The control group received saline gauze compression, group test 1 saline gauze compression and fibrinolysin-deoxyribonuclease and test 2 bismuth subgallate and fibrinolysin-deoxyribonuclease application. In group 1, the clot presence was lower at 3 and 7 day ($p < 0.05$). Control group showed greater necrosis presence in relation to others ($p < 0.05$). The linear measure was lower in 1 than in the control at 3 ($p = 0.04$) and 7 day ($p < 0.01$). The epithuelium vertical measurement did not present statistical difference between them. Then, topical fibrinolysin/deoxyribonuclease application promoted faster epithelial closure when compared to saline alone and its use alone or associated with the bismuth subgalate did not interfere with epithelial repair.

Introduction

In excisional surgeries, such as the donor areas of the palataline mucosa, the repair of the lesions occurs by second intention due to the greater loss of cells and tissues, which can lead to an increase in bleeding. Recently, bismuth subgalate has been reported as an important adjunct in reducing hemostasis time in the donor site of the free gingival graft. Clinically favorable result is observed in the operative bleeding control, marked decrease in surgery time, increase in safety against postoperative hemorrhages and greater tranquility for the patient and the operator (Kim, Tramontina, Papalexiou, & Luczyszyn, 2010). In addition, experimental studies in animals revealed that it did not interfere in the process of repairing the epithelial and connective tissues (Kim et al., 2012; Tramontina et al., 2002).

In an injured tissue, the clot establishes a physical and biological barrier against contamination and serves as a matrix for cell migration. However, rapid degradation of fibrin and fibrinous exudate followed by replacement of granulation tissue may accelerate tissue repair. For this purpose, a bovine debridant enzymatic substance composed of fibrinolysin / deoxyribonuclease has been used in cutaneous wounds (Falabella, Carson, Eagltein, & Falanga, 1998; Mekkes, Zeegelaar, & Westerhof, 1998; Westerhof, Jansen, Wit, & Falanga, 1987). Its fibrinolytic action is directed mainly against denatured proteins of devitalized tissues. The products resulting from the enzymatic breakdown are composed of large molecules that are not easily absorbed by the body. Thus, they do not produce local or general undesirable reactions. Deoxyribonuclease specifically hydrolyzes the main components of deoxyribonucleic acid, the main exudates constituents. Therefore, the simpler break in polynucleotides helps in the liquefaction of the exudate facilitating its removal of the wounds. In this way, the production of a smooth surface by the dissolution of the exudate and the necrotic tissues can stimulate the soft tissue healing (Falabella, 2006; Mekkes & Zeegelaar, 1999).

Although there are reports of the fibrinolysin / deoxyribonuclease efficacy in the tissue repair of cutaneous wounds, there are no studies about the use of this substance in palataline mucosa, combined or not with the bismuth subgalate. Based on this, this study aimed to evaluate by histological analysis the repair process repair of standardized wounds in rat palate in the presence of fibrinolysin / deoxyribonuclease associated or not to the bismuth subgalate.

Material and methods

The study was previously approved by the Committee of Ethics in the use of animals (565/2011). Wistar rats, male, young adults (300-350 g) were selected. The animals were randomly divided into 3 groups (control, test 1 and test 2) with 25 animals each and accommodated 5 out of 5 in cages. For the experiment, the animals were intramuscularly anesthetized using 80 mg Ketamine / kg + 8 mg Xylazine / kg, according to Brazilin Good practice guide on Euthanasia in animal-concepts and procedures recommended-Brasilia (2012). A standardized wound was then performed per animal in the central region of the third palatal fold with aid of a punch (Golgran - Brazil) of 3mm in diameter. All soft tissue was removed by 3S spatula (Duflex - Brazil) leaving the bone tissue exposed (Figure 1).

After a biopsy, hemostasis for the control group was obtained with compression aid with sterile gauze soaked in 0.9% physiological solution at room temperature. For test group 1, fibrinolysin / deoxyribonuclease cream (Fibrinase, Cristália, SP, BR) was applied to the clot with the aid of a cotton swab. For test group 2, the hemostasis obtained with a bismuth subgalate application (Dermatol, Farmanilquima, PR, BR) on a described wound methodology according to methodology described1, in a 0.9% saline solution that was added to the bismuth subgalate powder until a cream consistency was achieved. The cream was applied on a wound with the help of number 7 spatula (Duflex – Brazil) and compressed lightly with a dry sight. The excess material was then removed by washing with 0.9% physiological solution and the fibrinolysin / deoxyribonuclease cream was applied to the clot with the aid of a cotton swab. On the two days, at 24 h, the animals of the tested groups 1 and 2 were manually contained and received the application of fibrinolysin / deoxyribonuclease cream on the wound, totaling 3 applications (Figure 2).

On 3, 7, 14, 30 and 60 postoperative days, 5 animals from each group were submitted to euthanasia by overdose of intraperitoneal injection with sodium thiopental9. Whole-jaw biopsies were performed avoiding the palatine wound area laceration. The collected samples were kept in 10% buffered formalin solution for 72 h. They were then washed in running water and dipped in Paul Speight's descaling solution (University of Sheffield, England) reduced to 1 liter (820 ml of distilled water and 180 ml of 85% formic acid). After decalcification, the pieces were reduced in order to allow the microtomy in the transverse direction in the wound center. For each specimen, 55 µm thick sections were obtained and stained with hematoxylin and eosin for histological evaluation.

The images were taken from a camera coupled to the light microscope and to the computer at 40X and 400X magnification through the Leica Las V4.2 program (Leica Microsystems, Switzerland). The images obtained were analyzed by Image Pro Plus 4.0 (Media Cybernetics, USA) with previous calibration in millimeters. The histological evaluation included the dichotomous nominal scale with the presence or absence of variables usually present in the tissue repair: clot, necrosis, edema, foreign material, foreign body reaction, chronic inflammation, vascular proliferation and fibroblast cell proliferation (Kahnberg & Thinlander, 1982) (Figure 3).

The wound center was used as a reference for this analysis. The clot was evaluated at 3 and 7 day, while the other variables were up to 60 days. A single experienced and previously calibrated evaluator performed all the evaluation.

To evaluate the epithelial repair, linear measurements of the ulcerated areas were realized and vertical measurements of the repaired areas were performed. Centripetal epithelial proliferation measurement in the ulcer area, corresponding to the distance between the proliferating laterally basal layers, was defined as a linear measure on days 3 and 7. To standardize the sample, 2 vertical lines were initially drawn near the layers proliferation on each side and then a horizontal tracing (Figure 4).

In turn, a vertical epithelial measure was equivalent at a distance from the basal layer to the cornea to measure the thickness of epithelia on days 14, 30 and 60. The center of the nasal septum was defined as the reference point of the center of the repaired epithelial area. From this point, it was measured horizontally 1.5 mm bilaterally, delimiting with vertical lines an extension of 3 mm corresponding to the area to be evaluated. Vertical measurements from basal layer to the cornea. The total was 15 points, being 1 central and 7 more on each side. The keratin layer was excluded to avoid areas with artifact and displacement of the same (Figure 5).

The normality of the data was verified with the Kolmogorov-Smirnov test and the homogeneity of variances between the groups through the Levene test at a significance level of 5%. For the analysis of the data of the nominal dichotomous scale, the Pearson chi-square test and difference test between 2 proportions were applied; For the epithelial histomorphometric data, we used variance tests, Tukey HSD multiple comparisons tests for homogeneous variances and Games-Howell for heterogeneous variances at a significance level of 5%.

Results

In the periods of 3 and 7 days the simultaneous presence of degenerate and proliferating epithelium was observed. In these periods, the clot was present in all groups. Test group 1 had the lowest mean value of clot presence with a statistically significant difference in relation to the other groups ($p < 0.05$). The presence of necrosis was higher in the control group and with statistical significance in relation to the test groups ($p < 0.05$). Chronic inflammation was also higher in the control group, however, with significance only in relation to the test group 2. All groups showed a similar presence and no statistical significance of the variables: edema, foreign material, foreign body type reaction and fibroblast cell proliferation. In relation to vascular proliferation, control and test groups 2 were similar, whereas test group 1 presented the lowest percentage ($p < 0.05$) (Table 1).

In 3 and 7 day periods the incomplete wounds epithelial closure was observed in all groups. Only after 14 postoperative days, all wounds presented complete closure. The reduction in the linear epithelial measurement occurred between days 3 and 7 were, 26.61% for the control group, 85.71% for the test group 1 and 57.67% for the test group 2 (Table 2).

The multiple comparisons of the linear measures at 3 days showed a statistically significant difference between the control and test 1 groups ($p = 0.04$). At the same time, at 7 days, the differences remained statistically significant between these two groups ($p < 0,01$). In turn, test group 2 did not show any statistically significant differences with the other groups in any of the evaluated periods (Table 3).

For the vertical measurement of the epithelium in the periods of 14, 30 and 60 days, the means were similar between the groups. The mean values observed in these periods were: 0.13 ± 0.02 mm for the control group, 0.15 ± 0.04 mm for the test group 1 and 0.15 ± 0.04 mm for the test group 2 (Table 4).

The respective mean values were homogeneous and the analysis of variance showed no statistical difference regarding the vertical measurements of the epithelium in the groups and periods evaluated (Table 5).

Discussion

In an epithelial repair process, clot formation and fibrin deposition occur within the first 24 hours and peak phagocytosis activity of antigenic particles and foreign bodies within 24 to 48 hours (Kahnberg & Thinlander, 1982; Li & Kirsner, 2007). In this aspect, a widely used option for the removal of cellular debris in the wound bed, which may aid in tissue repair, is enzymatic debridement (Falabella et al., 1998; Falabella, 2006; Li & Kirsner, 2007; Mekkes et al., 1998; Perini et al., 2015; Pullen, Pop, Volkers, & Fulgen, 2002). The results of the present study showed that the debridant properties of fibrinolysin / deoxyribonuclease may have contributed to the lower percentage of clot in the test group 1 by direct action on the wound. The fact that the test group 2 did not present significant difference with the control group may be

related to the hemostatic action where the greater blood volume may have hindered the enzymatic action of fibrinolysin.

Epithelial necrosis was less present in the test groups than in the control group. In group 2 this fact can be explained by the astringent action of the bismuth subgalate, promoting protein precipitation and formation of a protective layer on the bare areas (Kim et al., 2012). Whereas, the debridement properties of topical application of fibrinolysin / deoxyribonuclease (Perini et al., 2015; Pullen et al., 2002) alone or on the bismuth subgalate layer may have decreased necrosis.

The greater presence of the inflammatory phase in the control group compared to the test group 2 may have been due to the slower centripetal epithelial closure, leading to extensive bacterial colonization and eventual traumas at the site (Cornelissen, Maltha, Von den Hoff, & Kuijpers-Jagtman, 1999a; Cornelissen, Maltha, Von den Hoff, & Kuijpers-Jagtman, 1999b; Kahnberg & Thinlander, 1982). Thus, bone longer exposure may also have influenced the repair of epithelial and connective tissues since the interaction between them is a prerequisite for healing (Kahnberg & Thinlander, 1982; Witte & Barbul, 1997). The subgalate astringent action, forming a protective layer on the wound, appears to have been essential for less pronounced inflammatory activity. All groups presented the presence of chronic inflammation underlying the injured area up to 60 days. An evidence that the repair process in fibrous connective tissue can take months to be dependent on a prolonged process of maturation and remodeling of collagen (Witte & Barbul, 1997).

On the other hand, in a favorable way, vascular proliferation was higher in the control group than in the test groups. This fact may also be explained by an interference in the events of the coagulation cascade that stimulate the migration and mitosis of the endothelial cells (Pullen et al., 2002; Tazima, Vicente, & Moriya, 2008; Weller, Foittzik, Paus, Syska, & Maurer, 2006; Witte & Barbul, 1997). Thus, the local debridant action for the reduction of clot volume may have reflected in the test groups, mainly in the test group 1 because it had a more direct action on the tissues (Perini et al., 2015). Despite this hypothesis, apparently unfavorable, the repair was superior in the test groups, especially for the test group 1.

The analysis of the epithelial closure was conditioned to the centripetal migration of the basal layer (Li et al., 2007; Kahnberg & Thinlander, 1982; Perini et al., 2015). Study of the repair evaluation in the rat palate, without the aid of any medications, and with the same diameter of this work (3 mm) showed that complete epithelization occurred after 14 days. However, another study using debridant substances showed that epithelial closure was better in periods of 8 to 15 days with the application of fibrinolysin when compared to saline alone (Perini et al., 2015). In this work, complete epithelization of the epithelium was observed in the 14-day post-operative samples. However, it may have occurred in the non-evaluated intervals (from the 8th to the 13th day).

In the linear measurements in the 3 and 7 day periods, it was observed that the test group 1 presented a faster epithelial regrowth rate and with significance in relation to the control group, corroborating with a previous study on the rats back (Perini et al., 2015). Despite the test group 2 did not present values with significant differences in comparison to the control group, the averages were smaller in relation to it. This finding may be relevant in preventing bacterial colonization with Bismuth Subgalate in ulcerated area on the first postoperative days, as well as the impact of physical aggressions on the wound (Cornelissen et al., 1999b). This result in the test group 2 also confirmed a better performance when compared to the study of application of bismuth subgalate isolated over the wound where the closure response was slower than the control group (saline) (Pullen et al., 2002). In this way, although it was not the object of this study to evaluate the subgalate alone, it seems that the action of associated fibrinolysin to subgalate may have provided a more favorable local response to epithelial closure.

However, regardless of the variation of the linear epithelial measurements in the periods and in the groups, the vertical epithelial measurements, related to the epithelial thickness in the operated area, did not differ among themselves, presenting similar values and without statistical significance.

About fibroblastic proliferation, the presence remained constant between the groups, without statistical differences. This data confirms results from previous studies reporting that the bismuth (Kim et al., 2010; Kim et al., 2012; Tramontina et al., 2002) subgalate and fibrinolysin (Cornelissen et al., 1999b; Li et al., 2007; Mekkes & Zeegelaar, 1999; Pullen et al., 2002; Perini et al., 2015Westerhof et al., 1987; Witte & Barbul, 1997) do not interfere in the final quality of the epithelial repair.

Conclusion

The findings of this research suggest that the application of fibrinolysin / deoxyribonuclease alone or combined with the bismuth subgalate may be of great clinical value in excisional periodontal surgical procedures. The acceleration in epithelial closure may have repercussions on less postoperative discomfort. However, in spite of the results obtained, other experimental and clinical evaluations are suggested for a better understanding of tissue repair with the application of fibrinolysin / deoxyribonuclease. Therefore, the topical application of fibrinolysin / deoxyribonuclease promoted a faster epithelial closure when compared to saline alone and its use alone or associated with the bismuth subgalate did not interfere with tissue repair.

Tables

Table 1. Mean values in percentage of the variables presence with the nominal dichotomous scale according to groups and application of the difference test between 2 proportions.

Variables	Evaluation Period (days)	Group					
		Control (%)		Test 1 (%)		Test 2 (%)	
Clot	3,7	96,77	a	54,05	b	87,5	a
Necrosis	3,7,14,30,60	50,7	a	32,9	b	28,1	b
Chronic Inflammation	3,7,14,30,60	91	a	81,6	a b	76,6	b
Edema	3,7,14,30,60	74,6	a	78,9	a	71,9	a
Strange material	3,7,14,30,60	37,3	a	31,6	a	32,8	a
Foreign body type reaction	3,7,14,30,60	4,5	a	10,5	a	3,1	a
Vascular proliferation	3,7,14,30,60	91	a	73,7	b	89,1	a
Fibroblast cell proliferation	3,7,14,30,60	76,1	a	75	a	81,3	a

Different letter represents statistical difference ($p < 0.05$).

Table 2. Descriptive statistics of the wounds linear measures in periods and groups.

Group	Period (days) Mean (mm)		Standard Deviation Default error		Confidence interval	
					Upper limit	Inferior limit
Control	3	2,03	0,38	0,17	1,55	2,5
	7	1,49	0,33	0,15	1,08	1,9
Test 1	3	0,7	0,64	0,29	-0,1	1,49
	7	0,1	0,15	0,07	-0,08	0,29
Test 2	3	1,63	0,3	0,13	1,26	2
	7	0,69	0,49	0,22	0,08	1,3

Table 3. Multiple comparisons of linear measures (ML) between groups and in the periods of 3 and 7 days.

Comparation	Average difference	Default error	P value	Confidence interval	
				Upper limit	Inferior limit

Control - 3 days	Test 1 – 3 days	1,3	0,3	0,04	0,0	2,6
	Test 2 – 3 days	0,4	0,2	0,5	-0,4	1,2
Control – 7 days	Test 1 – 7 days	1,4	0,2	0,00	0,7	2,0
	Test 2 – 7 days	0,8	0,3	0,13	-0,2	1,8
Test 1 – 3 days	Control – 3 days	-1,3	0,3	0,04	-2,6	-0,0
	Test 2 – 3 days	-0,9	0,3	0,15	-2,2	0,3
Test 1 - 7 days	Control – 7 days	-1,4	0,2	0,00	-2,0	-0,7
	Test 2 – 7 days	-0,6	0,2	0,27	-1,6	0,4
Test 2 – 3 days	Control – 3 days	-0,4	0,2	0,51	-1,2	0,4
	Test 1 – 3 days	-0,9	0,3	0,15	-0,3	2,2
Test 2 – 7 days	Control – 7 days	-0,8	0,3	0,13	-1,8	0,2
	Test 1 – 7 days	0,6	0,2	0,27	-0,4	1,6

P <0.05 (Games-Howell Test).

Table 4. Descriptive statistics of vertical measures (LVM) of the wound center in the groups.

		n	Mean	Standard Deviation	Default Error	95% Confidence interval for mean		Minimum	Maximum
						Upper limit	Inferior limit		
MVE	Control	15	0,13	0,02	0,00	0,11	0,14	0,09	0,18
	Test 1	15	0,15	0,04	0,01	0,13	0,17	0,05	0,22
	Test 2	15	0,15	0,04	0,01	0,12	0,17	0,09	0,24
	Total	45	0,14	0,03	0,00	0,13	0,15	0,05	0,24

Mean in mm

Table5. Variance analysis of the epithelium vertical measurements according to groups and periods.

Variable	Squares sum	Gl	Middle square	F	P value	Observed potency
Group	0,007	2	0,003	2,4	0,1	0,4
Period	0,003	2	0,001	0,9	0,4	0,2

Gl – Freedom degrees, F- Snedecor F test, b - computed using alpha = 0.05 Statistical difference (p <0.05).

Illustrations

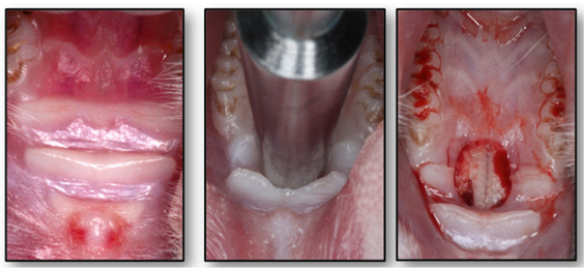

Figure 1. Representative image of the standardized wound on third fold palatine rat: (A) positioned punch, (B) standardized wound, (C) exposed bone tissue**.**

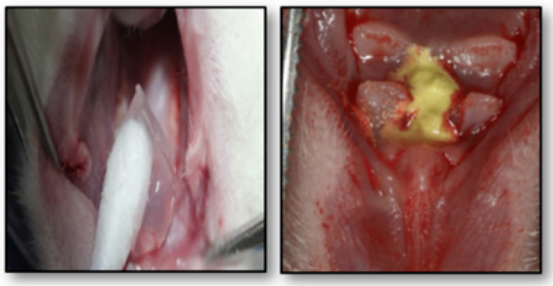

Figure 2. Representative image of fibrinolysin / deoxyribonuclease applications (A) and bismuth subgalate cream applications (B).

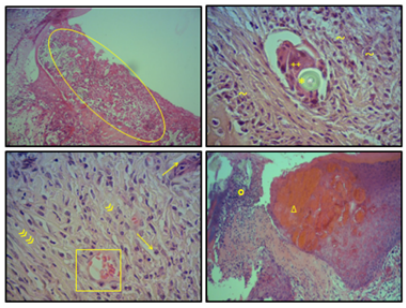

Figure 3. Representative image of histological variables: (A) clot, (B) foreign body *, reaction to foreign body ++, chronic inflammation~, (C) edema: arrow, fibroblastic proliferation «vascular proliferation ,(D) necrosisş and clotΔ . HE, 400X.

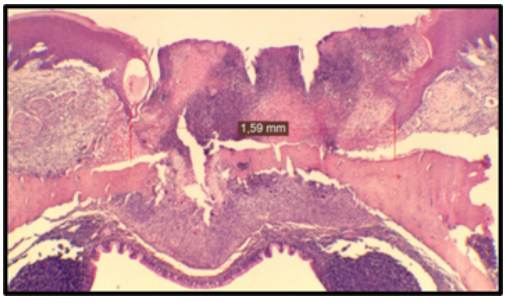

Figure 4. Representative image of the linear measure. Vertical traces parallel to the proliferating basal layer. Horizontal trace related to linear measurement in millimeters. HE, 40X. Image ProPlus4 program.

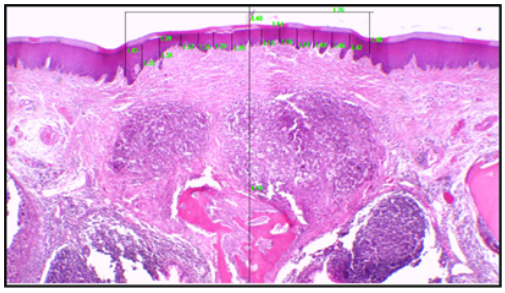

Figure 5. Vertical Measurement: representative general view of the region evaluated in the periods of 14, 30 and 60 days, HE, 40X. Image Pro-Plus4 program.

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