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LUCINARA IGNEZ TAVARES LUZZI

USO DE FIBRINOLISINA / DESOXIRRIBONUCLEASE E SUBGALATO DE BISMUTO NO REPARO DE FERIDAS DA MUCOSA PALATINA: ESTUDO HISTOLÓGICO EM RATOS

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos para obtenção do título de Doutor em Odontologia - Área de Concentração: Periodontia

Orientador: Prof. Dr. Sung Hyum Kim

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LUCINARA IGNEZ TAVARES LUZZI

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Orientador (a): Prof. Dr. Sung Hyun Kim
Programa de Pós-Graduação em Odontologia, PUCPR

Prof. Dr. Vinicius Augusto Tramontina
Programa de Pós-Graduação em Odontologia, PUCPR

Profª Drª Sônia Mara Luczyszyn
Programa de Pós-Graduação em Odontologia, PUCPR

Profª Drª Danielle Portinho Coutinho
Programa de Pós-Graduação em Odontologia, UNIOESTE

Prof. Dr. Marcio José Mendonça
Programa de Pós-Graduação em Odontologia, UNIOESTE

Curitiba, 18 de dezembro 2015.

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Colaboradores da PUCPR

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Misael Gomes Barbosa - Biólogo,Técnica Operatória/ e **Aline Ducate** – aluna PIBIC, fundamental auxílio na fase cirúrgica, contenção e cuidado com os animais.
Seigo Nagashima – Biólogo, Laboratório de Patologia/ incansável orientação e auxílio nos programas de análise de imagens e **Ana Paula Camargo Martins**, bióloga/ apoio no laboratório.

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Douglas Oliveira dos Santos, José Alcir dos Santos e Álvaro Roberto Gonçalces Machado – Técnicos de Laboratório/Biotério/Técnica operatória-transporte e cuidado com os animais.

Colaboradores da UNIOESTE

Carmen Isabel Rossoni - secretária geral/ processo de afastamento.
Profa. Dra Ana Lúcia Carrinho Ayrosa Rangel - coordenadora do Laboratório de Patologia / fase laboratorial.

Ms. Renata Delgado - colega /orientação e auxílio na captura e análise de imagens no Lab. de Patologia.

Marlene de Oliveira Baú – técnica /suporte técnico no processamento histológico no Laboratório de Patologia.

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FIBRINOLISINA/DESOXIRRIBONUCLEASE E SUBGALATO DE BISMUTO NO REPARO DE FERIDAS DA MUCOSA PALATINA: ESTUDO HISTOLÓGICO EM RATOS

Lucinara Ignez Tavares Luzzi, DDS; MSc[a]
Antônio Adilson Soares de Lima, DDS; MSc; PhD[b]
Vinicius Augusto Tramontina, DDS; MSc; PhD[c]
VulaPapalexiou, DDS; MSc; PhD[c]
Sônia Mara Luczsyzyn, DDS; MSc; PhD[c]
Sung Hyun Kim, DDS; MSc; PhD[c]

[a.] Aluna do programa de Pós-Graduação em Odontologia, Escola de Saúde e Biociências, Pontifícia Universidade Católica do Paraná

[b.] Professor do programa de Pós-Graduação em Odontologia, Universidade Federal do Paraná.

[c.] Professor do programa de Pós-Graduação em Odontologia, Escola de Saúde e Biociências, Pontifícia Universidade Católica do Paraná.

Correspondência para:

Prof. Dr. Sung Hyun Kim, Curso de Odontologia, PUCPR
Rua Imaculada Conceição, 1155 CEP 80215-901 Curitiba - Paraná, Brasil
e-mail: sung.kim@pucpr.br

RESUMO

Em áreas doadoras de enxerto gengival livre pode ocorrer maior sangramento e, o reparo das lesões ocorre por segunda intenção. Neste sentido, avaliou-se histologicamente o reparo na mucosa palatina de75 ratos *Wistar* divididos em 3 grupos de 25 animais cada. Feridas com *punch* de 3 milímetros foram realizadas no centro da terceira prega palatina. Para a hemostasia, o grupo controle recebeu compressão com gaze umedecida em soro fisiológico, o grupo teste 1, após hemostasia igualmente ao grupo controle,aplicação de fibrinolisina /desoxirribonuclease, e o grupo teste 2 hemostasia com subgalato de bismuto e aplicação de fibrinolisina/desoxirribonuclease. A análise histológica ocorreu aos 3,7,14,30 e 60 dias.Em todos os grupos observou-se epitélio ulcerado aos 3 dias, em franca proliferação aos 7 dias e fechamento epitelial completo aos 14 dias.No grupo teste 1 a presença de coágulo foi menor aos 3 e 7 dias (p<0,05). O grupo controle evidenciou maior presença de necrose em relação aos grupos testes 1 e 2 (p <0,05). A medida linear foi menor no grupo teste 1 em relação ao grupo controle aos 3 dias (p=0,04) e 7 dias (p<0,01).A medida vertical do epitélio não apresentou diferença estatística entre os grupos. Conclui-se que aplicação tópica da fibrinolisina /desoxirribonuclease promoveu um fechamento epitelial mais rápido quando comparada somente ao soro fisiológico e sua utilização isolada ou associada ao subgalato de bismuto não interferiu no reparo epitelial.

Palavras chaves: Fibrinolisina / desoxirribonuclease.Subgalato de bismuto. Mucosa palatina.Reparo. Ratos *Wistar*

Abstract

In donor areas of free gingival graft may lead an increase bleeding and the repair of injuries occurs by second intention. Thus, it was evaluated histologically palatine mucosa in the repairing of 75 Wistar rats divided into 3 groups of 25 animals each. Wounds were made with punch with 3 mm in diameter in the central region of the third palatal fold. For hemostasis, the control group received gauze compression with saline.Test group 1 received gauze compression with saline and fibrinolysin/deoxyribonuclease and the test group 2 received application of the bismuth subgallate and fibrinolysin/deoxyribonuclease. Histological analysis tissue was given at 3,7,14,30 and 60 days. In all groups we observed ulcerated epithelium at 3 days. At 7 days the epithelium showed good proliferation and complete epithelial closure at 14 days. In the test group 1 the presence of coagulum was less at 3 and 7 days ($p < 0.05$). The control group showed increased presence of necrosis in relation to the test groups 1 and 2 ($p < 0.05$). The linear measurement was lower in the test group 1 when compared with the control group at 3 days ($p = 0.04$) and 7 days ($p < 0.01$). The vertical extent of the epithelium showed no statistical difference between the groups. It is concluded that topical application of fibrinolysin/deoxyribonuclease promoted a faster epithelial closure when compared to the saline only. The use of fibrinolysin/deoxyribonuclease alone or in association with bismuth subgallate did not interfere on the epithelial wound healing.

Key words: Fibrinolisina/deoxyribonuclease.Bismuth subgalate.Palatal mucosa. Repair.*Wistar* rats.

SUMÁRIO

ARTIGO EM PORTUGUÊS

INTRODUÇÃO

Em cirurgias excisionais como nas áreas doadoras da mucosa palatina, o reparo das lesões ocorre por segunda intenção devido à maior perda de células e tecidos, podendo levar a um aumento no sangramento. Recentemente, o subgalato de bismuto foi relatado como um importante auxiliar na redução do tempo de hemostasia na área doadora do enxerto gengival livre. Constatou-se resultado clinicamente favorável no controle do sangramento operatório, diminuição acentuada no tempo de cirurgia, aumento da segurança contra hemorragias pós-operatórias e maior tranquilidade ao paciente e operador.[1] Além disso, os estudos experimentais em animais revelaram que o mesmo não interferiu no processo de reparação dos tecidos epitelial e conjuntivo.[2,3]

Em um tecido lesado, o coágulo estabelece uma barreira física e biológica contra a contaminação e serve de matriz para a migração celular. No entanto, a degradação rápida da fibrina e exsudato fibrinoso seguidos pela substituição de tecido de granulação pode acelerar o reparo tecidual. Para esta finalidade, tem-se utilizado em feridas cutâneas, uma substância enzimática debridante de origem bovina composta por fibrinolisina/desoxirribonuclease.[4-6]Sua ação fibrinolítica é direcionada principalmente contra proteínas desnaturadas de tecidos desvitalizados. Os produtos resultantes da quebra enzimática são compostos de moléculas grandes que não são facilmente absorvidos pelo corpo. Assim, não produzem reações indesejáveis locais ou gerais.A desoxirribonuclease hidrolisa especificamente os principais componentes do ácido desoxirribonucléico, principais constituintes dos exsudatos. Por isso, a quebra em polinucleotídeos mais simples ajuda na liquefação do exsudato facilitando sua remoção dos ferimentos. Desta maneira, a produção de uma superfície lisa pela dissolução do exsudato e dos tecidos necróticos pode estimular a cicatrização do tecido mole.[7,8]

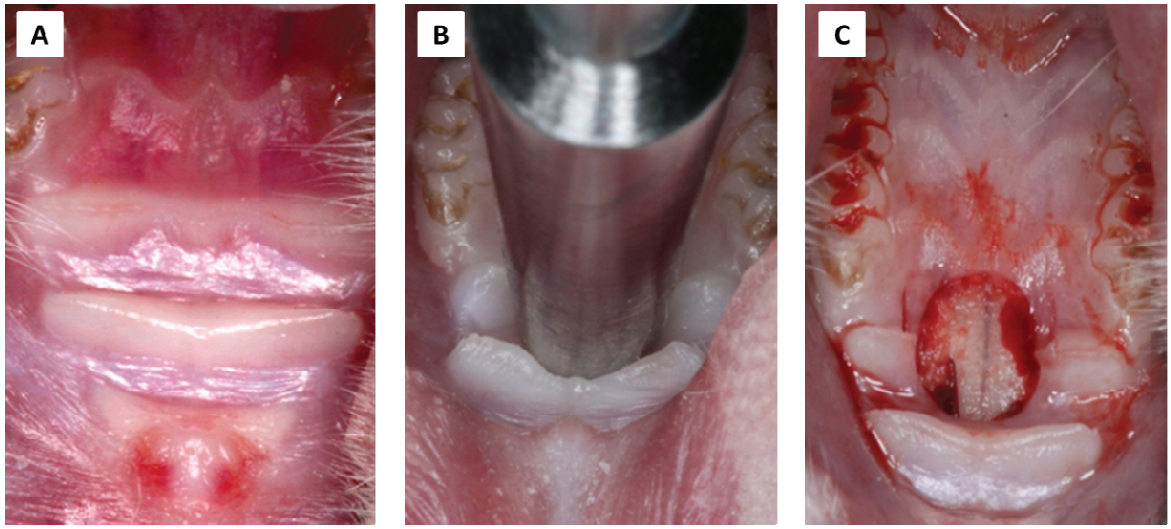
Embora haja relatos da eficácia da fibrinolisina/desoxirribonuclease no reparo tecidual de feridas cutâneas, não se encontram estudos da utilização desta substância em mucosa palatina, combinada ou não com o subgalato de bismuto. Com base nisso, este estudo teve como objetivo avaliar por meio de análise histológica, o processo de reparo de feridas padronizadas em palato de ratos, na presença de fibrinolisina/desoxirribonuclease associada ou não ao subgalato de bismuto.

MATERIAIS E MÉTODO

O estudo foi previamente aprovado pelo Comitê de Ética no uso de animais da Pontifícia Universidade Católica do Paraná, CEUA/PUCPR (656/2011).

Foram selecionados 75 ratos *Wistar,*machos, adultos jovens (300 a 350 g) oriundos do biotério da Pontifícia Universidade Católica do Paraná. Os animais foram divididos aleatoriamente em 3 grupos (controle, teste 1 e teste 2) de 25 animais cada e acomodados de 5 em 5 em gaiolas. Para o experimento, os animais foram anestesiados utilizando-se 80 mg de Cetamina/kg + 8 mg de Xilazina/kg via intramuscular.[9]Em seguida foi realizada uma ferida por animal, de forma padronizada, na região central da terceira prega palatina com auxílio de um *punch* de 3mm de diâmetro(CE R-806-9-3). Todo o tecido mole foi removido por uma espátula 3S (Duflex, SSWhite, Artigos Dentários Ltda, RJ, BR) deixando o tecido ósseo exposto (Figura 1).

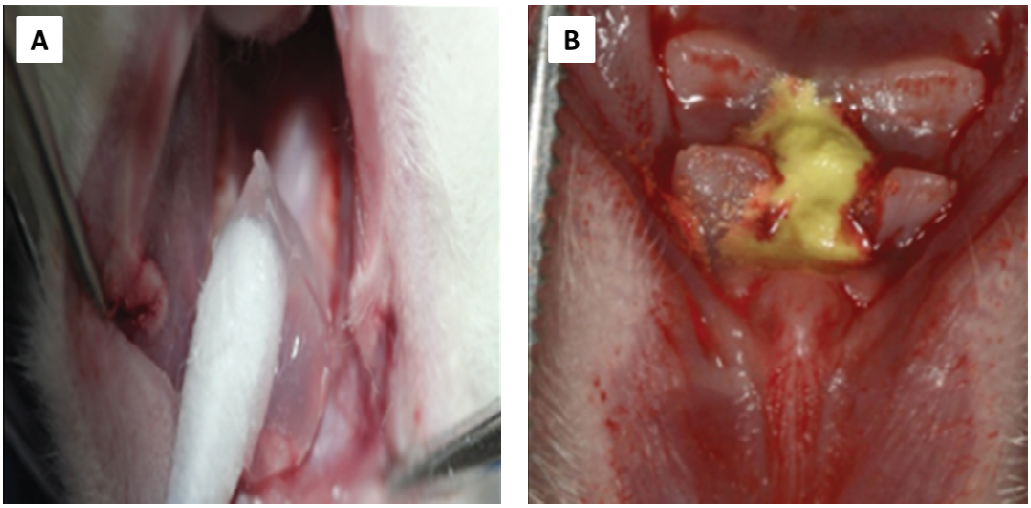
Figura 1. Imagem representativa da sequência para ferida padronizada:(A) pregas palatinas, (B) *punch* posicionado, (C) tecido ósseo exposto.



Após a biópsia, a hemostasia para o grupo controle foi obtida com auxílio de compressão com gaze estéril embebida em solução fisiológica a 0,9% à temperatura ambiente. Para o grupo teste 1, após a obtenção da hemostasia igualmente ao grupo controle, o creme de fibrinolisina /desoxirribonuclease (Fibrinase, Cristália, SP,BR) foi aplicado sobre o coágulo com auxílio de uma haste com algodão. Para o grupo teste 2, a hemostasia foi obtida com a aplicação de subgalato de bismuto (Dermatol, Farmanilquima, PR, BR) sobre a ferida conforme metodologia descrita[1] em que a solução fisiológica 0,9% foi adicionada

ao pó de subgalato de bismuto até obter a consistência de "creme". O creme foi aplicado sobre a ferida com auxílio de espátula nº7(Duflex, SSWhite, Artigos Dentários Ltda, RJ, BR) e comprimido levemente com uma gaze seca. Em seguida, removeu-se o excesso do material por lavagem com solução fisiológica 0,9% e o creme de fibrinolisina/ desoxirribonuclease foi aplicado sobre o coágulo com auxílio de uma haste com algodão. Nos dois dias seguintes à cirurgia, a cada 24h, os animais dos grupos testes 1 e 2 foram contidos manualmente e receberam aplicação do creme de fibrinolisina/ desoxirribonuclease sobre a ferida, totalizando 3 aplicações (Figura 2).

Figura 2. Imagem representativa das aplicações de (A) fibrinolisina/desoxirribonuclease e (B)creme de subgalato de bismuto.



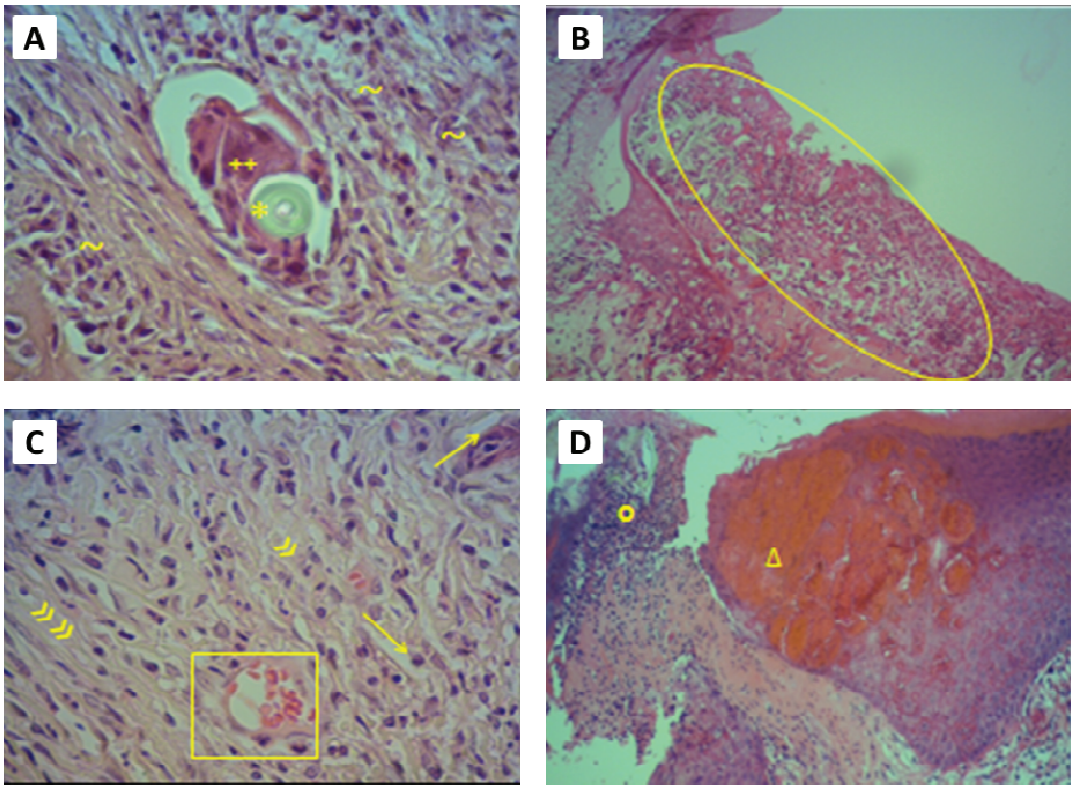
Nos dias 3, 7, 14, 30 e 60 pós-operatórios, 5 animais de cada grupo foram submetidos a eutanásia pela overdose de injeção intraperitoneal com Tiopental sódico.[9]Biópsias da maxila inteira foram realizadas, evitando a dilaceração da área da ferida palatina. As amostras coletadas foram mantidas em solução de formol tamponado a 10% durante 72 h. Em seguida, lavadas em água corrente e imersas em solução descalcificadora de Paul Speight (Universidade de Sheffield, Inglaterra) reduzida para 1 litro (820ml de água destilada e 180ml de ácido fórmico a 85%). Após a descalcificação, as peças foram reduzidas de forma a permitir a microtomia no sentido transversal no centro da ferida. Para cada peça, foram obtidos 5cortes com 5 µm de espessura e corados com hematoxilina e eosina para avaliação histológica.

A captura das imagens foi realizada a partir de uma câmera acoplada ao microscópio de luz e ao computador em 40X e 400X de aumento através do programa Leica Las V4.2(Leica Microsystems, Switzerland).As imagens obtidas foram analisadas pelo pro-

grama Image Pro Plus 4.0 (Media Cybernetics, EUA) com calibração prévia em milímetros.

A avaliação histológica incluiu a escala nominal dicotômica com o critério de presença ou ausência de variáveis usualmente presentes no reparo tecidual: coágulo, necrose, edema, material estranho, reação a corpo estranho, inflamação crônica,proliferação vascular e proliferação celular fibroblástica[10] (Figuras 3).

Figura 3. Imagem representativa das variáveis histológicas (A): corpo estranho*, reação a corpo estranho+,inflamação crônica ~,(B): coágulo,(C) edema →, proliferação fibroblástica «, proliferação vascular□,(D) necrose○ e coágulo△(HE, 400X).

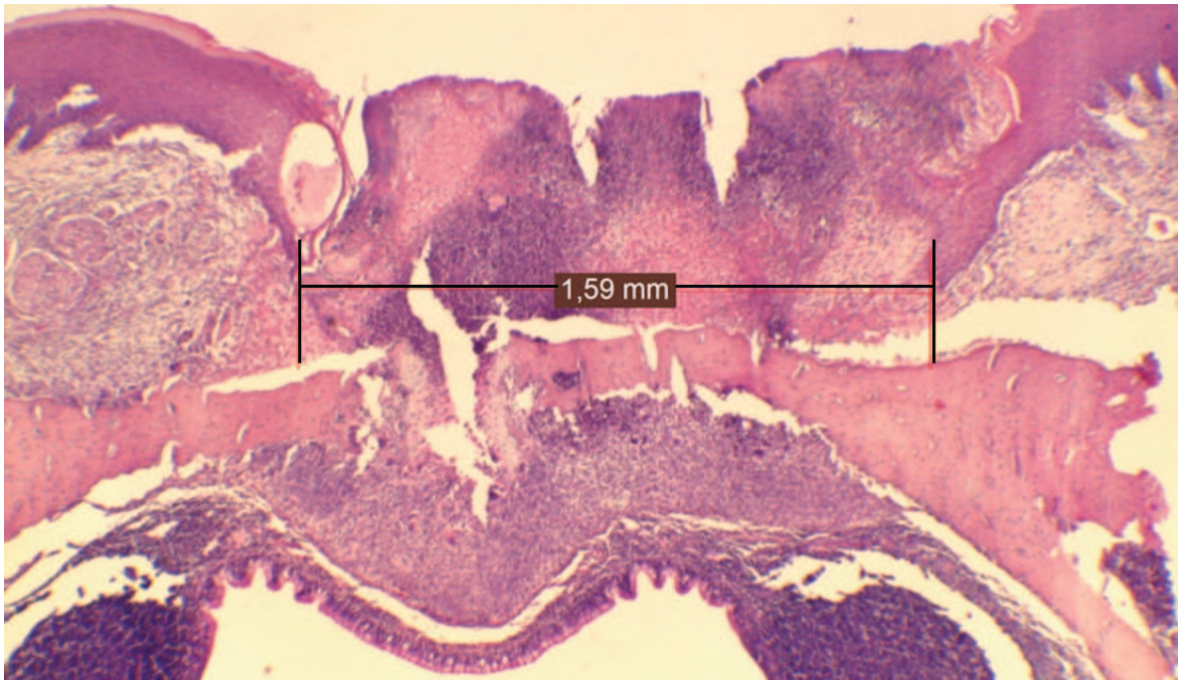


O centro da ferida foi utilizado como referência para esta análise. O coágulo foi avaliado aos 3 e 7 dias, enquanto as demais variáveis foram até 60 dias. Toda a avaliação foi executada por um único avaliador experiente e previamente calibrado.

Para avaliação do reparo epitelial foram realizadas mensurações lineares das áreas ulceradas e verticais das áreas reparadas. A mensuração da proliferação epitelial centrípeta na área da úlcera, correspondente à distância entre as camadas basais lateralmente em proliferação,foi definida como medida linear nos dias 3 e 7. Para a obtenção padronizada da

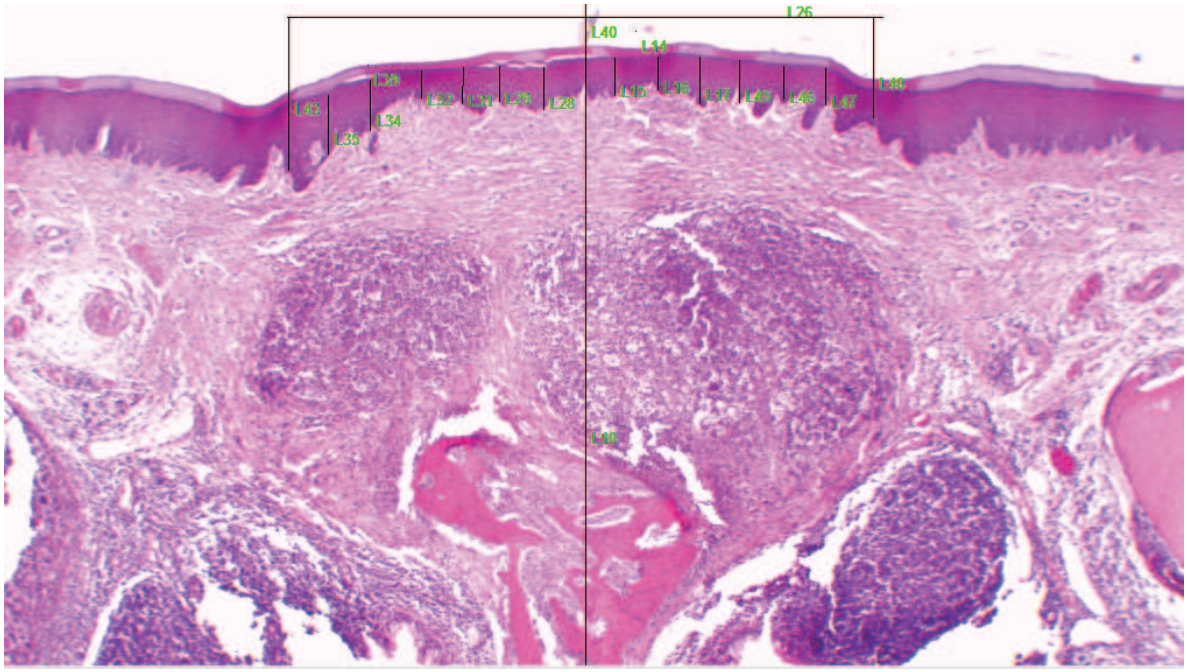
mesma, traçou-se inicialmente 2 linhas verticais junto às camadas basais em proliferação de cada lado e em seguida um traçado horizontal (Figura 4).

Figura 4. Imagem representativa da medida linear.Traçados verticais paralelos à camada basal em proliferação.Traçado horizontal referente à medida linear em milímetros. HE, 40X.Programa Image ProPlus4.



Por sua vez, a medida vertical epitelial correspondeu à distância da camada basal à córnea para mensurar a espessura dos epitélios nos dias 14, 30 e 60. O centro do septo nasal foi definido como ponto de referência do centro da área epitelial reparada. A partir deste ponto, mediu-se no sentido horizontal 1,5 mm bilateralmente, delimitando com traçados verticais a extensão de 3 mm correspondente a área avaliada. As medições verticais foram da camada basal à córnea. Ao total foram 15 pontos, sendo 1 central e mais 7 de cada lado. A camada de ceratina foi excluída para evitar áreas com artefato e deslocamento da mesma (Figura 5).

Figura 5. Medida Vertical: vista geral representativa da região avaliada nos períodos de 14, 30 e 60 dias, HE,40X. Programa Image Pro-Plus4.



A normalidade dos dados foi verificada com o teste de Kolmogorov-Smirnov e a homogeneidade de variâncias entre os grupos através do teste de Levene ao nível de significância de 5%. Para a análise dos dados da escala nominal dicotômica, aplicou-se o teste qui-quadrado de Pearson e teste de diferença entre 2 proporções.Por sua vez, para os dados histomorfométricos epiteliais foram utilizados teste de variâncias,testes de comparações múltiplas de Tukey HSD para variâncias homogêneas e Games-Howell para variâncias heterogêneas ao nível de significância de 5%.

RESULTADOS

Nos períodos de 3 e 7 dias foi observado a presença simultânea de epitélio degenerado e em proliferação. Nestes períodos, o coágulo esteve presente em todos os grupos. O grupo teste 1 apresentou o menor valor médio de presença de coágulo com diferença estatística significante em relação aos outros grupos ($p < 0,05$). A presença da necrose foi maior no grupo controle e com significância estatística em relação aos grupos testes ($p<0,05$). A inflamação crônica também foi maior no grupo controle, porém, com significância apenas em relação ao grupo teste 2. Todos os grupos mostraram a presença similar e sem significância estatística das variáveis: edema, material estranho, reação do tipo corpo

estranho e proliferação celular fibroblástica. Em relação à proliferação vascular, os grupos controle e teste 2 mostraram-se similares, enquanto que o grupo teste 1 apresentou o menor percentual ($p < 0,05$) (Tabela 1).

Tabela 1. Valores médios em porcentagem da presença das variáveis com a escala nominal dicotômica segundo grupos e aplicação do teste de diferença entre 2 proporções.

Variáveis	Período de avaliação (dias)	Grupo		
		Controle (%)	Teste 1 (%)	Teste 2 (%)
Coágulo	3,7	96,77 a	54,05 b	87,5 a
Necrose	3,7,14,30,60	50,7 a	32,9 b	28,1 b
Inflamação crônica	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
Corpo estranho	3,7,14,30,60	37,3 a	31,6 a	32,8 a
Reação do tipo corpo estranho	3,7,14,30,60	4,5 a	10,5 a	3,1 a
Proliferação vascular	3,7,14,30,60	91 a	73,7 b	89,1 a
Proliferação celular fibroblástica	3,7,14,30,60	76,1 a	75 a	81,3 a

Letra diferente representa diferença estatística (p <0,05).

Nos períodos de 3 e 7 dias foi observado o fechamento epitelial incompleto das feridas em todos os grupos. Somente a partir de 14 dias pós-operatório, todas as feridas apresentaram o fechamento completo. A redução da medida linear epitelial ocorrida entre os dias 3 e 7 foram, 26,61 % para o grupo controle, 85,71 % para o grupo teste 1 e 57,67 % para o grupo teste 2 (Tabela 2).

Tabela 2. Estatística descritiva das medidas lineares das feridas nos períodos e grupos.

Grupo	Período (dias)	Média (mm)	Desvio padrão	Erro Padrão	Intervalo de confiança	
					Limite superior	Limite inferior
Controle	3	2,03	0,38	0,17	1,55	2,5
	7	1,49	0,33	0,15	1,08	1,9
Teste 1	3	0,7	0,64	0,29	-0,1	1,49
	7	0,1	0,15	0,07	-0,08	0,29
Teste 2	3	1,63	0,3	0,13	1,26	2
	7	0,69	0,49	0,22	0,08	1,3

As comparações múltiplas das medidas lineares aos 3 dias mostraram diferença estatisticamente significante entre os grupos controle e teste 1 (p = 0,04).Igualmente ao período anterior, aos 7 dias, as diferenças continuaram estatisticamente significantes entre estes dois grupos (p < 0,01). Por sua vez, o grupo teste 2 não demonstrou nenhuma diferença estatisticamente significante com os demais grupos em nenhum dos períodos avaliados (Tabela 3).

Tabela 3. Comparações múltiplas das medidas lineares(ML) entre grupos e nos períodos de 3 e 7 dias.

Comparação		Diferença média	Erro padrão	Valor p	Intervalo de confiança	
					Limite superior	Limite inferior
Controle - 3 dias	Teste 1 – 3 dias	1,3	0,3	0,04	0,0	2,6
	Teste 2 – 3 dias	0,4	0,2	0,5	-0,4	1,2
Controle – 7 dias	Teste 1 – 7 dias	1,4	0,2	0,00	0,7	2,0
	Teste 2 – 7 dias	0,8	0,3	0,13	-0,2	1,8
Teste 1 – 3 dias	Controle – 3 dias	-1,3	0,3	0,04	-2,6	-0,0
	Teste 2 – 3 dias	-0,9	0,3	0,15	-2,2	0,3
Teste 1 - 7 dias	Controle – 7 dias	-1,4	0,2	0,00	-2,0	-0,7
	Teste 2 – 7 dias	-0,6	0,2	0,27	-1,6	0,4
Teste 2 – 3 dias	Controle – 3 dias	-0,4	0,2	0,51	-1,2	0,4
	Teste 1 – 3 dias	-0,9	0,3	0,15	-0,3	2,2
Teste 2 – 7 dias	Controle – 7 dias	-0,8	0,3	0,13	-1,8	0,2
	Teste 1 – 7 dias	0,6	0,2	0,27	-0,4	1,6

p <0,05 (Teste de Games-Howell).

Quanto à medida vertical do epitélio nos períodos de 14, 30 e 60 dias, as médias foram similares entre os grupos. Os valores médios observados nestes períodos foram: 0,13 ± 0,02 mm para o grupo controle, 0,15 ± 0,04 mm para o grupo teste 1 e 0,15 ± 0,04 mm para o grupo teste 2 (Tabela 4).

Tabela 4. Estatística descritiva das medidas verticais (MVE) do centro da ferida nos grupos.

		n	Média	Desvio padrão	Erro Padrão	Intervalo de confiança de 95% para média		Mínimo	Máximo
						Limite inferior	Limite superior		
MVE	Controle	15	0,13	0,02	0,00	0,11	0,14	0,09	0,18
	Teste 1	15	0,15	0,04	0,01	0,13	0,17	0,05	0,22
	Teste 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

Média em mm

Os respectivos valores médios mostraram-se homogêneos e a análise de variância não demonstrou diferença estatística quanto às medidas verticais do epitélio nos grupos e períodos avaliados (Tabela 5).

Tabela 5. Análise de variância das medidas verticais do epitélio segundo grupos e períodos.

Variável	Soma dos quadrados	Gl	Quadrado médio	F	Valor p	Potência observada[b]
Grupo	0,007	2	0,003	2,4	0,1	0,4
Período	0,003	2	0,001	0,9	0,4	0,2

Gl - graus de liberdade, F- teste F de Snedecor, b - computado usando alfa = 0,05

Diferença estatística (p<0,05).

DISCUSSÃO

Em um processo de reparo epitelial, a formação de coágulo e deposição de fibrina ocorrem nas primeiras 24 horas e o pico de atividade de fagocitose das partículas antigênicas e corpos estranhos em 24 a 48 horas. [10,11]Neste aspecto, uma opção amplamente utilizada para a remoção de detritos celulares no leito de ferida, que pode auxiliar na reparação tecidual, é o debridamento enzimático.[5-7,11-13] Os resultados do presente estudo, mostraram que as propriedades debridantes da fibrinolisina/desoxirribonuclease podem ter contribuído para o menor percentual de coágulo no grupo teste 1 pela ação direta na ferida.O fato do grupo teste 2 não apresentar diferença significante com o grupo controle pode estar relacionado à ação hemostática onde o maior volume sanguíneo pode ter dificultado a ação enzimática da fibrinolisina.

A necrose epitelial foi menos presente nos grupos testes em relação ao grupo controle. No grupo teste 2 este fato pode ser explicado pela ação adstringente do subgalato de bismuto, promovendo precipitação de proteínas e formação de uma camada protetora sobre as áreas desnudas.[3] Enquanto que, as propriedades debridantes da aplicação tópica da fibrinolisina/desoxirribonuclease[12,13] isoladamente ou sobre a camada de subgalato de bismuto podem ter diminuído a necrose.

A maior presença da fase inflamatória no grupo controle em relação ao grupo teste 2 pode ter sido em virtude do fechamento epitelial centrípeto mais lento, levando a extensa

colonização bacteriana e eventuais traumas no local.[10,14,15] Dessa forma, a exposição óssea por mais tempo, também pode ter influenciado a reparação dos tecidos epitelial e conjuntivo uma vez que a interação entre eles é um pré-requisito para a cura.[10,16] A ação adstringente do subgalato, formando uma camada protetora sobre a ferida, parece ter sido fundamental para uma atividade inflamatória menos pronunciada.Todos os grupos apresentaram a presença de inflamação crônica subjacente à área lesionada até os 60 dias. Uma evidência de que o processo de reparo no tecido conjuntivo fibroso pode levar meses por estar na dependência de um prolongado processo de maturação e remodelação do colágeno. [16]

Por outro lado, de maneira favorável, a proliferação vascular foi maior no grupo controle em relação aos grupos testes. Este dado, ainda, talvez possa ser explicado por uma interferência nos eventos da cascata de coagulação que estimulam a migração e a mitose das células endoteliais. [12,16-18] Dessa forma, a ação debridante local para a redução do volume de coágulo pode ter refletido nos grupos testes, principalmente, no grupo teste 1 por ter uma ação mais direta sobre os tecidos.[13] Apesar desta hipótese, aparentemente desfavorável, a reparação foi superior nos grupos testes, especialmente para o grupo teste 1.

A análise do fechamento epitelial esteve condicionada à migração centrípeta da camada basal. [10,11,13]Estudo da avaliação de reparo no palato de ratos, sem auxílio de quaisquer medicamentos, e com o mesmo diâmetro deste trabalho (3 mm) mostrou que a epitelização completa ocorreu a partir dos 14 dias.[10] Porém, outro estudo utilizando substâncias debridantes, mostrou que o fechamento epitelial foi melhor nos períodos de 8 a 15 dias com a aplicação de fibrinolisina quando comparada apenas ao soro.[13]Neste trabalho, a epitelização completa do epitélio foi observada nas amostras de 14 dias pós-operatório. Contudo, pode ter ocorrido nos intervalos não avaliados (do 8º ao 13º dia).

Nas medidas lineares nos períodos de 3 e 7 dias observou-se que o grupo teste1 apresentou uma velocidade de recobrimento epitelial mais rápida e com significância em relação ao grupo controle, corroborando com estudo prévio em dorso de ratos.[13]Apesar do grupo teste 2 não ter apresentado valores com diferenças significativa sem comparação ao grupo controle, as médias foram menores em relação ao mesmo. Este achado pode ter relevância ao se prevenir a colonização bacteriana com o SGB na área ulcerada nos primeiros dias pós-operatório, como também o impacto das agressões físicas na ferida[15] Este resultado no grupo teste 2, também confirmou melhor desempenho quando comparado ao estudo de aplicação de subgalato de bismuto isolado sobre a ferida onde a resposta de fechamento foi mais lenta em relação ao grupo controle (soro) [2]. Desta forma, apesar de não ter sido

objeto desse trabalho avaliar o subgalato isoladamente, parece que a ação da fibrinolisina associada ao subgalato pode ter propiciado resposta local mais favorável ao fechamento epitelial.

No entanto, independentemente da variação das medidas epiteliais lineares nos períodos e nos grupos, as medidas epiteliais verticais, relacionadas à espessura epitelial na área operada, não diferiram entre si, apresentando valores similares e sem significância estatística.

Quanto à proliferação fibroblástica, a presença se manteve constante entre os grupos, sem diferenças estatísticas. Este dado confirma resultados de estudos prévios que relatam que o subgalato de bismuto [1-3] e fibrinolisina[4-8,11-13, 15 16] não interferem na qualidade final do reparo epitelial.

Os achados desta pesquisa sugerem que a aplicação de fibrinolisina/ desoxirribonuclease isolada ou combinada com o subgalato de bismuto pode ser de grande valia clínica em procedimentos cirúrgicos periodontais excisionais. A aceleração no fechamento epitelial poderá repercutir em menor desconforto pós-operatório. Contudo, apesar dos resultados obtidos, sugere-se outras avaliações experimentais e clínicas para uma melhor compreensão da reparação tecidual com a aplicação da fibrinolisina/desoxirribonuclease.

CONCLUSÃO

A aplicação tópica da fibrinolisina/desoxirribonuclease promoveu um fechamento epitelial mais rápido quando comparada somente ao soro fisiológico e sua utilização isolada ou associada ao subgalato de bismuto não interferiu no reparo tecidual.

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WOUND REPAIR IN PALATINE MUCOSA WITH FIBRINOLYSIN/ DEOXYRIBONUCLEASE AND BISMUTH SUBGALATE: HISTOLOGICAL STUDY IN RATS

Lucinara Ignez Tavares Luzzi, DDS; MSc; [a]
Antônio Adilson Soares de Lima, DDS; MSc; PhD [b]
Vinicius Augusto Tramontina, DDS; MSc; PhD [c]
Vula Papalexiou, DDS; MSc; PhD [c]
Sônia Mara Luczsyzyn, DDS; MSc; PhD [c]
Sung Hyun Kim, DDS; MSc; PhD [c]

a. Student in the Dentistry post-graduation program, Biosciences and Health School, Pontifical Catholic University of Paraná.
b. Professor in the Dentistry post-graduation program, Federal University of Paraná.
c. Professor in the Dentistry post-graduation program, Biosciences and Health School, Ponfifical Catholic University of Paraná.

Correspondence to:
Prof. Dr. SungHyun Kim, Curso de Odontologia, PUCPR
Rua Imaculada Conceição, 1155 ZIP 80215-901 Curitiba - Paraná Brazil
e-mail: sung.kim@pucpr.br

Abstract

In donor areas of free gingival graft may lead an increase bleeding and the repair of injuries occurs by second intention. Thus, it was evaluated histologically palatine mucosa in the repairing of 75 Wistar rats divided into 3 groups of 25 animals each. Wounds were made with punch with 3 mm in diameter in the central region of the third palatal fold. For hemostasis, the control group received gauze compression with saline.Test group 1 received gauze compression with saline and fibrinolysin/deoxyribonuclease and the test group 2 received application of the bismuth subgallate and fibrinolysin/deoxyribonuclease. Histological analysis tissue was given at 3,7,14,30 and 60 days. In all groups we observed ulcerated epithelium at 3 days. At 7 days the epithelium showed good proliferation and complete epithelial closure at 14 days. In the test group 1 the presence of coagulum was less at 3 and 7 days ($p < 0.05$). The control group showed increased presence of necrosis in relation to the test groups 1 and 2 ($p < 0.05$). The linear measurement was lower in the test group 1 when compared with the control group at 3 days ($p = 0.04$) and 7 days ($p < 0.01$). The vertical extent of the epithelium showed no statistical difference between the groups. It is concluded that topical application of fibrinolysin/deoxyribonuclease promoted a faster epithelial closure when compared to the saline only. The use of fibrinolysin/deoxyribonuclease alone or in association with bismuth subgallate did not interfere on the epithelial wound healing.

Key words: Fibrinolisina/deoxyribonuclease.Bismuth subgalate.Palatal mucosa. Repair.*Wistar* rats.

Introduction

In excisional surgery as in the donor areas of the palatal mucosa, the repair of injuries occur by secondary intention due to increased loss of cells and tissues and may lead to an increased bleeding. Recently, bismuth subgallate was reported as an important aid in the reduction of hemostasis time in the donor area of the free gingival graft.It was found a clinically favorable result in control of operative bleeding, marked decrease in operative time, increased security against postoperative bleeding and more tranquility to the patient and surgeon.[1] In addition, experimental studies in animals have shown that it does not interfere in the repair process of epithelial and connective tissues.[2,3]

In an injured tissue, a blood clot establishing a physical and biological barrier against contamination and serves as a matrix for cell migration. However, the rapid degradation of the fibrin and fibrinous exudate followed by replacement of granulation tissue can accelerate tissue repair. For this purpose, it has been used in wound healing an enzymatic debridement substance composed by bovine fibrinolysin /deoxyribonuclease.[4-6]Its fibrinolytic action is mainly directed against denatured proteins of devitalized tissue. The products resulting from the enzymatic breakdown are composed of large molecules which are not easily absorbed by the body. Thus, do not produce local or general adverse reactions. Thereby, the breakdown into simpler polynucleotides helps exudate liquefaction facilitating removal of injuries.In this way, the production of a smooth by dissolving the exudate and necrotic tissue may stimulate the soft tissue healing.[7,8]

Although there are reports of efficacy of fibrinolysin/deoxyribonuclease on tissue repair of skin wounds, there are no studies of the use of this substance in palatine mucosa or in combination with bismuth subgallate. Based on this, our study aimed to evaluate through histological analysis, the repair process of the standardized wound palate in mice in the presence of fibrinolysin/deoxyribonuclease with or without the bismuth subgalate.

Materials and method

The project was approved by the Ethics Committee on the use of animals at the Pontifical Catholic University of Parana, CEUA / PUCPR (565/2011).

75 male *Wistar* rats were selected. It was young adults (300 to 350g) coming from the vivarium of the Pontifical Catholic University of Parana. The animals were randomly divided into 3 groups (control, test 1 and test 2) of 25 animals each and accommodated in cages with five rats in each one.For the experiment, the animals were anesthetized intramuscularly with Ketamina 80 mg / kg + 8 mg of xylazine / kg.[9] Then, standardized wounds were made with a punch of 3 mm diameter (CE R-806-9-3)in the central region of the third fold palate. It was held one wound per animal. All soft tissue was removed with a 3S spatula(Duflex, SSWhite, ArtigosDentáriosLtda, RJ, BR) exposing the bone tissue (Figure 1).

After the biopsy, hemostasis for the control group was obtained by sterile gauze compression and saline 0.9% at room temperature.For the test group 1, after obtaining hemostasis also the control group, the fibrinolysin/deoxyribonuclease cream (Fibrinase, Cristália, Itapira, Brazil) was applied to the clot with the help of a cotton swab. For the test group 2, haemostasis was achieved with the application of bismuth subgalate (Dermatol-Farmanilquima, Brazil) on the wound according to the methodology describe[1],in which 0.9% saline was added to the subgalate powder to obtain the consistency of "toothpaste".The cream was applied to the wound with a spatula number 7(Duflex, SSWhite, ArtigosDentáriosLtda, RJ, BR) and lightly compressed with a dry gauze. then the excess material was removed by washing with saline 9% and thefibrinolysin/deoxyribonucleasecream was applied over the clot with the aid of a cotton swab. In the next two days of the surgery, every 24 hours, the animals of test groups 1 and 2 were contained mannualy and received application of fibrinolysin/deoxyribonuclease cream on the wound, totaling three applications (Figure 2).

On postoperative days 3, 7, 14, 30 and 60, 5 animals from each group were euthanized with an overdose of Thiopental by intraperitoneal injection.[9] Then the biopsies were performed for all jaw, avoiding dilaceration of the palatal wound area. The samples colected were maintained in buffered of10% formaldehyde solution for 72 hours and then washed in water and immersed in a Paul Speight decalcifying solution(Universidade de Sheffield, Inglaterra), reduced to 1 liter (820mL distilled water and 180 ml of 85% formic acid). After decalcification, the specimens were reduced to permit microtomy in the trans-

verse direction at the center of the wound. For each piece, there was obtained 5 sections of 5 micrometers and stained with hematoxylin and eosin for histologic evaluation.

The capture of images was performed from a camera attached to a light microscope and computer in 40x and 400x magnification. The images was captured using the Leica Las V4.2 program (Leica Microsystems, Switzerland) and analyzed by Image Pro Plus 4.0 (Media Cybernetics, USA) with previous calibration in millimeters.

Histological evaluation included the dichotomous nominal scale with criterion of presence or absence of variables usually present in tissue repair: clot necrosis, edema, foreign body, foreign body reaction, chronic inflammation, vascular proliferation, and fibroblast cell proliferation[10] (Figures 3).

The center of the wound was used as reference for this analysis. The clot was evaluated at 3 and 7 days, while the other variables were analyzed within 60 days. All assessment was performed by a single experienced and previously calibrated evaluator.For evaluation of epithelial repair, linear and vertical measurements were held,respectively from ulcerated and repaired areas. The measurement of the centripetal epithelial proliferation in the area of the ulcer, corresponding to distance between the basal layers laterally proliferating,was defined as a linear measured on days 3 and 7. For the obtaining of these standard measurement, was traced initially two vertical lines along the layers basal proliferation in each side and then a horizontal route (Figure 4).

In turn, epithelial vertical measurement corresponded to the distance from the basal layer to the cornea to measure the thickness of the epithelium on days 14, 30 and 60. The center of the nasal septum was defined as a reference point of the center epithelial repaired area. From this point, measured in the horizontal direction 1.5 mm bilaterally, with vertical strokes delimiting the extent of 3 mm corresponding text area evaluated.The vertical measurements were the basal layer of the cornea.In total were 15 points, 1 central and 7 more on each side. The keratin layer was excluded to avoid areas with artifacts and movement there of (Figure 5).

Data normality was verified with the Kolmogorov-Smirnov test and homogeneity of variances between groups through Levene test at the 5% significance level. For the analysis of the dichotomous nominal scale data it was applied the chi-square test and Pearson test of difference between 2 proportions. In turn, to the epithelial morphometric data were used variances test, multiple comparison test Tukey HSD for homogeneous and Games-Howell variances for heterogeneous variance at the 5% significance level.

Results

During periods 3 and 7 days was observed simultaneous presence of degenerated epithelial and proliferation. In these periods, the presence of clot was constant in all groups. The test group 1 showed the lowest average value of coagulation with a statistically significant difference compared to other groups ($p < 0.05$). The presence of necrosis was higher in the control group and statistical significance in relation to testing groups ($p <0.05$). Chronic inflammation was higher in the control group but with significance only for the test group 2.All the groups showed similar and not statistically significant presence of edema variables, discreet presence of foreign material, foreign body reaction and fibroblast cell proliferation. Regarding the vascular proliferation, control and test groups two were similar, while the test group 1 showed the lowest percentage ($p < 0.05$) (Table 1).

In periods of 3 and 7 days was observed incomplete epithelial close wounds in all groups. Only after 14 days postoperatively, all wounds showed complete closure. The reduction of epithelial linear measurement took place between 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).

Multiple comparisons of linear measurements for three days showed statistically significant difference between control and test groups 1 ($p = 0.04$).Similarly to the previous period, at 7 days, the differences remained statistically significant between the two groups ($p < 0,01$). On the other hand, the test group 2 showed no statistically significant difference with the other groups in all evaluated periods (Table 3).

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

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

Discussion

In an epithelial healing process, the fibrin clot formation and deposition occur within the first 24 hours and the peak phagocytic activity of antigenic particles and foreign bodies within 24 to 48 hours.[10,11] In this aspect, a widely used option to remove cell debris in the wound bed, which may aid in tissue repair is the enzymatic debridement.[5-7, 11-13] The results of this study showed that debridantes properties fibrinolysin/deoxyribonuclease may have contributed to the lower percentage of clot in the test group 1 by the direct action on the wound. The fact that the test group 2 did not present significant difference to the control group may be related to the hemostatic action where the increased blood volume may have hindered the enzymatic action of fibrinolysin.

Epithelial necrosis was less present in the test group compared to the control group. In the test group 2 this fact can be explained by the astringent action of bismuth subgalate, promoting protein precipitation and formation of a protective layer on the wounds.[3] .While the debridantes properties of the topical application of fibrinolysin/desoxirribonuclease[12,13] alone or on the bismuth subgallate layer can be 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 epithelial closing slower centripetal, leading to extensive bacterial colonization and possible trauma on site.[10,14,15] Thus, the exposed bone longer, may also have influenced the repair of the epithelium and connective tissues as the interaction between them is a prerequisite for cura.[10,16] The astringent action of subgalate forming a protective layer over the wound, it seems to have been key to a less pronounced inflammatory activity. All groups showed the presence of underlying chronic inflammation to the injured area up to 60 days. That is evidence that the repair process in fibrous connective tissue can take months to be the responsibility of a long process of maturation and remodeling of collagen.[16]

Moreover, favorably, vascular proliferation was higher in the control group compared to the test groups. This fact, although not elucidated, might be explained by an interference in the coagulation cascade of events that stimulate the migration and mitosis of endothelial cells.[12,16 -18] Thus, debridement local action to reduce clot volume may have reflected in the test groups, mainly in the test group 1 to have a more direct effect on the tissues.[13] Despite this case, apparently unfavorable repair tests was higher in groups, especially for the test group 1.

The analysis of the epithelial closure was conditioned at centripetal migration basal layer.[10,11,13] repair evaluation study in palate of rats without the aid of any medications, and the same diameter of the work (3mm) showed complete epithelialization took place from 14 dias.[10] However, another study using debridantes substances, showed that epithelial closure was best for the periods 8-15 days with the application of fibrinolysin compared only to soro.[13]In our work, complete epithelialization of the epitheliumIt was observed in the samples 14 days post-operatively. However, may have occurred at intervals not evaluated (from the 8th to 13th day).

In the linear measurements in periods of 3 and 7 days it was observed that the test1 group showed an epithelial covering speed faster and significance compared to the control group, confirming previous study in ratos.[13]Despite the test group 2 did not present values with significant differences compared to the control group, the means were lower compared to the same. This finding may have relevance to preventing bacterial colonization with bismuth subgallate in ulcerated area in the earlier postoperative days, but also the impact of physical agression on the wound.[15] This outcome in the test group 2 also confirmed better performance when compared to the study application isolated bismuth subgallate over the wound where the closing response was slower compared to the control group (saline).[2] In this way, despite not having been the object of this study was to evaluate the subgalate alone, it seems that the action of associated fibrinolysin the subgalate may have provided local response more favorable to the epithelial closure.

However, regardless of the variation of the linear epithelial measures in periods and groups, vertical epithelial measures, related to epithelial thickness in the operated area, not different, with similar and not statistically significant values.

As for fibroblast proliferation, the presence remained constant between the groups, with no statistical differences. This finding confirms results from previous studies reporting that the bismuth subgallate 1-3 and fibrinolysin[4-8,11-13, 15 16] do not interfere with the final quality of epithelial repair.

The findings of this research suggest that the application of fibrinolysin / deoxyribonuclease alone or in combination with bismuth subgallate can be of great clinical value in excisional periodontal surgical procedures. The acceleration in epithelial closure will pass in less postoperative discomfort. However, despite these results, it is suggested that other experimental and clinical evaluations for a better understanding of tissue repair in the application of fibrinolysin/deoxyribonuclease.

Topical application of fibrinolysin/deoxyribonuclease promoted faster epithelial closure compared to only saline and its use alone or associated with bismuth subgalate did not interfere with tissue repair.

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Table 1. Mean values as a percentage of the presence of variables with dichotomous nominal scale into caller groups and application of difference test between 2 proportions.

Variables	Evaluation period (days)	Groups		
		Control(%)	Test 1 (%)	Test 2 (%)
Clor	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
Foreignbody	3,7,14,30,60	37,3 a	31,6 A	32,8 a
Foreign body 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
Fibroblastic cell proliferation	3,7,14,30,60	76,1 a	75 A	81,3 a

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

Table 2. Descriptive Statistics of linear measurements of wounds in periods and groups

Group	Period (days)	Mean(mm)	Standard Deviation	Standard Error	Confidence Interval	
					Superior 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 measurements (ML) between groups and in the periods of 3 and 7 days to the Games-Howell test.

Comparison		MeanDifference	Standard Error	PValue	ConfidenceInterval	
					SuperiorLimit	SuperiorLimit
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 as statisticallysignificant.

Table 4. Descriptive statistics of the measurements of the vertical (VM) center of the wound in groups (mm).

		n	Mean	Standard Deviation	Standard Error	ConfidenceInterval 95% to mean		Minimum	Maximum
						Inferior Limit	Superior Limit		
VM	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

Table 5. Analysis of variance (ANOVA) of vertical measurements of the epithelium into caller groups and periods. Gal - degrees of freedom, F - F Snedecor test, b - computed using alpha = 0.05

Variable	Sum ofsqua-res	Gl	Mean Squa-re	F	P Value	Power Ob-served[b]
Group	0,007	2	0,003	2,4	0,1	0,4
Period	0,003	2	0,001	0,9	0,4	0,2

p <0.05 as statistically significant

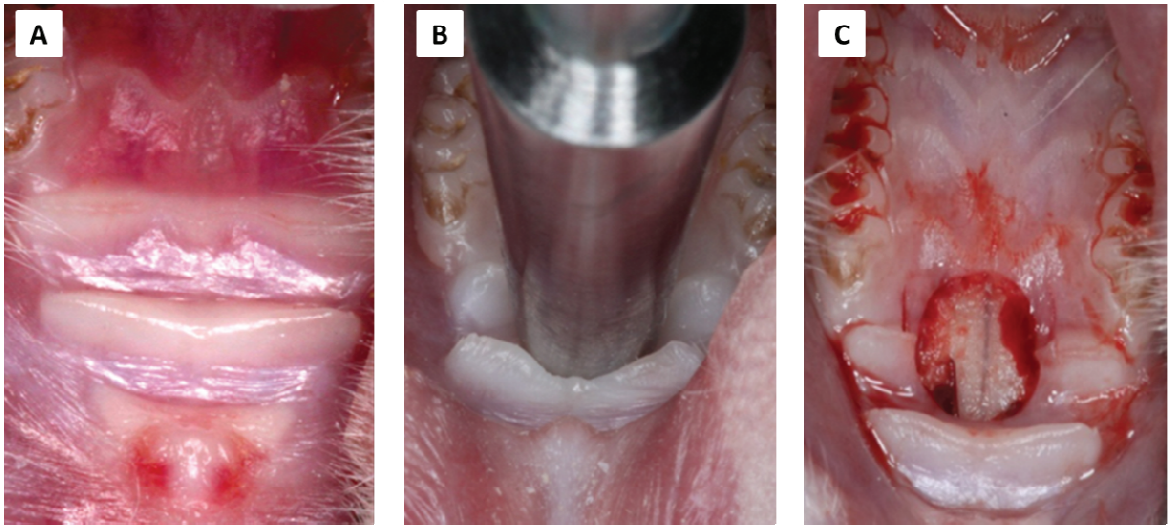

Figure 1. Representative image of the standardized wound in 3rd palatal folds of rats: (a) punch positioned, (b) standardized wound, (c) exposed bone.

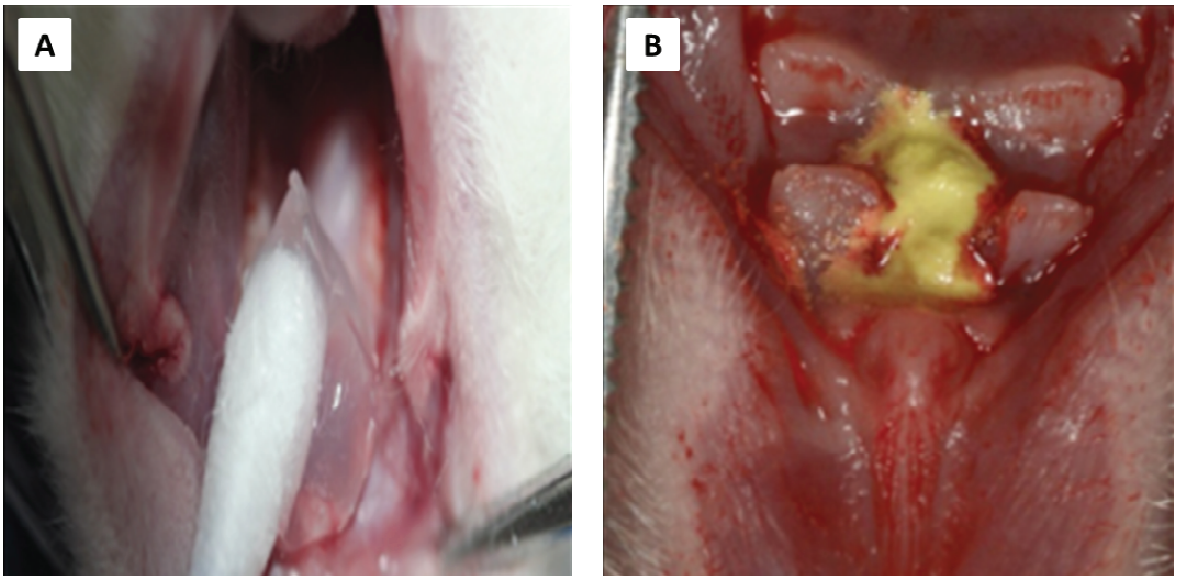

Figure 2. Representative image of the standardized wound in 3rd palatal folds of rats: (a) punch positioned, (b) standardized wound, (c) exposed bone

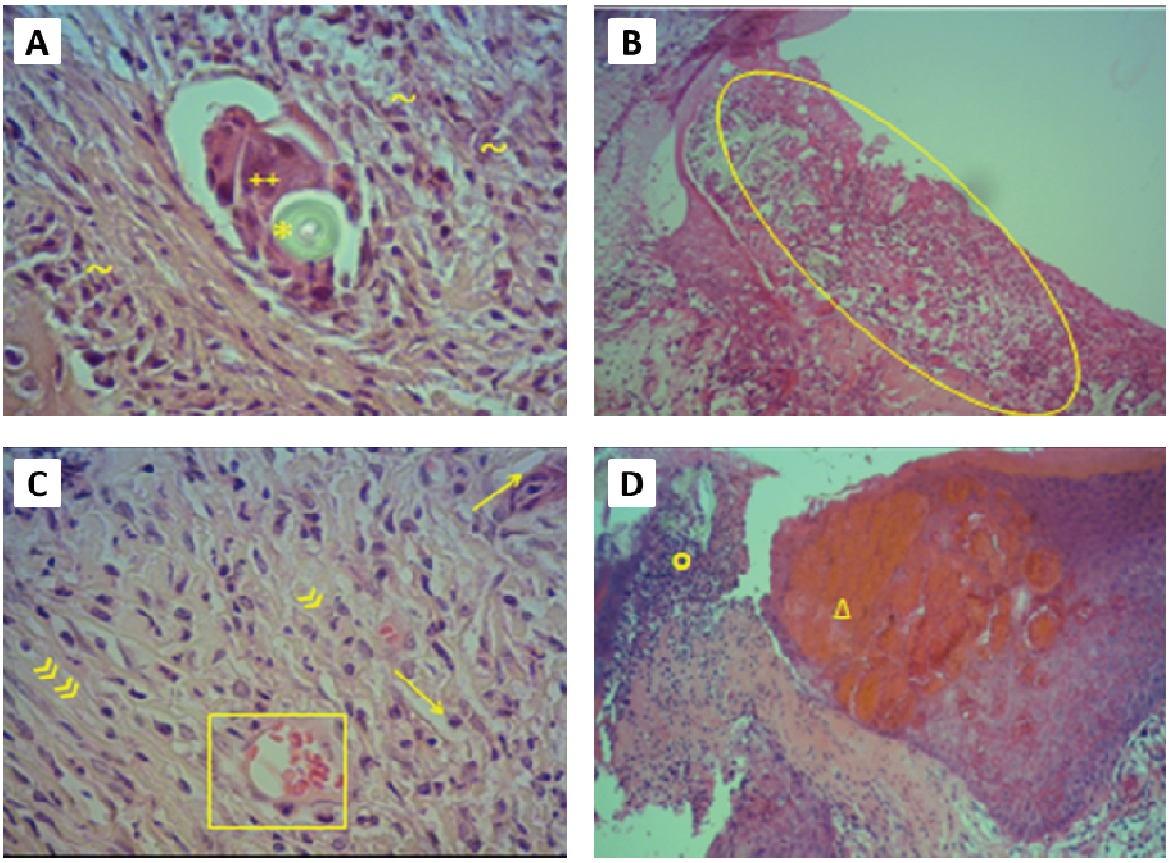

Figure 3. Representative image of the histological variables (A): clot, (B) foreign body*, foreign body reaction+, chronic inflammation~,(C) edema →, fibroblast proliferation «, vascular proliferation□, (D) necrosis○ e clot□(HE, 400X).

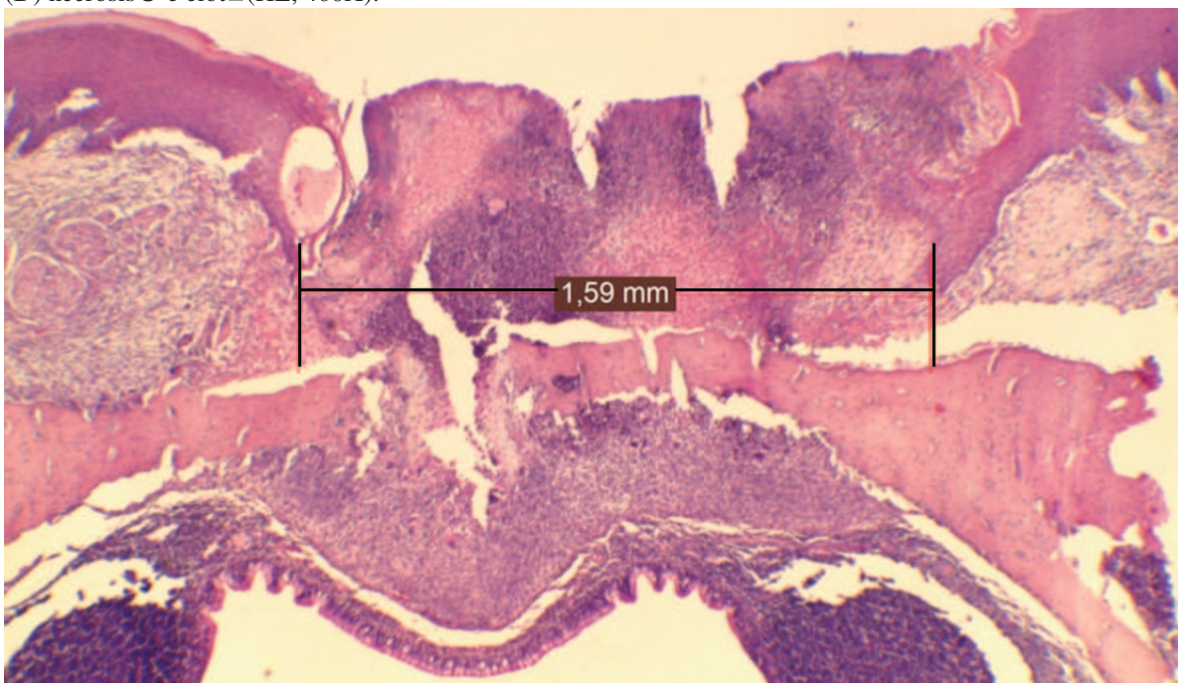

Figure 4. Representative image of the linear measurement. Tracings parallel vertical to the basal layer proliferating. Strokerefers to linear horizontal in millimeters(HE, 40X). Programa Image ProPlus4.

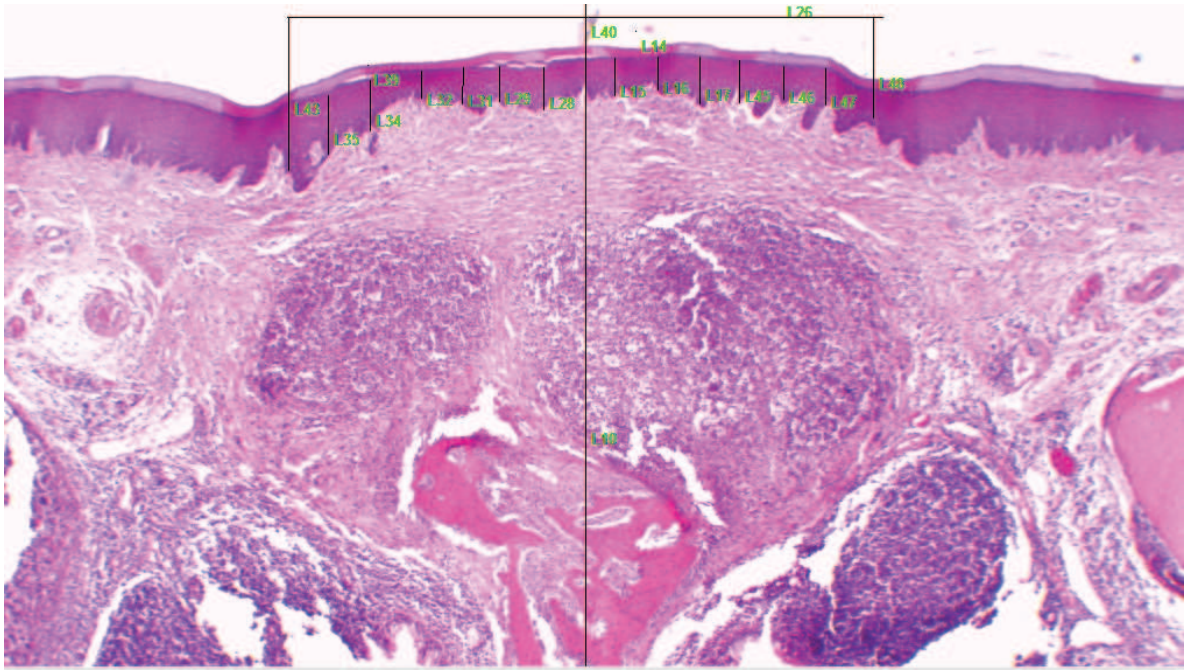

Figure 5. Measuring the vertical:representative overview of the assessed region in periods of 14, 30 and 60 days(HE, 40X). Image Pro-Plus 4.

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PARECER DE PROTOCOLO DE PESQUISA

REGISTRO DO PROJETO: 656 – 2ª versão

TÍTULO DO PROJETO: Avaliação histológica do subgalato de bismuto e fibrolisna desoxirribonuclease no palato de ratos

PESQUISADOR RESPONSÁVEL: Lucinara Ignez Tavarez Luzzi

EQUIPE DE PESQUISA:

Lucinara Ignez Tavarez Luzzi, Eduardo Ferrucio, Aline Ducate, Sung Hyun Kim

INSTITUIÇÃO:

Pontifícia Universidade Católica do Paraná

CENTRO / CURSO:

CCBS / Odontologia

ESPÉCIE DE ANIMAL	SEXO	IDADE / PESO	CATEGORIA	QUANTIDADE
Ratos *Wistar* Albinos	Macho	Adultos / 300 g - 350 g	C	144

O colegiado do CEUA em reunião no dia 08/12/2011, avaliou o projeto e emite o seguinte parecer: **APROVADO.**

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEUA-PUCPR de forma clara e sucinta, identificando a parte do protocolo a ser modificado e as suas justificativas.

Se a pesquisa, ou parte dela for realizada em outras instituições, cabe ao pesquisador não iniciá-la antes de receber a autorização formal para a sua realização. O documento que autoriza o início da pesquisa deve ser carimbado e assinado pelo responsável da instituição e deve ser mantido em poder do pesquisador responsável, podendo ser requerido por este CEUA em qualquer tempo.

Comitê de Ética no Uso de Animais PUCPR

Lembramos ao pesquisador que é obrigatório encaminhar o relatório anual parcial e relatório final da pesquisa a este CEUA.

Atenciosamente,

Profª Gracinda Maria D'Almeida e Oliveira
Coordenadora Adjunta
Comitê de Ética no Uso de Animais