FLÁVIA FIGUEIREDO AZEVEDO

EFFECT OF TOPICAL INSULIN ON CUTANEOUS WOUND HEALING IN DIABETIC RATS

EFEITO TÓPICO DA INSULINA EM LESÕES CUTÂNEAS EM RATOS DIABÉTICOS

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Orientadora: Profa. Dra. Maria Helena Melo Lima

EFEITO TÓPICO DA INSULINA EM LESÕES CUTÂNEAS EM RATOS DIABÉTICOS

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RESUMO

Objetivo: Caracterizar o modelo animal de ferida por queimadura de segundo grau e avaliar o efeito cicatrizante do creme enriquecido com insulina em ratos com diabetes induzido por estreptozotocina.

Método: Ratos machos foram divididos em quatro grupos: ratos controles e ratos diabéticos que receberam o tratamento tópico com o creme enriquecido com insulina e o creme placebo. A queimadura foi realizada com um molde aquecido. Nos dias 1º, 7º, 14º, 26º e 32º pós-lesão amostras do tecido da ferida do animal foram extraídas para análise histológica e immunoblotting.

Resultados: Confirmado que a lesão induzida corresponde a uma queimadura de segundo grau no 1º dia pós-lesão. O tratamento tópico com insulina foi capaz de reverter o parâmetro das células inflamatórias nos animais diabéticos a valores semelhantes aos animais controle (diâmetro da ferida no 26ºd: vehice control, topical insulin control e topical insulin STZ-DM 0.0 mm ± 0.1, vehicle STZ-DM 5.2 mm ± 0.5, $P < 0.01$; de acordo com 2-way (ANOVA) e o pós-teste de Bonferroni, $P<0.05$). A análise de tecidos submetidos ao immunoblotting nos 7º e 14º dias após a injúria mostraram uma maior expressão das proteínas AKT e ERK1/2 nas feridas de queimaduras de animais diabéticos (topical insulin STZ-DM) e não diabéticos tratados topicamente com insulina (topical insulin control).

Conclusão: O uso tópico do creme enriquecido com insulina acelera a cicatrização em feridas de queimadura de 2º grau em ratos diabéticos, diminuindo a cronicidade da fase inflamatória, com capacidade de ativar a via de crescimento celular da AKT e ERK, exercendo um importante papel no processo de cicatrização.

Descritores: Queimaduras, Diabetes Mellitus, Cicatrização de Feridas, Insulina.

Linha de Pesquisa: Estudo do processo cicatricial e implicações terapêuticas em lesões**.**

ABSTRACT

Objective: In order to characterize the animal model of second-degree burn wound and evaluate the healing effect of the topical insulin-enriched cream in streptozotocin-induced diabetic rats.

Methods: Male rats were divided into four groups: control rats and diabetic rats, receiving treatment with topical insulin-enriched cream or placebo cream. The burn was done using a 1-cm^2 mould. The 1^{st}, 7^{th}, 14^{th}, 26^{th} and 32^{nd} days post-wound samples of the burning tissue were extracted for histological and immunoblotting analysis.

Results: It was confirmed that the wound corresponded to a second-degree burn. Topical insulin was able to revert the parameter of inflammatory cells in diabetic animals to levels similar to the morphology of control animals (wound's diameter on 26^{th}: vehicle control, topical insulin control and topical insulin STZ-DM 0.0 mm ± 0.1, vehicle STZ-DM 5.2mm ± 0.5, $P < 0.01$, two-way (ANOVA) and Bonferroni post-test, $P<0.05$). The analysis of tissues subjected to immunoblotting at 7 and 14 days after injury showed increased protein expression of AKT and ERK1/2 in burn wounds of diabetic animals and control animals, treated with insulin.

Conclusion: The use topical insulin cream accelerates wound healing of burns in diabetic animals, suggesting that AKT and ERK pathways may have an important role in wound healing.

Keywords: Burns, Diabetes Mellitus, Wound Healing, Insulin.

AKT serina/treonina quinase

ANOVA análise de variância

BSA burn surface area

DM Diabetes Mellitus

DNA ácido desoxiribonucléico

ECM matriz extracelular

ERK 1 quinases reguladoras de sinalização extracelular 1

ERK 2 quinases reguladoras de sinalização extracelular 2

EV endovenosa

GRB2 proteína ligante do receptor para fator de crescimento

GRB2/mSos complexo da proteína ligante do receptor para fator de crescimento e o fator permutador de guanina

GSK3 glicogênio sintetase quinase 3

H&E hematoxilina e eosina

HIF-1 fator induzido por hipóxia-1

IGF fator de crescimento semelhante à insulina

IL-1 interleucina-1

IL-10 interleucina-10

IL-6 interleucina-6

IM intra-muscular

IP intra-peritoneal

IR receptor de insulina

IRS-1 substrato-1 de receptor de insulina

IRS-2 substrato-2 de receptor de insulina

IRS-3 substrato-3 de receptor de insulina

IRS-4 substrato-4 de receptor de insulina

MAP-K quinase ativadora da atividade mitogênica

MMP-9 metaloproteinase 9

p 21 Ras ativador da proteína GTPase

PI3-K fosfatidilinositol 3-quinase

Rac-1 Ras relacionada ao substrato C3 da Toxina botulínica 1

RNA ácido ribonucléico

SCQ superfície corpórea queimada

SHC molécula adaptadora e substrato do receptor de insulina

STZ estreptozotocina

STZ-DM diabetes induzido por estreptozotocina

TGF fator de transformação de crescimento

TGFβ fator de transformação de crescimento β

TGFβ 1 fator de transformação de crescimento β1

TGFβ 2 fator de transformação de crescimento β2

TGFβ 3 fator de transformação de crescimento β3

TNFα fator de necrose tumoral-α

VEGF fator de crescimento endotelial vascular

VO via oral

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INTRODUÇÃO GERAL

1. INTRODUÇÃO GERAL

Queimaduras são apontadas entre as causas acidentais mais frequentes em todo o mundo, sendo responsáveis por aproximadamente 300.000 mortes a cada ano, o que as torna um grave problema de saúde (1, 2).

A Organização Mundial de Saúde estima que 95% dos acidentes acontecem em países de baixa e média renda. Nos Estados Unidos aproximadamente dois milhões de pessoas são vítimas de queimadura, 80.000 são hospitalizadas e 6.500 morrem a cada ano (1, 2). No Brasil as queimaduras aumentam as taxas de morbimortalidade por causas externas e cerca de 63 milhões são gastos na rede do Sistema Único de Saúde (SUS) (3, 4).

Dados do Ministério da Saúde apontam que 27% dos acidentes com queimaduras tem como vítimas crianças menores de nove anos e que 91,6% dos acidentes acontecem em domicílio, o que evidencia e reforça a magnitude epidemiológica do problema (3).

Trata-se de extremos ferimentos causados por curta ou longa exposição a um agente de origem térmica, elétrica, radioativa ou química (3).

A gravidade da queimadura está associada à etiologia do agente, extensão e profundidade da superfície corpórea queimada (SCQ). As taxas de morbidade e mortalidade nos pacientes queimados aumentam conforme o tamanho e grau da ferida (5).

As queimaduras podem ser classificadas também quanto ao comprometimento tecidual em primeiro, segundo e terceiro grau. As queimaduras de primeiro e segundo grau são também denominadas queimaduras parciais da espessura da pele e as queimaduras de terceiro grau, totais ou completas da espessura da pele (6).

As queimaduras de primeiro grau atingem apenas a epiderme, tendo como características eritema e ardor da pele. Histologicamente apresentam ausência de adesão intercelular da epiderme. Nas queimaduras de segundo grau há dano da epiderme e parte

da derme, caracterizada por eritema, presença de flictemas e são extremamente dolorosas. Histologicamente apresentam vasodilatação acentuada na derme e necrose coagulativa. As queimaduras de terceiro grau promovem a necrose total da epiderme e da derme e dano dos anexos dérmicos. Nesse tipo de queimadura de espessura total, a pele se apresenta com aspecto esbranquiçado ou marmóreo, com redução da elasticidade tecidual, tornando-se rígida (7).

Apesar dos avanços no cuidado às queimaduras, são evidentes as complicações como imunossupressão, aumento da susceptibilidade para sepse, complicações na cura da ferida e falência múltipla dos órgãos. Entretanto a infecção é a principal causa de mortalidade em pacientes queimados, em virtude do comprometimento do sistema tegumentar, principal barreira de defesa contra infecções (1, 4, 5, 8).

O processo de cicatrização é complexo e regulado por mecanismos celulares e bioquímicos que interagem entre si para restabelecer o tecido lesionado. Esses eventos celulares e bioquímicos são divididos em três fases, que não ocorrem isoladamente e se sobrepõem: inflamatória, proliferativa e remodelamento (9-11).

O processo de cicatrização envolve componentes da matriz extracelular, células residentes (queratinócitos, fibroblastos, células endoteliais, células nervosas), leucócitos (neutrófilos, macrófagos/monócitos, linfócitos), mediadores de natureza lipídica (prostaglandinas, leucotrienos, fator de agregação plaquetária) e proteínas (citocinas, quimiocinas, fatores de crescimento, receptores, proteases e seus inibidores) (9-12).

Os fatores de crescimento são os mediadores do processo de cicatrização que controlam a interação célula-célula e matriz celular e ocupam papel de destaque na reparação tecidual (13, 14). Esses fatores têm sido estudados em modelos experimentais que permitem compreender e promover avanços no processo de cura de feridas (8, 10).

A fase inflamatória é de extrema importância para o reparo tecidual. Os macrófagos e neutrófilos acumulam-se no leito da ferida com o propósito de fagocitar microorganismos,

remover restos celulares e debris da matriz. Essas células brancas são essenciais para o sucesso do processo de cicatrização e secretam citocinas e fatores de crescimento (10, 15). Nesta etapa da cicatrização os fatores de crescimento, citocinas e as quimiocinas são responsáveis pelo movimento e infiltração celular necessária para a reparação do tecido e regulam aspectos importantes da inflamação da ferida (10).

Esta fase inicial é modulada por fatores de crescimento da superfamília TGF (TGFβ; TGFβ1; TGFβ2; TGFβ3) e citocinas pró-inflamatórias como interleucina 1 (IL-1), interleucina 6 (IL-6) e o fator de necrose tumoral alfa (TNF-α) (16).

A fase proliferativa é caracterizada pela neo angiogênese, produção de colágeno pelos fibroblastos e intensa migração celular, principalmente de queratinócitos promovendo a reepitelização. Nesta fase, o fator de crescimento vascular endotelial (VEGF) é o principal regulador da vasculogênese e angiogênese durante o desenvolvimento do tecido cicatricial. Sob estímulo dos fatores de crescimento e de outros mediadores, as células endoteliais do interior de capilares intactos nas margens da ferida passam a secretar colagenase e ativar o plasminogênio. Estas substâncias permitem a migração das células endoteliais em direção a região da ferida, o que favorece a formação do tecido de granulação, e lentamente este tecido é enriquecido com mais fibras de colágeno dando à região lesada a aparência de cicatriz devido ao acúmulo de massa fibrosa (16).

A conclusão do processo de reparo tecidual dá-se na fase reparadora, responsável pela remodelação e contração da ferida, onde há substituição do tecido de granulação por tecido conjuntivo denso e a recomposição celular da epiderme (16).

Estudo em roedores, que compara o processo de cicatrização de feridas de queimaduras térmicas e feridas comuns, demonstrou uma diferença da resposta celular e do infiltrado inflamatório entre ambas, apontando uma resposta inflamatória mais atenuada nas queimaduras, o que explica o processo de cicatrização atrasado e comprova o risco de infecções e complicações aos pacientes queimados (15).

Este mesmo estudo, através de cultura de células isoladas de ferimentos de queimaduras demonstrou evidências da desregulação dos macrófagos na resposta inflamatória e consequente nível suprimido dos mediadores inflamatórios como a Interleucina 6 (IL-6) e a Interleucina 10 (IL-10). Esse aspecto contribui para a redução da infiltração celular e resulta em complicações como cicatrização atrasada ou infecção, justificando a resposta inflamatória deficitária nas queimaduras e reforça as complicações clínicas da cicatrização dessas lesões (15).

O processo de cicatrização das queimaduras pode se tornar ainda mais comprometido quando associado ao Diabetes Melittus (DM) (5).

O Diabetes é caracterizado por uma menor produção de insulina ou uma sinalização insulínica deficiente, glicose plasmática elevada e predisposição a complicações crônicas envolvendo vários tecidos (17).

A ausência absoluta ou relativa da insulina/ação da insulina é um marcador da diabete e a ação defeituosa da insulina na pele é descrito como um mecanismo que contribui para uma limitada cicatrização das feridas nesta doença (18).

As feridas em indivíduos com diabetes apresentam prolongada inflamação, pobre angiogênese e uma menor deposição de matriz, quando comparado com a cicatrização de feridas comuns (19). Em análise histológica comparando as taxas de contração da ferida e o tempo de completa epitelização demonstrou-se um menor tempo para a completa regeneração do tecido nos ratos não diabéticos comparando-se às taxas de epitelização dos ratos com diabetes induzido (20).

Essas lesões apresentam na fase inflamatória, atraso na ativação dos macrófagos e leucócitos e consequentemente menor taxa de citocinas anti-inflamatórias como IL-10 e fatores de crescimento como TGF-β, VEGF e IGF (19), um elevado nível de citocinas pró-inflamatórias TNF-α e IL-6 (21).

Durante a reepitelização há uma inibida proliferação e migração dos queratinócitos. A redução na proliferação celular e uma menor contração da ferida explicam-se em virtude das altas taxas de citocinas inflamatórias que estimulam o aumento de níveis de proteases como MMP-9, que são capazes de destruir TGF-β e ECM (matriz extracelular), nas feridas de animais com diabetes induzido com STZ (22). Além disso, a fase de remodelamento é marcada por uma reduzida deposição de colágeno em virtude da menor proliferação dos fibroblastos (20).

A insulina é um hormônio anabólico com ações metabólicas e de regulação do crescimento. Exerce um importante efeito metabólico e mitogênico celular, mediado através do receptor de insulina (IR), que está presente em tecidos de vertebrados em diferentes concentrações, de acordo com o tecido. Os efeitos metabólicos são observados na regulação da homeostase da glicose enquanto os efeitos de crescimento e diferenciação celular ocorrem por meio da modificação da atividade de enzimas e sistema de transporte proteico, levando à estimulação da síntese de DNA, RNA e proteínas e inibição da degradação de proteínas (17, 23, 24).

A insulina pode induzir o fechamento mais rápido das feridas em virtude da regulação do metabolismo da glicose e a regulação de citocinas inflamatórias. Em síntese o aumento das taxas de cicatrização das feridas está associado com a diminuição da inflamação e o aumento do depósito do colágeno no sítio das feridas. O colágeno é produzido pelos fibroblastos presentes na matriz, onde há evidências de que a insulina também induz a síntese dessa proteína (25).

Os efeitos biológicos da insulina iniciam-se com a sua ligação ao receptor proteico específico, que sofre autofosforilação em tirosina e aumenta a atividade da tirosina quinase em outras moléculas intermediárias incluindo substrato do receptor de insulina 1 (IRS-1), substrato do receptor de insulina 2 (IRS-2), substrato do receptor de insulina 3 (IRS-3), substrato do receptor de insulina 4 (IRS-4) e intermediários de Shc (23). Os níveis

aumentados de glicose afetam a regulação da expressão de vários genes incluindo o gene do receptor de insulina, provocando uma atenuada sinalização insulínica (26).

Durante a interação com o receptor de insulina, as proteínas IRS são fosforiladas em vários resíduos tirosina pelo receptor, criando sítios de ligação para proteínas com domínio SH2(18). A enzima fosfatidilinositol 3-quinase (PI3-quinase) foi a primeira molécula estudada com domínio SH2 a associar-se ao IRS-1, assim como ao IRS-2 (27, 28).

Dentre as quinases dependentes de PI3K, destaca-se a AKT, que após o estímulo do fator de crescimento, esta enzima que se localiza perto da membrana plasmática, após fosforilação, tem a capacidade de translocar-se para o núcleo. A AKT tem a capacidade de fosforilar proteínas que regulam a síntese lipídica, de glicogênio e de proteínas, além da sobrevivência celular (23).

A PI3K apresenta papel importante, pois está diretamente relacionada em processos metabólicos e mitogênicos regulados pela insulina como síntese proteica geral e crescimento. Sustentando as evidências que sugerem que pode ser essa uma das vias pelas quais a insulina induz a proliferação celular na pele (23).

Como outros fatores de crescimento, a insulina também estimula a proteína quinase ativadora da mitogênese (MAPK). Esta via envolve a fosforilação de uma proteína adaptadora Grb2, recrutando o fator permutador de guanina, chamado mSOS (son-of-senveless). Desta forma o complexo Grb/mSOS ativa a p21 Ras que se trata de uma proteína intracelular com importante papel no controle de crescimento celular e metabolismo (23).

Estudo que investiga a cicatrização de ratos diabéticos revela uma expressão aumentada tanto da MAPK quanto da AKT no tecido cicatricial de ratos normais, enquanto a expressão destes encontra-se diminuída em ratos diabéticos, justificando o comprometimento do processo de cicatrização no Diabetes Mellitus (23).

O uso tópico de insulina tem sido utilizado no tratamento de feridas em seres humanos e ratos com diabetes, mostrando uma aceleração do processo de cicatrização (29). Recente estudo demonstrou um menor tempo no processo de cicatrização de lesões de ratos com diabetes tratados topicamente com creme enriquecido em insulina, associado à recuperação da expressão de proteínas da via de sinalização de insulina como os IR, IRS-1, IRS-2, Shc, MAP-K e AKT demonstrando fortes evidências da participação desse hormônio nos eventos celulares e moleculares na reconstrução tecidual (30).

Desta forma as evidências confirmam que a insulina estimula o crescimento e a diferenciação de diferentes tipos celulares e interfere na proliferação, migração e secreção de queratinócitos, células endoteliais e fibroblastos (25, 31, 32), sugerindo que a insulina funcione como um hormônio de crescimento (33). Entretanto não há evidências dessa ação da insulina em outros modelos de feridas, como as queimaduras.

2

OBJETIVOS

2. OBJETIVOS

2.1 Objetivo geral

Analisar o efeito do creme enriquecido com insulina na cicatrização de queimaduras de 2º grau em ratos Wistar diabéticos.

2.2 Objetivos específicos

- Caracterizar o modelo de queimadura experimental de 2º grau em ratos Wistar.
- Avaliar as alterações macroscópicas da área da lesão, ao longo da evolução da cicatrização da queimadura.
- Avaliar as alterações histológicas nos diferentes grupos e respectivos tempos de seguimento quanto ao infiltrado inflamatório, formação de novos vasos e do tecido epitelial, em cortes histológicos corados com hematoxilina e eosina.
- Demonstrar a presença de fibras colágenas em cortes histológicos, através da microscopia Confocal.
- Avaliar a expressão das proteínas AKT e ERK, através de sua dosagem no sobrenadante do macerado das lesões dos diferentes grupos pela técnica de immunoblotting.

3

ARTIGO

3. ARTIGO

EFFECT OF TOPICAL INSULIN ON CUTANEOUS WOUND HEALING IN DIABETIC RATS

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Introduction

Burnings are the most frequent accidental cause of death worldwide, being responsible for an average 300,000 deaths per year (1, 2).

The Word Health Organization estimates that 95% of burn accidents happen in low and medium income countries and that they increase the taxes of morbimortality caused by external causes, being 27% of the victims children under the age of nine and occurring 91.6% of the accidents at home, which evidence and reinforce the epidemiological magnitude of the problem (3).

Burns are severe wounds caused by short or long exposure to a thermic, electric, radioactive or chemical agent (3).

The graveness is associated with the agent aetiology and to the burned corporeal extension and deepness (5), classified as first, second and third degree burn (6).

Growth factors are mediators of the healing process that control cell-cell and cell-matrix interactions and have an important role in tissue repair (13, 14). These factors have been studied in experimental models that allow us to comprehend and promote advances in the wound cure process (8, 10).

Studies in rodents that compare the wound-healing process of thermic burns and common healings, demonstrated a difference in the cell response and the inflammatory infiltrate between them, i.e. there was a more attenuated inflammatory response in burns, by virtue of the macrophages dysregulation and consequential suppressed level of inflammatory mediators such as interleukin-6 (IL-6) and interleukin-10 (IL-10), which explains the delayed healing process and confirms the risk of infections and complications to burned patients (15).

The burn healing process can become even more compromised when associated with Diabetes Mellitus (5).

A recent study demonstrated that tissue expression of IR, IRS-1, IRS-2, Shc, MAP-K and AKT are increased in wound healing tissue, compared to intact skin and an insulin cream administered on the wound skin of diabetic animals improved wound healing and reversed the reductions observed in proteins of the insulin signalling pathways, showing strong evidence of the insulin enrolment in the cellular and molecular events of the tissue reconstruction (30).

The evidence confirms that insulin stimulates the growth and differentiation of various cell types and interferes with proliferation, migration and secretion of keratinocytes, endothelial cells and fibroblasts (25, 30-32), suggesting that the insulin works like a growth hormone (33).

The purpose of this study was to investigate the effect of topical insulin-enriched cream on wound healing of second-degree burns in control and streptozotocin-induced diabetic rats and to analyse the histologic characteristics and protein expression that may elucidate the wound-healing process.

Materials and Methods

Materials

Anti-phospho-extracellular signal-regulated protein kinase (ERK)-1/2 and anti-rabbit IgG-peroxidase-conjugated antibodies were from Santa Cruz Technology (Santa Cruz, CA, USA). Anti-serine-threonine kinase (AKT) antibody was from Cell Signaling Technology (Beverly, MA, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless specified elsewhere. Materials for immunostaining were from Vector Laboratories Inc. (Burlingame, CA, USA).

Animals

Male Wistar rats were provided by the University of Campinas Central Breeding Centre. Eight-week-old male rats were divided into four groups: control rats treated with topical insulin-enriched cream (topical insulin control), control rats treated with placebo cream (vehicle control), diabetic rats treated with topical insulin-enriched cream (topical insulin STZ-DM) and another diabetic group treated with placebo cream (vehicle STZ-DM). All groups received standard rodent chow and water *ad libitum.* This study was approved by the Ethical Committee for Animal Use of the University of Campinas (ID protocol: 2581-1). The approval is available as supporting information; see Approval S1.

Second-degree burns and use of topical insulin-enriched cream

The four groups of animals were submitted to second-degree burns. To achieve the burning procedure the animals were anaesthetized with 30 mg/kg of ketamine chlorhydrate (Ketalar, Parke-Davis, Brazil) and 15 mg/kg of intraperitoneal (IP) xylazine chlorhydrate (National Pharmaceutical Chemistry Union S/A, Brazil) (12, 15). Having confirmed the anaesthesia through the corneal reflexes, a trichotomy in the distal dorsal region was performed and the rats were subjected to a procedure of thermic burn via a mould of 1 cm^2 heated to 120ºC and placed on the skin for 20 seconds, provoking a second-degree burn.

The burned surface area (TBSA) was based in *Meeh* formula, where $A=KW^{2/3}$ (A= area in cm^2; K=8.95; W= corporeal weight in grams) (34, 35). The burn model done corresponded to 4% of the burned corporeal surface to the adult animals that were part of this study.

After the burn procedure, animals received 50 mg/kg of intramuscular (IM) tramadol chlorhydrate (Medley Pharmaceutical Industry) and 6.2 mg/kg of oral sodium dipyrone, which was added to drinking water. The added analgesic was maintained until day 5 post-wound Studies that appraised the pain in post-surgery demonstrated that rats that received analgesic in this phase presented a reduction in the pain and discomfort, quicker recovery and increased food ingestion (8).

Rats were resuscitated with Ringer's lactate solution (4 mL x kg(-1) x 1% TBSA(-1), following the *Parkland formula* 30 minutes after the injury (11).

The insulin-enriched cream or placebo cream was applied topically to cover the whole burned area immediately after the burn (day 0) and reapplied daily until the end of the experiment when complete re-epithelization of the groups was reached.

The insulin cream used was prepared with regular insulin (0.5 U/g cream) in the hospital pharmacy of our University, with patent number PI 0705370-3 (Campinas State University, Brazil) (30).

Streptozotocin treatment

Overnight-fasted rats were rendered diabetic by a single intravenous injection of streptozotocin (STZ) (Sigma). These animals received 65 mg/kg STZ, diluted in citric buffer, pH 4.2, administered via the vein of the penis (12). Animals of control groups received an equivalent volume of citric buffer, pH 4.2, also administered by intravenous injection. Rats were used in the experiments 10 days after receiving STZ or citric buffer injection, when blood glucose reached stable levels over 250 mg/dl or $\leq$110 mg/dl, respectively (36). Plasma glucose levels were determined by the glucose oxidase method using blood samples collected from the animal tail before the experiments were performed, by glucometer (Accu-chek Active).

Wound measurement

To evaluate wound closure, the wounds were photographed daily with a Sony Cyber Shot (model DSC-W310 12.1MP 4_Optical zoom) by the same examiner. After digitization, the wound area was measured using ImageJ software (National Institutes of Health, Bethesda, MD). Wound closure was defined as a reduction of wound area and results were expressed as percentage (%) of the original wound area.

Histology and morphometric analysis

Control rats and diabetic rats, receiving treatment with topical insulin-enriched cream or placebo cream. The wounds were photographed on the 1st, 7th, 14th, 26th and 32nd days post-wound for measurement of wound area and samples of the burning tissue were extracted for histological analysis. Samples were fixed in 4% formaldehyde solution for 8h at room temperature and processed by Paraplast® embedding. Transversal 7-µm thick sections were stained with haematoxylin and eosin (H&E). For the morphological analysis (HE staining), the tissue was observed using a x10 objective. Data were compared by ANOVA and Bonferroni's post-test ($p<0.05$).

Analyses of the collagen fibres

Skin wounds from wounded control and diabetic rats treated with or without topical insulin-enriched cream, on the 26th day after experimental wounding, were excised and processed for analysis of the collagen fibres through Microscope Zeiss LSM 780 Multifóton.

Tissue extraction and immunoblotting

Rats from each group were anaesthetised with sodium amobarbital (15 mg/kg body weight) (IP) and were used 10-15 min later, as soon as anaesthesia was assured by the loss of pedal and corneal reflexes. For evaluation of protein expression and activation of signal transduction pathways, the wounded skin of anaesthetised rats was excised and immediately homogenised in extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM EDTA, 10 mM sodium orthovanadate, 2 mM PMSF and 0.1 mg aprotinin/ml) at 4ºC with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 seconds. The extracts were centrifuged at 15,000 rpm at 4ºC in a Beckman 70.1 Ti rotor (Palo Alto CA) for 45 min to remove insoluble material, and the supernatant of these tissues was used for immunoblotting with antibodies against AKT (1:1000) and ERK (1:1000). Whole-tissue extracts from all animals were mixed with Laemmli buffer and similar-sized aliquots (20 µg protein) were subjected to SDS-PAGE. Following transfer to nitrocellulose, blots were subsequently incubated with peroxidase-conjugated anti-rabbit and anti-mouse antibodies. For measurement and analysis of the expression of said proteins, membranes were normalized to Ponceau staining (21, 22). The tissue extraction for immunoblotting was performed on the 7^{th} and 14^{th} days after the burn, unless specified elsewhere.

Statistical analysis

Wound dimensions were calculated and analysed for homogeneity and significance using Prisma, version 5.0. Comparisons between groups were made

using two-way ANOVA analysis of variance and Bonferroni multiple-comparisons post-test. The significance was set at $P<0.05$.

Results

The animals were submitted to burn wound and monitored for 32 days, and received the treatment with topical insulin-enriched cream or placebo cream until wound closure. **Figures 1 (A)** and (**B)** show that topical insulin cream or its placebo cream did not alter the plasma glucose levels for either normoglycaemic animals (controls) or hyperglycaemic animals (streptozotocin-induced diabetic).

There was a progressive reduction of wound area on the 7th, 14th and 26th days post-burn on insulin control animals compared to the vehicle control animals ($p<0.01$; **Fig. 1B** and **C)**, and the total closure of the wound area happened on the 26th day after the beginning of the treatment ($p<0.01$; **Fig 1B** and **C)**. The diabetic animals presented delayed tissue repair, and therefore the diabetic animals that received topical insulin cream presented a reduction of the wound area from the 14th day, with complete resolution on the 26th day post burn ($p<0,001$; **Fig. 1B** and **C)**. During this phase, the wound area reduction of diabetic animals treated with topical insulin cream was similar to the control group treated with topical insulin cream and placebo cream (**Fig. 2D, sections a, c, d).**

Histological analyses of wounds on the 1st day post-injury was able to confirm if the burn was a second-degree burn, by virtue of the commitment of the skin layers, epidermis and dermis, independently of the physiological state and treatment to which the animals were submitted (**Fig. 2A, section a-c, b-d)**. On the 1st day post-injury, the diabetic groups were observed less presence of inflammatory cells on the wound area when compared to control groups.

The skin tissue samples were extracted on the 7th, 14th, 26th and 32nd days post-burn for histological analyses. During the period from the 7th to the 14th day post-burn, no alterations in cellular infiltration of control animals treated with topical

insulin cream or placebo cream were seen (**Fig. 2B, section a-c** and **Fig. 2C, section a-c)**. On the 26th day, re-epithelization of the wound area with a reduction in cellular infiltration for control group animals treated with topical insulin cream and placebo cream was observed (**Fig. 2D, section a-c)**. Therefore, for the diabetic group treated with topical insulin cream during the period from 7th to the 14th day post-burn a higher presence of inflammatory cells and new vessel formation (on the 14th day post-burn) on the wound area was observed compared to the diabetic group treated with placebo cream (**Fig. 2B** and **2C, section b-d)**.

The wound area of diabetic animals treated with topical insulin cream was completely re-epithelized on the 26th day and presented a reduction of the cellular infiltration compared with the diabetic group that received the treatment with placebo cream (**Fig. 2D, section b-d)**. Wound area in the diabetic animals that received treatment with placebo cream was not completely re-epithelized until the 32th day post-burn and the wound area maintained an elevated number of inflammatory cells when compared to topical insulin control, topical insulin STZ-DM and vehicle control group (**Fig. 2E, section b-d)**.

On the 26th day post-wound, the topical insulin or vehicle control group presented a higher number of collagen fibres, and this increase was similar to the topical insulin STZ-DM group when compared to the vehicle STZ-DM group (**Fig. 3, section a-d)**.

The AKT and ERK were quantified by immunoblotting on the 7th and 14th days post-wound (**Fig. 4A,** $p<0.01$ and **5A**, $p< 0.05$). When the ERK 1 protein in wound tissue of control animals was investigated, there was an improvement in these proteins on the 7th day post-burn for the topical insulin control group related to vehicle control group (**Fig. 4B**, $p<0.05$).

In the diabetic group (STZ-DM) treated with topical insulin cream, there was an improvement of AKT protein in wound tissue when compared to the vehicle STZ-DM group on the 7^{th} and 14^{th} day post-burn (**Fig. 4A**, $p<0.01$ and **5A**, $p<0,05$). During this same period (7–14 days) there was a higher expression of ERK 1 and 2 protein for the topical insulin STZ-DM group compared to the vehicle STZ-DM group in the wound tissue post-burn (**Fig 5B** and **C,** $p<0.05$).

Discussion

The results of the present study demonstrate that healing of second-degree burn wounds on the skin of diabetic or control animals is accelerated by topical insulin cream. Previous studies have demonstrated that the topical insulin improves tissue repair through the keratinocytes' migration stimuli pathway PI3-K/AKT-Rac1 (37); insulin signaling pathway with improvement of IRS-1, PI3-K, AKT, GSK-3 and VEGF expression in the skin of diabetic animals that received the topical insulin treatment (30), which confirms this hormone's capacity to stimulate a variety of cellular functions important to tissue repair.

Wound healing is a complex process involving various cells and matrix components (9-11). Burn wound tissue repair is different to incisional or excisional wound repair. In burns, the initial necrosis can expand and compromise the deepest layers of the skin. After the initial wound, this fact leads to the delay of the inflammatory phase beginning and consequently to retarded re-epithelization. The wound deepness is closely related to the wound healing process. In the superficial or first-degree burn, there is only epidermis commitment and the healing occurs in a short period with keratinocyte migration, without scar formation. However, in second-degree burns or those of partial thickness, there is a commitment of epidermis and dermis, as well as of appendixes, and the tissue repair process is prolonged with scar formation (7). Our results demonstrate that the experimental second-degree burn was confirmed through the morphologic analyses, where the epidermis and dermis commitment was verified.

Through macroscopic follow-up, we observed that from the 7^{th} day post-wound, the control animals that received the topical insulin-enriched cream treatment presented a significant area reduction compared to the other groups. However, the

wound outcomes happened on the 26th day for control animals with the topical insulin treatment or placebo cream and for diabetic animals treated with topical insulin, when compared to the diabetic animals treated with placebo cream. These results agree with other studies that demonstrated the insulin's capacity to accelerate the acute wound-healing repair (30, 37).

Our study demonstrated satisfactory histological results indicating a beneficial effect in the healing process, suggesting that the topical insulin-enriched cream was able to accelerate the tissue repair with properties that contribute to re-epithelization, as well as the collagen fibre production. In the analysed periods, i.e. the 7th and 14th days post-burn, the wounds of diabetic and control animals that received the topical insulin-enriched cream presented an elevated number of inflammatory cells and new vessel formation, similar to the morphology of control animals that received placebo cream. In contrast, in these same periods, the diabetic animals treated with placebo cream presented an attenuated inflammatory infiltrate when compared to the other groups. On the 26th day post-burn, the wounds of diabetic animals treated with topical insulin-enriched cream presented completed tissue repair, inflammatory cell number reduction equivalent to the control group. The topical treatment with insulin cream was able to revert the inflammatory cell parameters in diabetic animals to values similar to the control animal group. Nevertheless, the morphology analyses on the 26th day post-injury of the diabetic group that was subjected to the topical treatment with placebo cream, demonstrated an increased number of inflammatory cells, which remained until the 32nd day post-injury, when compared to the control group animals, which emphasizes that there was a delay in the inflammatory response of this group.

In the local treatment for second-degree burns, it is necessary to maintain a humid microenvironment with the goal of stimulating the granulation tissue formation and consequently the wound re-epithelization, as well as neutralising the microorganisms' development, which is capable of delaying or avoiding the healing biological phenomena (38). Our results suggest that the topical insulin-enriched cream appliance has a direct effect on the tissue repair of second-degree burns in diabetic and control animals.

According to a previous study (20), cutaneous wounds of diabetic animals treated topically with insulin cream had the healing process accelerated, with a higher cell migration to the epidermis and better dermis revascularization. The topical insulin cream was also able to stimulate the keratinocyte migration and proliferation, as well as the vascular endothelium cell migration (37).

A recent study (30) demonstrated that the insulin signalling pathway expression is diminished in acute wounds of STZ-induced diabetic animals, and after the topical insulin treatment there is a significant improvement in this via protein expression, with tissue repair accelerated compared to animals without the treatment, suggesting the improvement of wound outcome through this receptor activation.

During the inflammatory response, the neutrophils and macrophages have an important role, initially for the debridement and later as a source of multiple cytokines and growth factors that are essential to initiate other kinds of cell participation, which has an important role in tissue repair.

We observed in this study that the healing process in burn wounds of diabetic animals that received the topical insulin-enriched cream re-epithelized in a shorter

time (26th day) compared to the diabetic animals with placebo cream treatment (32th day). The histology results showed a thick epidermis layer in diabetic animals that received topical insulin (26th day), similar to the control animals with or without the topical insulin treatment (26th day), these results were confirmed by a planimeter.

Healing is the wound's final phase, i.e. the cell matrix maturation and remodelling occurs in this phase. It is during this phase that the scar acquires its maximum tensile resistance, the most important characteristic of this repair phase is the large and accelerated collagen deposition in the wound region. The collagen fibres are initially thin and parallel to the skin surface during the wound's acute phase. However, with the healing-phase progression, the collagen fibres become thicker and organised through the wound tension line (39). Our results showed that there was a greater presence of collagen fibres in the group of diabetic animals that received topical treatment with insulin, similar to the control group that received treatment with placebo cream. According to the literature (40), the collagen fibres' presence, as well as its structure, can be indicators of healing inside the expected patterns.

Our results show that AKT and ERK1/2 proteins are better expressed on the burn wounds of diabetic and control animals treated topically with insulin cream on the 7th and 14th days post-wound. The AKT is able to phosphorylate other proteins that regulate lipid synthesis, glycogen synthesis, cell survival and protein synthesis (23, 41, 42). Recent studies have demonstrated that AKT is an important step in VEGF release (43, 44). On the other hand, NO increases the VEGF production through the activation of AKT kinase, followed by the induction of various transcription factors, in particular hypoxia inducible factor (HIF-1) (45) ERK can translocate to the nucleus and activate transcription factors that initiate the cellular

proliferation and differentiation (46). The results of this study confirm that the topical insulin-enriched cream in second-degree burn wounds acts by diminishing the inflammatory phase chronicity, with capacity to activate cellular growth, which leads to healing in a smaller period.

Conclusion

The topical insulin enriched-cream treatment was able to revert the wound inflammation parameters of diabetic animals, because on the 26th day after second-degree burns, there was a lower number of inflammatory cells, higher formation of new vessels, higher presence of collagen fibres and complete wound re-epithelization in diabetic animals that received the topical insulin-enriched cream, similar to patterns of control animals. We demonstrated that the topical insulin acts on second-degree burns by diminishing the chronicity of the inflammatory phase, with the capacity to activate the cell growth pathways, through AKT and ERK, which leads to a lower wound healing duration.

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Figure legends

Figure 1 – Blood glucose levels of control or diabetic animals before and after burn and macroscopic wound closure in control and diabetic rats treated with or without topical insulin enriched cream. (A) The glycaemic values are represented in mg/dl for the diabetic and control groups before and after topical insulin cream or placebo cream treatment. **(B)** Representative images of wound evolution on the 1st day of the burn until the period end (32nd day), of the diabetic and control animals with topical insulin or placebo cream treatment. **(C)** The wound area was quantified on the 1st, 7th, 14th, 26th and 32nd days post-burn and are expressed as percentages (n=5~10). The data from the 7th day is shown with the median ± SEM, *p<0.01 *vs.* vehicle control, #p<0.01 *vs.* topical insulin control and *vs.* topical insulin STZ-DM; on the 14th day post-burn, *p<0.05 *vs.* topical insulin control; 26th day post-burn, *p<0.001 *vs.* other groups and on the 32nd day post-burn, *p<0.001 *vs.* other groups, according to two-way ANOVA and Bonferroni's post-test .

Figure 2 – Treatment with topical insulin improves the inflammatory phase during wound healing of the diabetic animals. (A) Confirmation of second-degree burn or partial thickness of skin, with epidermis and dermis commitment. **(B)** and **(C)** Representation of haematoxylin-eosin sections on the 7th and 14th days after burn showing an inflammatory cell number increase, new blood vessels formation (on the 14th days after burn) in diabetic animals that received the topical insulin cream (topical insulin STZ-DM), similar to the control group animals that received the placebo cream (vehicle control) and insulin (topical insulin control) **(sections d, a, c). (D)** Representation of haematoxylin-eosin sections on the 26th day post-burn showing an inflammatory infiltrate reduction and complete wound re-epithelization in the

diabetic animal group that received the insulin cream (topical insulin STZ-DM), similar to the control group animals that received the placebo cream (vehicle control) and control group treated with insulin cream (topical insulin control) **(sections d, a, c). (E)** Representation of haematoxylin-eosin sections on the 32nd day post-burn showing an inflammatory cell number increase in the diabetic animal group that received the placebo cream (vehicle STZ-DM), when compared to the other groups of animals **(sections b, a, c, d).** DE= Dermis; EP= Epidermis; AP= Apendixes; SCAB= Scab; FAT=Fat Tissue; Arrow head= Inflammatory Cells. H&E stain. Magnification bar in D= 83 µm.

Figure 3 – Treatment with topical insulin increase the collagen fibre presence in the healing wounds of diabetic animals. Representation of the microscopy sections on the 26th day post-burn showing an increase in the number of collagen fibres in the diabetic animals that received the insulin cream (topical insulin STZ-DM), similar to the control group animals that received the placebo cream (vehicle control) and control group treated with insulin cream (topical insulin control) **(sections d, a, c).**

Figure 4 – Time-course of AKT and ERK expression following skin wounding in control and diabetic animals treated with or without topical insulin-enriched cream. Tissue extracted on the 7th day post-burn presented higher protein expression in groups **(A)** anti-AKT antibody, $^{*}p<0.01$ *vs.* other groups. **(B)** anti-ERK 1 antibody, *p<0.05 *vs.* other groups; **p<0.01 *vs.* other groups. **(C)** anti-ERK 2

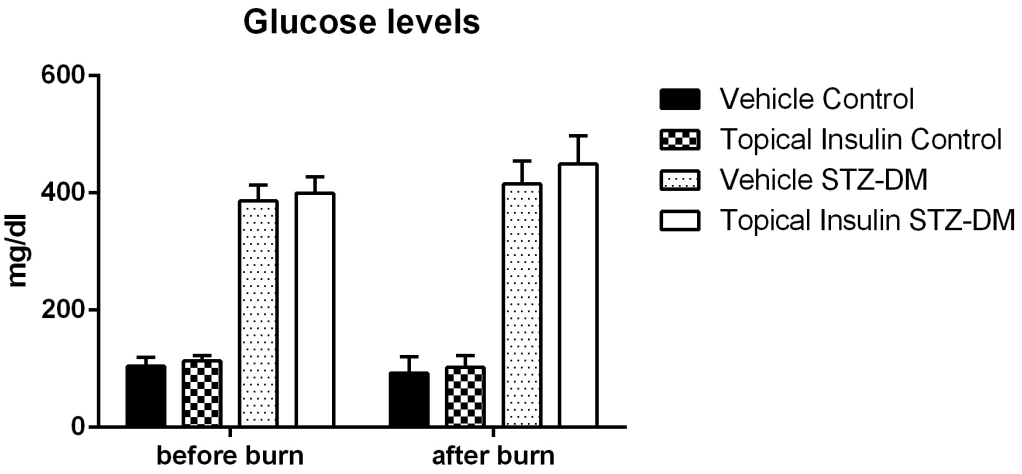
antibody, there is no difference between the groups. Ponceau is the control lane loading samples and the unit is arbitrary relationship.

Figure 5 – The topical insulin-enriched cream accelerates the wound healing of diabetic animals through the AKT and ERK pathways. Tissue extracted on the 14th day post-burn presented a higher expression in the group **(A)** anti-AKT antibody, *p<0.05 *vs.* vehicle STZ-DM and **p<0.05 *vs*. vehicle control **(B)** anti-ERK1 antibody, *p<0.05 *vs.* vehicle STZ-DM and **p<0.05 *vs*. vehicle control, **(C)** anti-ERK2 antibody, *p<0.05 *vs.* vehicle STZ-DM and **p<0.05 *vs.* vehicle control. Data presented as the median ± SEM (n=5) and compared according to two-way ANOVA and Bonferroni's post-test. Ponceau is the control lane loading samples and the unit is arbitrary relationship.

Figures

Figure 1

(A)



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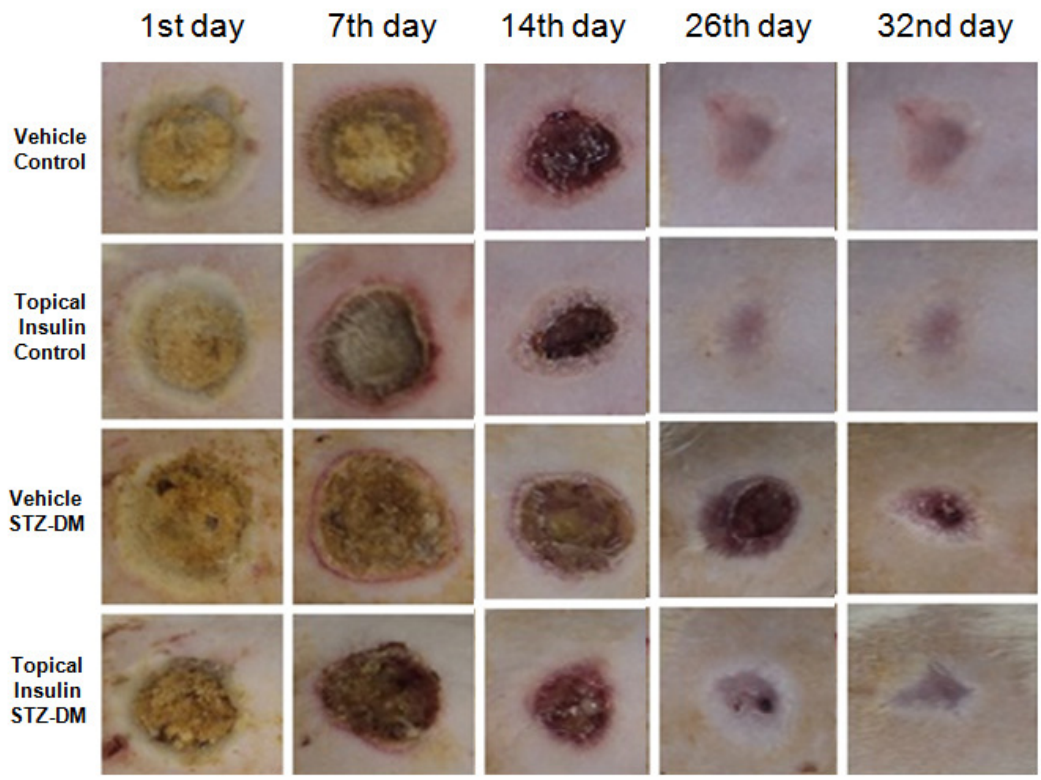

1st day
7th day
14th day
26th day
32nd day
Vehicle Control
Topical Insulin Control
Vehicle STZ-DM
Topical Insulin STZ-DM

(C)

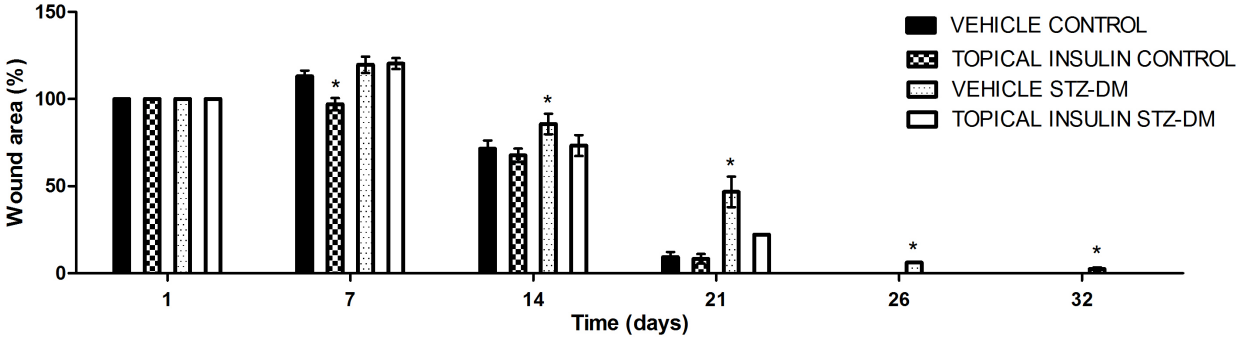
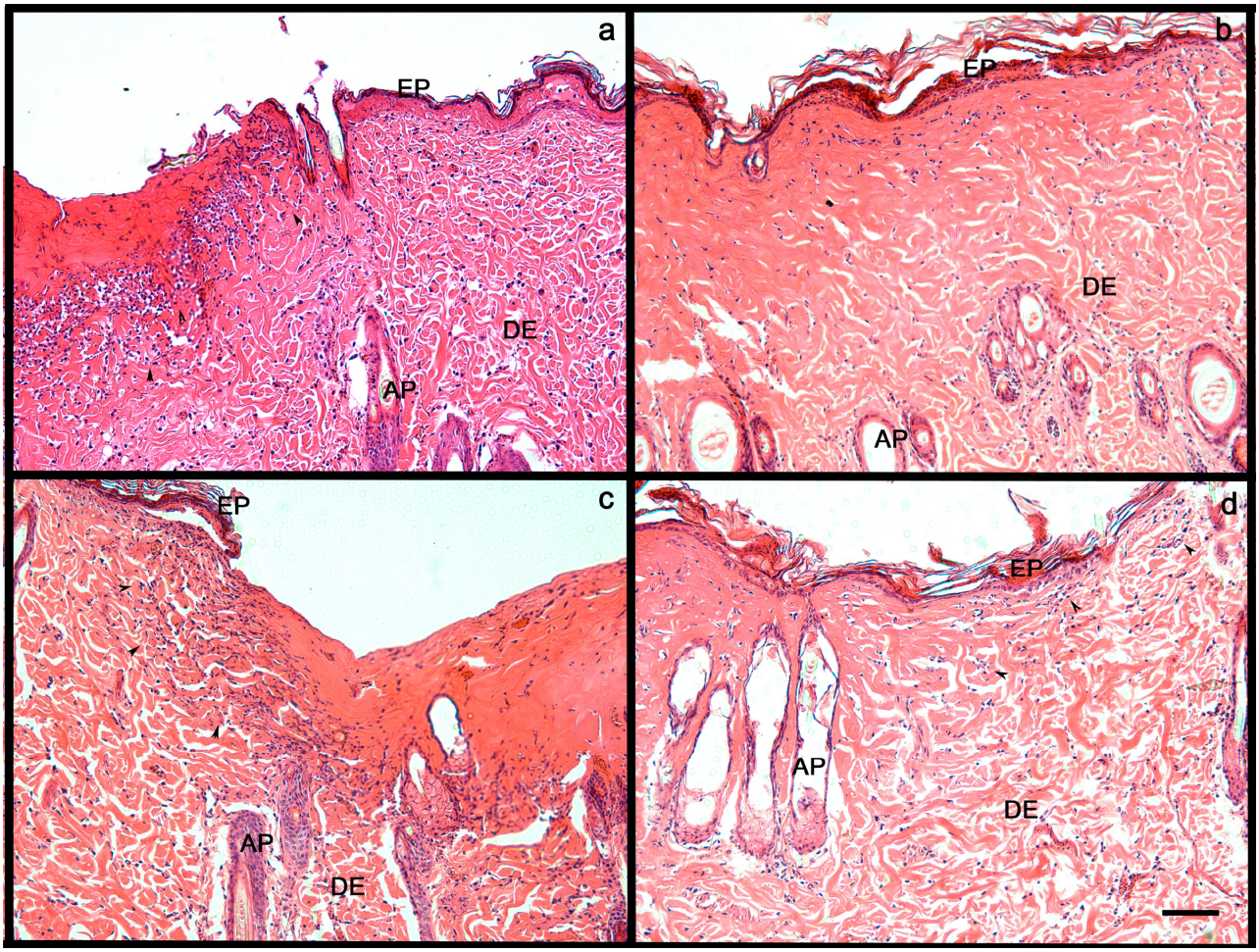
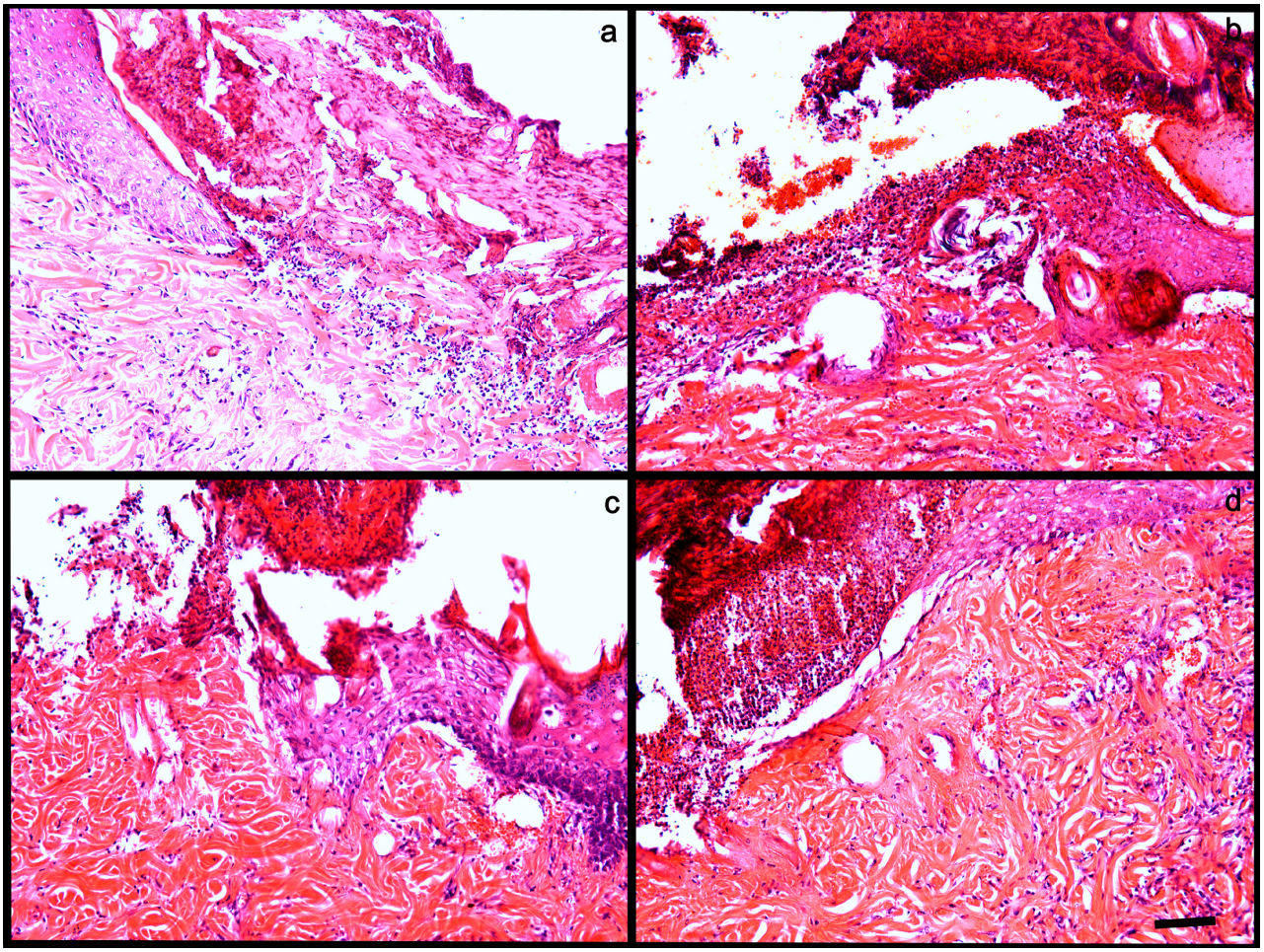

Wound area (%)
150
100
50
0
1
7
14
21
26
32
Time (days)
VEHICLE CONTROL
TOPICAL INSULIN CONTROL
VEHICLE STZ-DM
TOPICAL INSULIN STZ-DM

Figure 2

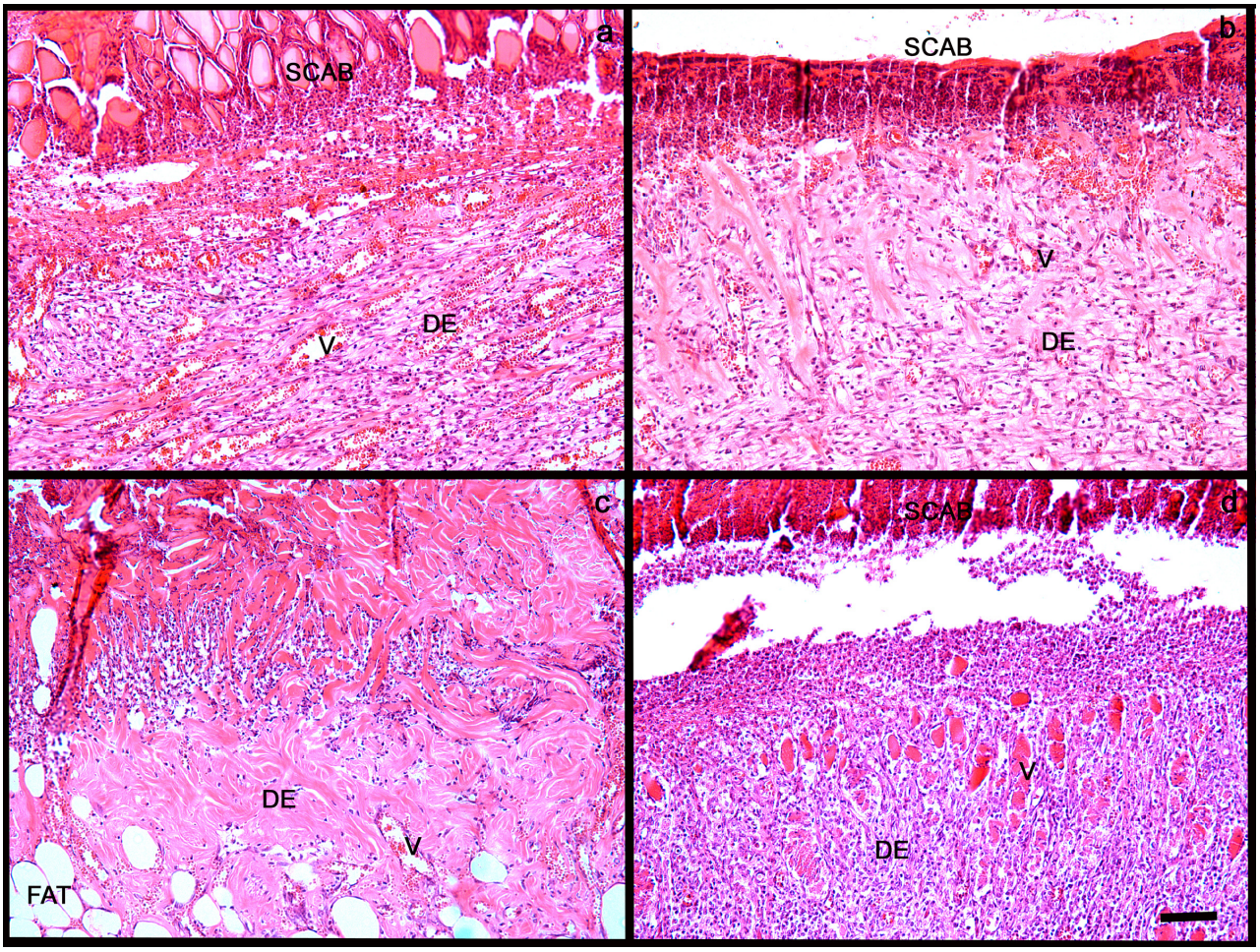
(A)



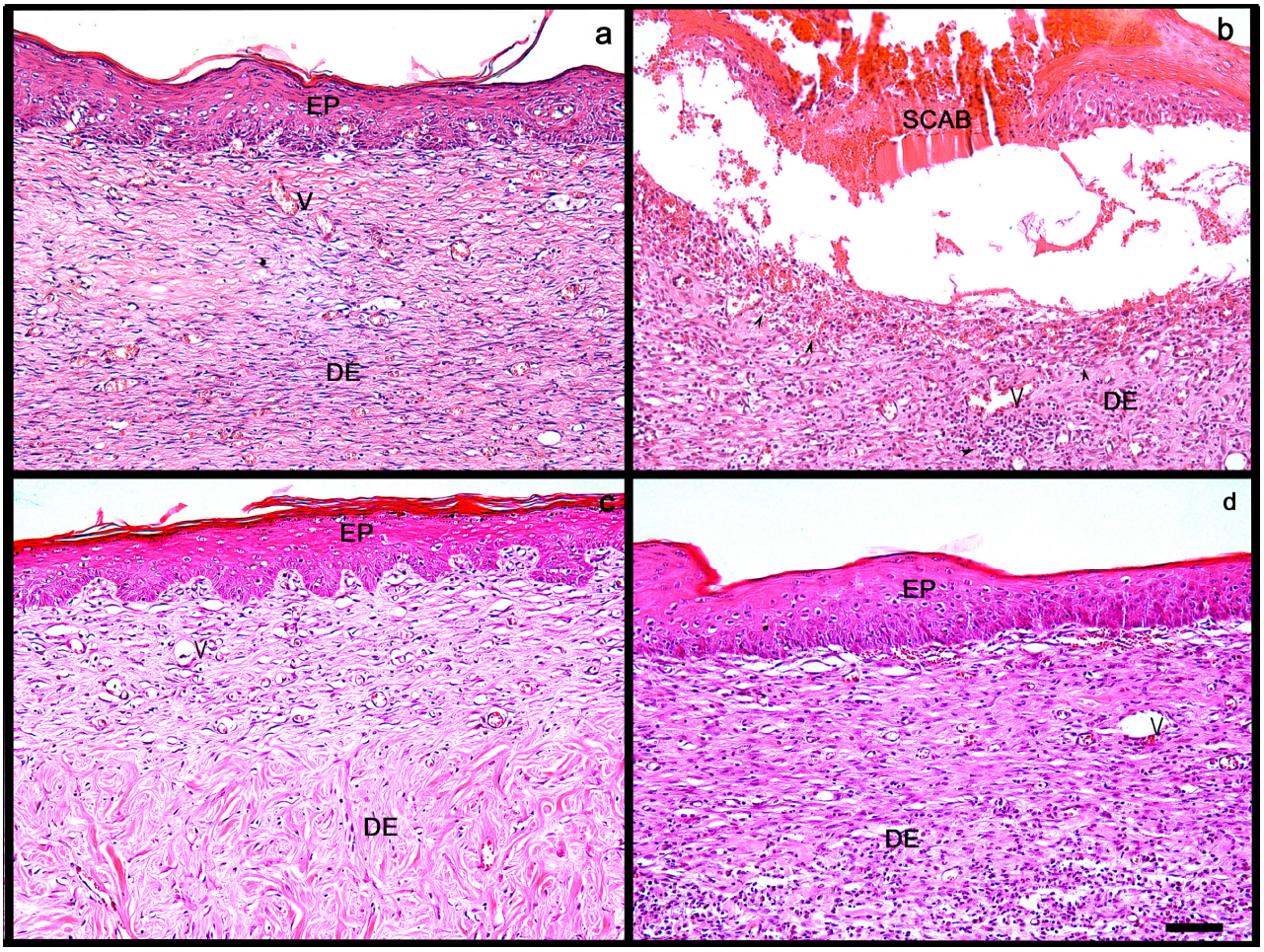
(B)



(C)



(D)



(E)

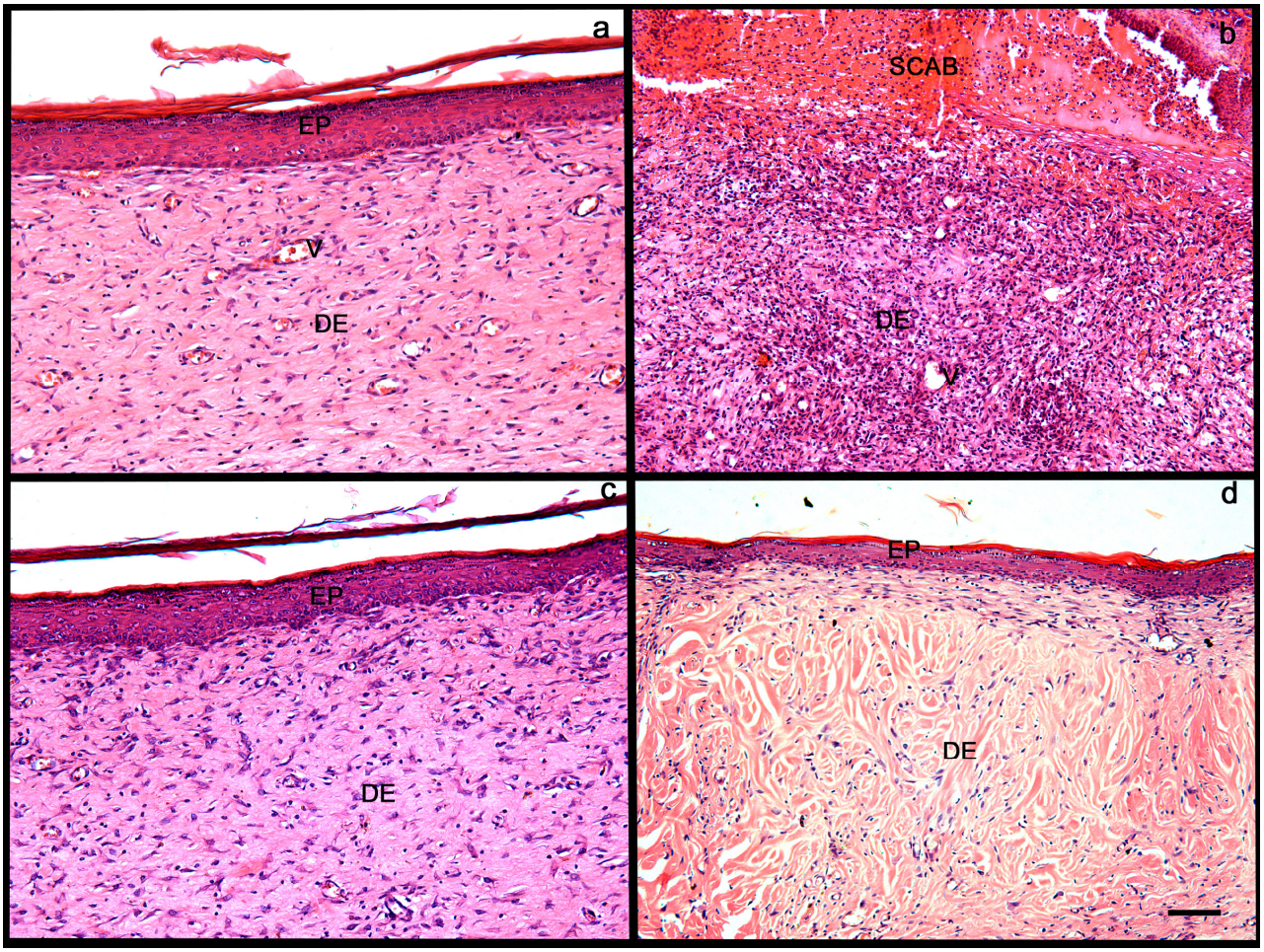


Figure 3

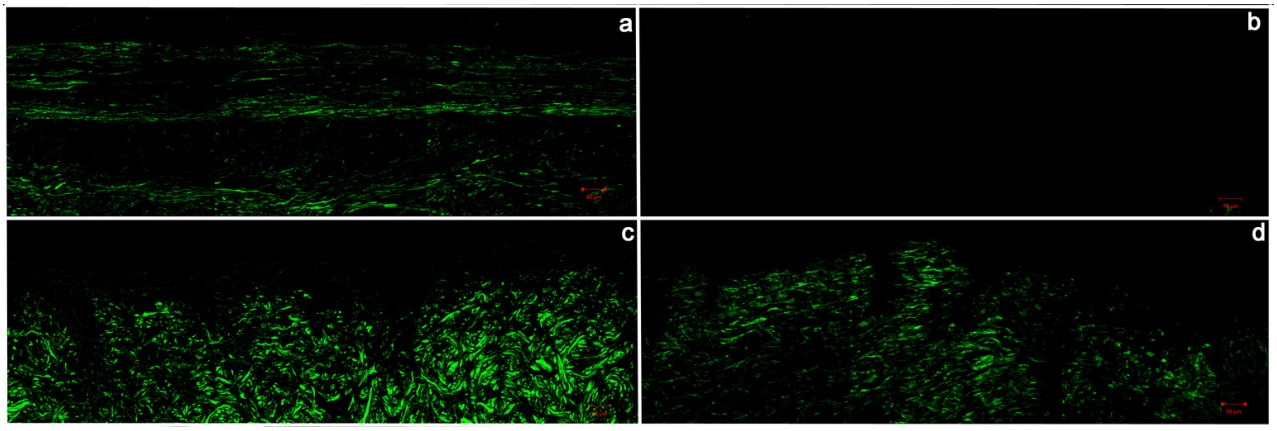


Figure 4

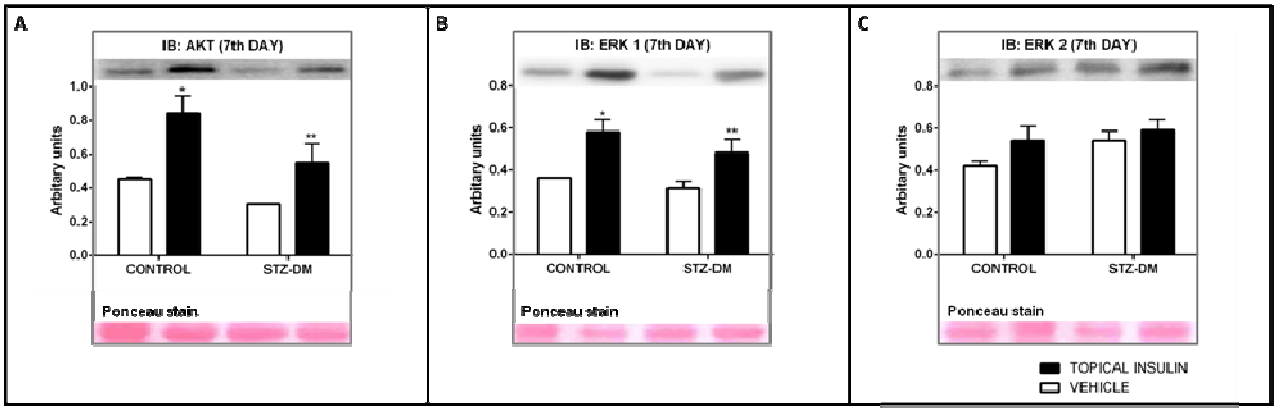
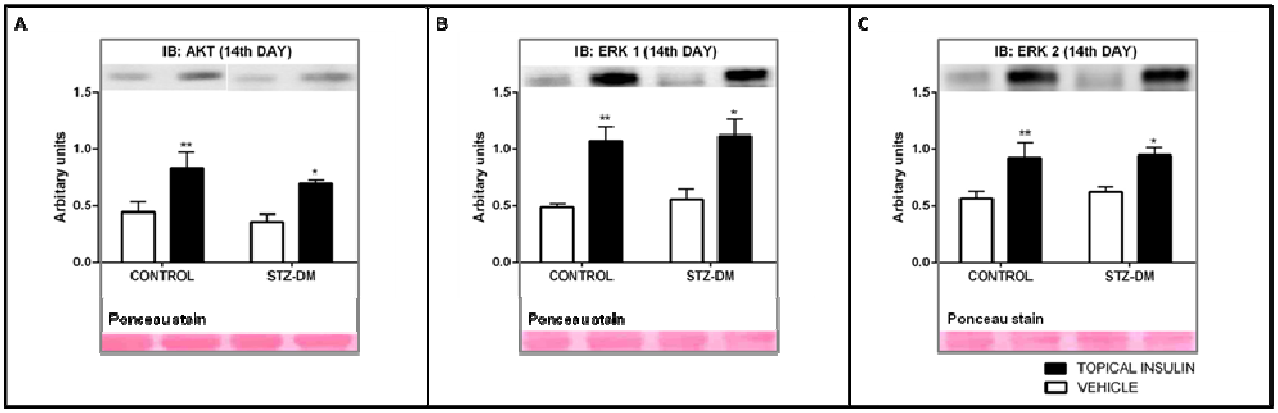

A
IB: AKT (7th DAY)
Arbitary units
1.0
0.8
0.6
0.4
0.2
0.0
*
**
CONTROL
STZ-DM
Ponceau stain
B
IB: ERK 1 (7th DAY)
Arbitary units
0.8
0.6
0.4
0.2
0.0
*
**
CONTROL
STZ-DM
Ponceau stain
C
IB: ERK 2 (7th DAY)
Arbitary units
0.8
0.6
0.4
0.2
0.0
CONTROL
STZ-DM
Ponceau stain
TOPICAL INSULIN
VEHICLE

Figure 5


A
IB: AKT (14th DAY)
Arbitary units
1.5
1.0
0.5
0.0
**
*
CONTROL
STZ-DM
Ponceau stain
B
IB: ERK 1 (14th DAY)
Arbitary units
1.5
1.0
0.5
0.0
**
*
CONTROL
STZ-DM
Ponceau stain
C
IB: ERK 2 (14th DAY)
Arbitary units
1.5
1.0
0.5
0.0
**
*
CONTROL
STZ-DM
Ponceau stain
TOPICAL INSULIN
VEHICLE

4

CONCLUSÃO GERAL

4. CONCLUSÃO GERAL

- O modelo experimental confirmou a queimadura de 2º grau em roedores normoglicêmicos e hiperglicêmicos, com comprometimento de epiderme e derme, verificado no 1º dia pós-injúria.

- Tratamento tópico com insulina demonstrou acelerar o processo de cicatrização de lesões em ratos diabéticos.

- A insulina tópica participou ativamente da fase inflamatória do processo de cicatrização, com recrutamento de elevado número de células inflamatórias entre os dias 7 e 14 pós-injúria, sendo capaz de reverter os parâmetros dessa fase nos animais diabéticos.

- A insulina tópica proporcionou forte estímulo à formação de novos vasos, produção de fibras colágenas e regeneração do tecido epitelial, no 26º dia pós-queimadura, alcançando a cicatrização total da ferida no mesmo período que os animais controle.

Contudo, o uso tópico do creme enriquecido com insulina demonstrou acelerar a cicatrização em feridas de queimadura de 2º grau em ratos diabéticos, diminuindo a cronicidade da fase inflamatória, com capacidade de ativar a via de crescimento celular da AKT e ERK, exercendo um importante papel no processo de cicatrização.

5

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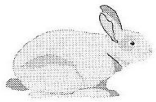

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O,

Certificamos que o projeto **"EFEITO DO CREME ENRIQUECIDO COM INSULINA NA CICATRIZAÇAO DE QUEIMADURAS DE SEGUNDO GRAU EM RATOS DIABÉTICOS"** (protocolo nº **2581-1**), sob a responsabilidade de **PROFA. DRA. MARIA HELENA MELO LIMA / FLÁVIA BACURAU FIGUEIREDO**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **12 de dezembro de 2011**.

Campinas, 12 de dezembro de 2011.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

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