LÉLIA LELIS FERREIRA DE ABREU

EFEITO COMBINADO DE FENOFIBRATO E CREME ENRIQUECIDO COM INSULINA NA CICATRIZAÇÃO DE LESÕES DE ANIMAIS DIABÉTICOS

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EFEITO COMBINADO DE FENOFIBRATO E CREME ENRIQUECIDO COM INSULINA NA CICATRIZAÇÃO DE LESÕES DE ANIMAIS DIABÉTICOS

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SUMÁRIO

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AKT	serina/treonina quinase
ATP	trifosfato de adenosina
DNA	ácido desoxirribonucléico
ECM	matriz extracelular
eNOS	NOS endotelial
EPCs	células progenitoras endoteliais
ERK	quinases reguladoras da sinalização extracelular
GDP	difosfato de guanosina
GSK-3	glicogênio sintetase quinase 3
Grb2	proteína ligante do receptor para fator de crescimento
GTP	trifosfato de guanosina
IGRF	receptor de fator de crescimento semelhante à insulina
IR	receptor de insulina
IRS-1	substrato-1 do receptor de insulina
IRS-2	substrato-2 do receptor de insulina
MAPK	quinase ativadora da atividade mitogênica
NOS	óxido nítrico sintetase
PI3K	fosfatidilinositol 3-quinase
PIP3	fosfatidilinositol 3-fosfato
PLGF	placenta Growth Factor

PPAR-α	receptores alfa ativados de proliferação dos peroxissomas
RNA	ácido ribonucléico
SDF-1α	célula estromal derivada do fator -1alfa
SHC	molécula adaptadora e substrato do receptor de insulina
SH2	segunda homologia ao Src
Src	oncogene originalmente definido como produto da sarcoma vírus Rous
SOS	fator ativador do Ras, apresenta homologia à Drosophila son-of-sevenless
TGF	fator de transformação de crescimento
TNF-α	fator de necrose tumoral-alfa
VEGF	fator de crescimento endotelial vascular

RESUMO

Amputações e úlceras no pé de pacientes diabéticos, além de comprometer a qualidade de vida, são também um problema de saúde pública. A insulina tem sido usada topicamente para tratar feridas diabéticas e mostrou acelerar o processo de cicatrização tecidual. Um estudo recente demonstrou que o fenofibrato está associado à um menor número de amputações de membros inferiores em pacientes com diabetes do tipo 2, provavelmente através de mecanismos não-lipídicos. O objetivo deste estudo foi investigar a associação e os efeitos do fenofibrato sistêmico em relação à insulina tópica na cicatrização de feridas de ratos diabéticos induzidos por estreptozotocina. O diabetes foi induzido em ratos utilizando estreptozotocina. Depois de quatro dias, foi realizada uma lesão no dorso dos animais diabéticos e estas lesões foram tratadas com insulina ou fenofibrato ou insulina mais fenofibrato ou placebo como controle. Nos dias 3 e 9 os animais foram sacrificados e as lesões foram extraídas. Portanto, os resultados deste estudo indicam que o tratamento sistêmico com fenofibrato por via oral em doses terapêuticas não acelera a taxa de cicatrização de feridas em ratos diabéticos, porém existe uma maior ativação do IR, ERK-1 na fase inflamatória; e do IR e GSK-3 na fase proliferativa, além da melhora da expressão de TGF-ß e TNF-α. O tratamento combinado do fenofibrato com o creme enriquecido de insulina apresentou uma aceleração da taxa de cicatrização, promovendo a ativação da via da PI3-K, além do aumento da expressão de citocinas (TNF-α e SDF1-α) e fatores de crescimento (VEGF e TGF-ß) na fase inflamatória; e melhora de TGF-ß e TNF-α durante a fase proliferativa. Neste sentido, podemos concluir

que o uso do creme enriquecido de insulina mostrou-se mais eficaz na aceleração da cicatrização quando utilizada sozinha, pois houve um aumento do infiltrado inflamatório, da maturação das fibras de colágeno e da angiogênese na derme, bem como a ativação da sinalização de insulina e melhora da expressão de SDF-1α, VEGF, TGF-ß e TNF-α tanto na fase inflamatória quanto na proliferativa.

Palavras-chave: diabetes mellitus, cicatrização, insulina, fenofibrato

Linha de Pesquisa: Processo de Cuidar em Saúde e Enfermagem

ABSTRACT

ABSTRACT

Amputations and foot ulcerations in patients with diabetes impairs their quality of life and is also a problematic public health issue. Insulin has been topically used to treat diabetic wounds and has shown acceleration of the healing process. A previous study of fenofibrate was associated with fewer lower-limb amputations in patients with type 2 diabetes, probably through non-lipid mechanism. The aim of this study was to investigate the association and the effect of systemic fenofibrate in relation to topical insulin on wound healing in streptozotocin-diabetic rats. Diabetes was induced in rats using streptozotocin. After 4 days, diabetic animals were wounded and the wounds were treated with insulin or fenofibrate or insulin plus fenofibrate or a placebo as the control. At days 3 and 9 animals were sacrificed and wounds were excised. Therefore, the results of this study indicate that systemic treatment with oral fenofibrate in therapeutic dose does not accelerate the rate of wound healing in diabetic rats, however there is a greater activation of IR, ERK-1 in the inflammatory phase, and IR and GSK-3 in the proliferative phase, besides the improvement of the expression of TGF-ß and TNF-α. The combination of fenofibrate with insulin enriched cream showed an increased rate of wound healing by promoting the activation of the PI3-K, as well as increased expression of cytokines (TNF-α and SDF1-α) and growth factors (VEGF and TGF-ß) in the inflammatory phase, and enhanced TGF-ß and TNF-α during the proliferative phase. We can conclude that the use of the insulin enriched cream was more effective in accelerating the healing when used alone, demonstrated by

an increased inflammatory infiltrate, the maturation of collagen fibers in the dermis and angiogenesis, as well as activation of insulin signaling and the enhanced expression of SDF-1α, VEGF, TGF-ß and TNF-α in both the proliferative and inflammatory phases.

Key-words: diabetes mellitus, wound healing, insulin, fenofibrate

Research Line: Caring process in health and nursing

INTRODUÇÃO GERAL

O diabetes melito (DM) é uma doença crônica com manifestações de hiperglicemia e intolerância à glicose, que ocorre quando o pâncreas não produz insulina suficiente ou quando a efetividade da ação da insulina está prejudicada nos tecidos, ou ambos (1).

A prevalência mundial do diabetes melito em 2010 é de 280 milhões de adultos e estima-se que este número irá crescer para 439 milhões de pessoas até o ano de 2030. No Brasil são 7,6 milhões de adultos com diabetes em 2010 e calcula-se que existirão 12,7 milhões de diabéticos até 2030 (2).

Vários estudos clínicos e experimentais têm mostrado que o processo de cicatrização de feridas está prejudicado em pacientes com DM (3). Apesar do tratamento insulínico e minucioso controle diabético, aproximadamente 15% de todos os pacientes com diabetes terão, em algum momento, feridas de difícil cicatrização e 50-70% deste número é responsável por todas as amputações não-traumáticas (4, 5).

Diferentes mecanismos patogênicos têm sido sugeridos no desenvolvimento das complicações dérmicas do diabetes, incluindo a microangiopatia, envelhecimento prematuro dos fibroblastos da pele, processos imune-mediados e mudanças estruturais e funcionais na membrana basal das células (6-10). Verificou-se que os distúrbios na cicatrização estão localizados na fase de inflamação e/ou proliferação e dependem das interações entre diferentes tipos celulares e a matriz extracelular (11). A matriz extracelular é predominantemente sintetizada pelos fibroblastos. A literatura mostra que existe um decréscimo na taxa de proliferação de fibroblastos em úlceras de diabéticos (8) e que a falta de insulina (12, 13) e um déficit na produção ou secreção de fatores de

crescimento específicos na lesão podem, posteriormente, determinar distúrbio na função dos fibroblastos (14). Muitos estudos mostram que vários fatores de crescimento e citocinas são chaves reguladoras do processo cicatricial (15, 16). A formação de novos vasos é essencial para o processo cicatricial e proteínas sinalizadoras como as proteínas da família do fator de crescimento endotelial vascular (VEGF) estão envolvidas neste processo. Atualmente a família do VEGF inclui o VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFE e o PLGF. O VEGF-A está sendo identificado como o principal regulador da vasculogênese e angiogênese durante o desenvolvimento celular (17). Em resposta a hipóxia causada pela lesão o VEGF é liberado pelos macrófagos, fibroblastos e células epiteliais, que induzem à fosforilação e ativação da eNOS na medula óssea resultando no aumento de NO, os quais recrutam a mobilização das células progenitoras endoteliais (EPCs) para circulação (18). As EPCs, essenciais na vasculogênese e na cicatrização tecidual, estão diminuídas em 50% em ratos diabéticos quando comparados com ratos não diabéticos. Acredita-se que isto ocorre devido à diminuição do nível da célula estromal derivada do fator -1α (SDF-1α) (19). A expressão SDF-1α está reduzida em ferida cutânea periférica de diabéticos. Fisiologicamente, SDF-1α é uma das primeiras quimiocinas responsáveis pela mobilização e recrutamento das EPCs na isquemia tecidual (20-22).

A superfamília do fator de crescimento transformante (TGF)-ß além exercer efeitos sobre a proliferação de fibroblastos, queratinócitos e a deposição de matriz extracelular participa também da diferenciação de miofibroblastos e do processo de angiogênese (23).

Estudos mostram que linhagens celulares de queratinócitos expressam receptor de insulina (IR) e receptor de fator de crescimento semelhante à insulina (IGRF) (24-26), que o receptor de insulina funcional está expresso em cultura de queratinócitos de pele de murinos (10), e que queratinócitos de pele humanos são dependentes de insulina para seu crescimento (27). Demonstrou-se também, com a utilização de camundongos sem receptor de insulina, que a insulina regula, via IR, a diferenciação e o transporte de glicose em queratinócitos da pele (28). Pelegrinelli et al (29) demonstraram a presença do receptor de insulina em queratinócito da epiderme e em folículo piloso e que o uso agudo de insulina aumenta a ativação das proteínas da via de sinalização de insulina em pele íntegra de animais diabéticos. Possivelmente a insulina age sozinha ou em conexão com outros polipetídeos promotores de crescimento, orquestrando diferentes eventos bioquímicos que culminam na restauração da integridade funcional do tecido em cicatrização. Em estudo recente, observa-se uma aceleração no processo cicatricial de lesões de ratos diabéticos tratados com creme enriquecido com insulina, como também melhora na expressão do IR, IRS-1, IRS-2, SHC, MAP-K e AKT, sugerindo que a insulina participe dos eventos celulares e moleculares na reconstituição do tecido (dados não publicados/patente 500 da Universidade Estadual de Campinas). É possível que a sinalização insulínica anormal, como demonstrado na pele em cicatrização dos diabéticos, possa afetar a função da pele.

A insulina é um hormônio anabólico com poderosas ações metabólicas e de promoção de crescimento. Os efeitos metabólicos são observados na regulação da homeostase da glicose, principalmente em tecidos hepáticos e periféricos (músculo e

tecido adiposo), apresentando também ação integrada no metabolismo dos carboidratos, proteínas e lípides (30). Além disso, a insulina promove o crescimento e a diferenciação celular, estes efeitos ocorrem através da modificação da atividade de uma variedade de enzimas e sistemas de transporte protéico, levando à estimulação na síntese de RNA e DNA, estímulo à síntese e inibição da degradação de proteínas (31-33).

Os efeitos biológicos característicos da insulina iniciam-se com a sua ligação ao seu receptor protéico específico, que por sua vez está presente, praticamente, em todos os tecidos de mamíferos, porém em concentrações diferentes dependendo do tecido (34). Na sua conformação nativa, o receptor de insulina é formado por duas subunidades α e duas subunidades β covalentemente ligadas por pontes dissulfeto (35). Ambas as subunidades são glicoproteínas com cadeias de carboidratos complexos (34). A subunidade β é responsável pela transmissão do sinal, sendo uma proteína de localização transmembrana (30, 32).

O receptor de insulina pertence à grande família de receptores de fatores de crescimento com atividade tirosina quinase intrínseca. Proteínas-quinases são enzimas envolvidas na transferência de grupos fosfato do ATP para aminoácidos específicos de proteínas (32). A ativação da capacidade quinase do receptor de insulina resulta na fosforilação em tirosina de uma família de proteínas, que são substratos de receptor de insulina (IRS) e são responsáveis pela formação de um complexo de multi-subunidades de sinalização (31). Durante a interação com o receptor de insulina, as proteínas IRS são fosforiladas em vários resíduos tirosina

pelo receptor, criando sítios de ligação para múltiplas proteínas com domínio SH2 (segunda homologia ao Src) (36). A primeira molécula com domínio SH2 que se relatou associar-se ao IRS-1 foi a enzima fosfatidilinositol 3-quinase (PI 3-quinase) (37, 38). Demonstrou-se também que a PI 3-quinase igualmente se associa ao IRS-2 (39). Estudos demonstram que a ativação da PI 3-quinase é essencial para o transporte de glicose, estimulado pela insulina (40). Dentre as quinases dependentes de PIP3, a Akt (um produto do proto-oncogene Akt) tem recebido muita atenção. Após estímulo de fator de crescimento, a Akt localiza-se perto da membrana plasmática, onde se torna fosforilada. A enzima ativada tem a capacidade de translocar-se para o núcleo (41). A Akt tem a capacidade de fosforilar proteínas que regulam a síntese lipídica (42), a síntese de glicogênio (43, 44), a sobrevivência celular (45) e a síntese protéica (46). Este mecanismo oferece uma ligação direta entre a sinalização do receptor de insulina e os efeitos biológicos. Assim, não está claro se a Akt exerce um papel único ou redundante na ação insulínica. A insulina inibe a produção e liberação de glicose no fígado através do bloqueio da gliconeogênese e glicogenólise. A insulina estimula o acúmulo de glicogênio através do aumento do transporte de glicose no músculo e síntese de glicogênio em fígado e músculo. Este último efeito é obtido via desfosforilação da glicogênio-sintetase. Após estímulo com insulina a Akt fosforila e inativa a GSK-3 (glicogênio sintetase quinase 3), o que diminui a taxa de fosforilação da glicogênio-sintetase aumentando sua atividade (44). Como outros fatores de crescimento, a insulina estimula a proteína quinase ativadora da mitogênese (MAP-K) ERK (quinase extracelular reguladora do sinal). Essa via

inicia-se com a fosforilação das proteínas IRS e/ou Shc (substrato do receptor de insulina e molécula adaptadora), que interagem com a proteína Grb2 (proteína ligante do receptor para fator de crescimento) (30). A Grb2 está constitutivamente associada à SOS (son-of-sevenless), proteína que troca GDP por GTP da Ras ativando-a. Uma vez ativada, Ras estimula a fosforilação em serina da cascata da MAPK e ERK que leva à proliferação e diferenciação celulares. A ERK ativada pode se translocar para o núcleo, onde catalisa a fosforilação de fatores transcricionais como $p62^{TCF}$ (fator de transcrição p62), iniciando um programa de transcrição que leva a proliferação ou diferenciação celular (47). O bloqueio desta via com mutantes dominantes negativos ou inibidores farmacológicos previne o estímulo insulínico ao crescimento celular, mas não afeta as ações metabólicas deste hormônio (48).

Os fibratos são fármacos derivados do ácido fíbrico e são clinicamente usados para o tratamento de dislipidemias, principalmente a hipertrigliceridemia (49). Recentemente, Kushwin Rajamania et al. (50), demonstraram que o tratamento com fenofibrato foi associado com baixo risco de amputações de pacientes portadores de diabetes melito tipo 2, provavelmente através de mecanismos não lipídicos, e que estes achados poderiam colaborar com mudanças no tratamento e na prevenção de amputações em portadores de diabetes.

Em resumo, lesões de ratos diabéticos tratadas com pomada de insulina apresentam aceleração no processo cicatricial e o tratamento com fenofibrato melhora a incidência de amputações. Entretanto, nenhum estudo procurou ainda investigar a

associação e os efeitos da aplicação sistêmica do fenofibrato em relação à insulina tópica na cicatrização de lesões de ratos diabéticos.

OBJETIVOS

Objetivo geral

Investigar a associação e os efeitos do uso sistêmico do fenofibrato em relação à aplicação da insulina tópica na cicatrização de lesões de ratos diabéticos.

Objetivos específicos

- Avaliar morfologicamente as lesões epiteliais de ratos diabéticos que receberam tratamento oral com fenofibrato ou aplicação tópica de creme enriquecido de insulina ou a associação de ambos.

- Avaliar a expressão e a ativação das proteínas da via de sinalização de insulina (IR, AKT, GSK-3 e ERK), além da expressão do VEGF, TGF-ß, TNF-α e SDF1-α em lesões de ratos diabéticos que receberam tratamento oral com fenofibrato ou aplicação tópica de creme enriquecido de insulina ou a associação de ambos.

ARTIGO

EFFECT OF TREATMENT WITH FENOFIBRATE AND INSULIN ON CUTANEOUS WOUND HEALING IN STREPTOZOTOCIN-DIABETIC RATS

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Running Title: insulin and fenofibrate in diabetic wound healing

Key words: diabetes mellitus, wound healing, insulin, fenofibrate.

ABSTRACT

Amputations and foot ulcerations in patients with diabetes impairs their quality of life and is also a problematic public health issue. Insulin has been topically used to treat diabetic wounds and has shown acceleration of the healing process. A previous study of fenofibrate was associated with fewer lower-limb amputations in patients with type 2 diabetes, probably through non-lipid mechanism. The aim of this study was to investigate the association and the effect of systemic fenofibrate in relation to topical insulin on wound healing in streptozotocin-diabetic rats. After 4 days, diabetic animals were wounded and the wounds were treated with insulin or fenofibrate or insulin plus fenofibrate or a placebo as the control. At days 3 and 9 animals were sacrificed and wounds were excised. Therefore, the results of this study indicate that systemic treatment with oral fenofibrate in therapeutic dose does not accelerate the rate of wound healing in diabetic rats, however there is a greater activation of IR, ERK-1 in the inflammatory phase, and IR and GSK-3 in the proliferative phase, besides the improvement of the expression of TGF-ß and TNF-α. The combination of fenofibrate with insulin enriched cream showed an increased rate of wound healing by promoting the activation of the PI3-K, as well as increased expression of cytokines (TNF-α and SDF1-α) and growth factors (VEGF and TGF-ß) in the inflammatory phase, and enhanced TGF-ß and TNF-α during the proliferative phase. We can conclude that the use of the insulin enriched cream was more effective in accelerating the healing when used alone, demonstrated by an increased inflammatory infiltrate, the maturation of collagen fibers in the dermis and angiogenesis, as well as activation of insulin signaling and the enhanced expression of SDF-1α, VEGF, TGF-ß and TNF-α in both the proliferative and inflammatory phases.

Introduction

Current data from the International Diabetes Federation (IDF) and the World Health Organization (WHO) show a dramatic worldwide increase in diabetes mellitus (DM). In 2010, nearly 285 million people were affected by DM, and this number will likely increase to 439 million by 2030 (1).

Despite insulin treatment and a meticulously-controlled diet, foot ulceration affects at least 15% of all diabetic patients and is responsible for 50-70% of all non-traumatic amputations (2, 3). Many physiological factors contribute to wound healing deficiencies in diabetic patients, including decreased or impaired growth factor production (4), angiogenic response (5), macrophage function (6), collagen accumulation, epidermal barrier function, keratinocyte , fibroblast migration and proliferation (2), granulation tissue (7), reduction in wound tensile strength (8) and balance between the accumulation of extracellular matrix (ECM) components and their remodeling by matrix metalloproteinases (MMPs) (9).

The impairment of the insulin signaling is a hallmark of diabetes and it has been proposed as an important mechanism contributing to wound healing defects in this disease (10). Pellegrinelli et al. (11) reported the presence of insulin receptors in keratinocytes of the epidermis and in hair follicles, and identified signaling pathways through which acute insulin can promote skin growth. We have known that diabetic rats induced by streptozotocin have decreased insulin signaling in wound healing compared to normal rats, and also, tissue expression of IR, IRS-1, IRS-2, SHC, ERK and AKT, is increased in the wound healing tissue, compared to intact skin, suggesting that the insulin signaling pathway may have an important role in wound healing (unpublished data).

Since the 1960's, insulin has been topically used to treat diabetic wounds in humans and rats and has shown acceleration of the healing process (12-21).

Experiments with cultured cells have shown that insulin increases the growth rate of fibroblasts (22), suggesting that insulin can function as a growth hormone (23). The molecular mechanisms by which insulin accelerates wound healing in diabetes seem to be multiple and they are far from being understood.

Fenofibrate is a fibric acid derivative indicated for use in the treatment of primary hypercholesterolaemia, mixed dyslipidaemia and hypertriglyceridaemia in adults and has also a nonlipid effect (24). It has been identified as a potential skin therapeutic agent involved in the early inflammation phase of the healing (25). Systemic administration of fenofibrate was associated with fewer lower-limb amputations in patients with type 2 diabetes and without large-vessel disease, probably through non-lipid mechanism (26). Diabetic mice treated with fenofibrate showed an improved vasodilatatory function of small vessels compared with non-treated fenofibrate mice (27). Therefore, further studies are necessary to clarify if fenofibrate may improve wound healing in diabetic animals.

In this regard, the aim of this study was to investigate the effect of systemic fenofibrate and/or its association to topical insulin on wound healing in streptozotocin-diabetic rats.

Materials and Methods

Antibodies and Chemicals Reagents for SDS-PAGE and immunoblotting were from Bio-Rad. HEPES, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol, and bovine serum albumin (fraction V) were from Sigma. Protein A-Sepharose 6MB was from GE Healthcare, and nitrocellulose paper (BA85, 0.2 μm) was from Amersham Biosciences. The reagents for the chemiluminescence labeling of proteins in blots were from Amersham Biosciences. Ketamine hydrochloride was from Parke-Davis (São

Paulo, Brazil), xylazine was from Bayer (São Paulo, Brazil) and thiopethal was from Cristália (Itapira, SP, Brazil). Streptozotocin was from Sigma. Antibodies against β-actin (mouse monoclonal, sc-8432), phospho [Tyr1162/1163]-Insulin Receptor (rabbit polyclonal, sc-25103), Insulin Receptor-ß (rabbit polyclonal, sc-711), phospho [Ser 473]-AKT (rabbit polyclonal, sc-7975), phopho-ERK (mouse monoclonal, sc-7383), ERK-1 (rabbit polyclonal, sc-93), VEGF (rabbit polyclonal, sc-507), SDF1-α (rabbit polyclonal, sc-28876), were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), AKT1 (rabbit polyclonal, Cell-9272), phospho [Ser 21/9]- GSK-3 (rabbit polyclonal, Cell-9331), GSK-3 (rabbit polyclonal, Cell-9315), TNF-α (rabbit polyclonal, Cell-3707), TGF-ß (rabbit polyclonal, Cell-3711) were from Cell Signaling Technology (Beverly, MA, USA).

Animal model Male Wistar rats (n=68), six weeks-old, weighing 180-250g were provided by the State University of Campinas Central Breeding Center. These rats were maintained on a 12:12 h artificial light-dark cycle and housed in individual cages and received standard rodent chow and water *ad libitum*. The investigation followed the University guidelines for the use of animals in experimental studies and conforms to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication No. 85-23 revised 1996). This study was approved by the Ethical Committee for Animal Use of the State University of Campinas, number 1941-1.

Streptozotocin-Induced Diabetes Overnight-fasted rats were rendered diabetic by a single intraperitoneal injection of streptozotocin (100 mg/Kg in citric buffer, pH 4.5). Skin wounds were created four days after receiving streptozotocin injection. The body weight of the animals was recorded and diabetes was confirmed by blood glucose >250 mg/dl. Plasma glucose levels were determined

by the glucose oxidase method using blood samples collected from the animal tail before the experiments were performed.

Skin wound creation The advantages of using full-thickness skin wounds in rats include (1) wound with loss of tissue as often observed in clinical setting; (2) wounds can be accurately reproduced and available for multiple investigations; (3) wounds with controlled shape and size can be created; (4) wounds can be continuously observed and monitored; and (5) time required to obtain useful results, justify use of this model. The animals were anesthetized with an intraperitoneal injection of 100 mg/kg of ketamine hydrochloride (100mg/kg) and xylazine chlorhydrate (2mg/Kg) and used 0.2 mL per 100 g body weight, intraperitoneally and used 10–15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes a tricotomy was made in the dorsal surface of the rats using a blade. After shaving, the skin of the dorsal region was cleaned and disinfected with 70% alcohol. In each animal a 6mm skin excision wound was made in the dorsal region with a biopsy punch instrument, including the panniculus carnosus and exposing the underlying dorso lateral skeletal muscle fascia. Following surgery, the wound was immediately treated with a topical application of insulin cream or placebo, covering all the wound healing area. Wounds were left without dressing and allowed to heal by second intention.

Treatment The animals were randomized into four groups: diabetic control (DP); diabetic treated with topical insulin cream (DI); diabetic treated with fenofibrate (DF); diabetic treated with insulin plus fenofibrate (DFI).

The insulin cream administered once a day was prepared with regular insulin in the pharmacy of our University Hospital and holds the patent number, PI 0705370-3 (University of Campinas, Brazil). The vehicle cream was without insulin. The fenofibrate (20.55mg/kg) (28) was dissolved in absolute alcohol, followed by water

dilution, in proportion 1:5; the placebo was absolute alcohol diluted in water. The wounds were cleaned with saline and gauze before being treated. The treatment with fenofibrate was realized by gavage once a day. The tissues were extracted on day 3 and day 9 of drug administration.

Wound closure measurements Wound-healing rate was calculated by wound contraction rate and complete epithelialization time. The wound healing area (height (↕) x width (↔)) was measured using a paquimeter of 0.01mm precision (Vonder, Brazil) every day until the extraction and was photographed (digital camera Power Shot SX120 IS, Canon) concomitantly. Wound closure was reported as percentage closure and calculated using the formula % closure: (29) [(area on day 0-open area on day n) / area on day 0] x 100; n represents each of the measurement days. Presence of complete epithelialization was monitored by visual observation throughout the experiment. Complete epithelialization was reached when the scar fell off the skin, leaving no raw wound behind.

Collection and preparation of wound samples for structural analysis In 3 and 9 days after the injury, three animals in each group were killed under anesthesia, the total area of the wound was removed and submitted to structural and morphometric analysis. Each sample was removed and fixed in 10% formalin in Millonig buffer, pH 7.4, for 24 h at room temperature. Next, the specimens were washed in buffer and processed for embedding in ParaplastTM (Merck). Longitudinal sections (6 μm) were stained with Toluidine blue for basophilic analysis, with picrosirius-hematoxylin for the observation and quantification of collagen fibers. The specimens were examined and documented using light and polarized light microscopy.

Morphometric analysis Cross-sections of the mid-region of the experimental wound were used for the determination of the following

morphometric parameters in repair tissue: mature collagen fibers deposition (% of tissue area), total number of cells (fibroblastic and inflammatory cells) (n/10^4 µm2), number of newly formed blood vessels (n/10^4 μm^2), and thickness of the regenerating epithelium (µm). For this purpose, five samples were randomly selected among the sections obtained. All images were captured and digitalized using photomicroscope. The measurements were made on the digitalized images using the Leica Image Measure™ and Sigma Scan Pro 6.0™ programs.

Tissue extraction and Immunoblotting Rats were anesthetized by intraperitoneal injection of sodium thiopental and used 10–15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. For evaluation of protein expression and activation of signal transduction pathways, the skin wound (days 3 and 9) of anesthetized rats were excised and immediately homogenized in solubilization buffer at 4ºC [1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM PMSF and 0.1 mg aprotinin/ml) with a polytron PTA20s (Brinkmann Instruments modelo PT 10/35). Insoluble material was removed by centrifugation for 40 min at 11,000 rpm in a 70.Ti rotor (Beckman) at 4ºC. The protein concentration of the supernatants was determined by the Biuret dye method. Aliquots of the resulting supernatants containing 50 µg of protein extracts were separated by SDS-PAGE (X% bis-acrilamide) in a Bio-Rad miniature slab gel apparatus (mini-Protean) transferred for 90 min at 120V to nitrocellulose membranes and blotted with antibodies against β-actin, phospho [Tyr1162/1163]-Insulin Receptor, Insulin Receptor-ß, phospho [Ser 473]-AKT, phopho-ERK, ERK-1, VEGF, SDF1-α, AKT1, phospho [Ser 21/9]- GSK-3, GSK-3, TNF-α, TGF-ß. In the immunoblotting experiments, proteins were denaturated by boiling in Laemmli buffer (Laemmli 1970). For immunoblotting, the sample buffer

contained 100 mM dithiothreitol (DTT). Specific bands were labeled with a chemioluminescence kit and visualization was performed by exposure of the membranes to X-ray films.

Statistical analysis Specific protein bands present on the blots were quantified by densitometry. All results are expressed as mean ± S.E.M. For statistical analysis, the groups were compared by One-way ANOVA with the Bonferroni test for post hoc comparisons. A $p < 0.05$ was accepted as statistically significant.

Data from the structural analyses was obtained for the different experimental groups, stored in electronic spreadsheets and compared by One-way ANOVA with the Tukey post test. The level of significance was set at 5% using the Biostat for Windows XP™ program.

Results

Macroscopic analyses

As illustrated in Fig. 1A, the wound area was completely reepitheliazed in the insulin cream treated group (DI) 9 days after wounding. The control group (DP) and the fenofibrate treated group (DF) showed less reepithelization than the DI group and the insulin plus fenofibrate treated animals (DFI). As shown on day 3 on the wound healing process graph (Fig. 1B) the DI and DFI groups had a 20% better healing rate than the DP and DF groups. On day 9 the DI wounds healed 15% faster than the wounds of the DP and DF animals and the DFI group healed 10% faster than the DP and the DF groups.

Structural analysis

Each group was studied within the 9-day observation period by comparing inflammatory processes (leukocytosis, hemorrhage and exudate), proliferative

processes (fibroblastic hyperplasia, epithelization and angiogenesis), and tissue reorganization. Structural analysis was performed on days 3 and 9.

In the DP group there was a small amount of inflammatory infiltrates observed in the repair area in the period of analysis (3 to 9 days). On the other hand, the presence of fibroblasts was already observed in large quantities in samples from day 3. Note that the density of these cells is higher at the edges of the repair area and in the original tissue preserved. The repair of the epithelium takes place gradually from the day 3 of analysis. The proliferation of keratinocytes occurs from the basal cells of the preserved tissue on the edges of the lesion. On Day 9, the entire repair area is covered by a new epidermis, including the presence of newly formed skin appendages. The presence of blood vessels has been detected since the 3rd day of analysis. A large numbers of arterioles and venules were observed in the middle portion of the area under repair. The structural characteristics of the fibrous matrix were also evaluated in the different groups. A predominance of thin and poorly compacted collagen fibers was observed in samples collected on day 3 after experimental injury in all groups. However, medium-thick and compact fibers were observed on day 9 when compared to intact tissue at the border of the wound (Figs. 2, 3 and 4). These results were supported by morphometric analysis comparing the different groups (Figs. 5, 6, 7 and 8).

There were not important qualitative differences found between the experimental groups tested. However, morphometric analysis showed some significant quantitative differences between them.

With respect to the total number of cells on day 3 (3d) after injury the DI group had a significant increase in the number of cells compared to the other groups. On day 9 (9d), there was not a significant difference between the groups (Fig. 5).

For the total number of newly formed vessels the same pattern was observed. A larger number of vessels, inside the wound area, were observed in the DI group on day 3 after experimental injury, when compared to the other groups. In the 9th day, there was not a significant difference between the groups, but the number of vessels was significantly less than observed on day 3 after experimental injury (Fig. 6).

Additionally, no difference was noted in relation to the thickness of newly formed epithelium on day 3 and day 9 of treatment. However, the values detected on day 9 were significantly higher in all groups (Fig. 7).

Morphometric evaluation of the mature collagen fibers showed a significant increase between day 3 and day 9. Moreover, only the DI group presented higher content on both days (Fig. 8).

Insulin signaling proteins in wounded skin

In the day 3 characterized by skin inflammation process we observed an increase in insulin cream-induced tyrosine phosphorylation of the insulin receptor (IR) in the wounded skin of the DI and DF groups compared to the DP group ($p<0.05$). The DI animals had an increase in the phosphorylation of IR compared to DFI group ($p<0.05$; Fig. 9A). On the other hand, in the day 9, characterized by cell proliferation, the insulin cream-induced tyrosine phosphorylation of the IR in the wounded skin had an increase in the DI and DF ($p<0.05$) and also DFI ($p<0.001$) animals compared to the DP group (Fig. 10A). In the 3 and 9 days the insulin cream-induced serine phosphorylation of AKT was increased in the DI group compared to the DP rats ($p<0.001$) and increased the DFI group compared to the DP rats ($p<0.01$ in the day 3 and $p<0.001$ in the day 9). The DF group had a dramatically decreased in the phosphorylation of AKT compared with the DI ($p<0.001$ in the day 3 and

$p<0.05$ in the day 9) and DFI ($p<0.05$) animals, but did not change the phosphorylation of this protein compared to the DP rats (Figs. 9B and 10B). In the day 3 the phosphorylation of glycogen synthase kinase-3 (GSK-3) showed an increase in the DI ($p<0.001$) and DFI ($p<0.05$) groups compared to the DP group. The activation of GSK-3 decreased in the DF and DFI groups compared to the DI animals ($p<0.05$; Fig. 9C). However, in the day 9 the phosphorylation of GSK-3 had an increase in the DI, DF and DFI groups compared to the DP group ($p<0.05$). There was not a significant difference concerning the phosphorylation of GSK-3 between the DI and DFI animals (Fig. 10C).

In the inflammatory phase (day 3) the phosphorylation of the extracellular signal-regulated kinase (ERK)1/2 was increased in the DI ($p<0.05$) and DF ($p<0.01$) groups compared to the DP animals. The phosphorylation of (ERK)1/2 has decreased in the DFI rats compared to the DI ($p<0.05$) and DF ($p<0.001$) animals (Fig. 9D). Nevertheless, in the proliferative phase (day 9) the wounded skin of DI animals had an increase in the phosphorylation of (ERK)1/2 compared to the DP group ($p<0.05$). There was not another significant difference between the groups concerning the phosphorylation of (ERK)1/2 (Fig. 10D).

Cytokines and Growth factors expression in the wounded skin

Growth factors, chemokines and cytokines are crucial for coordinating multiple cell types during the healing process, resulting in the reestablishment of the skin's barrier function. During the inflammatory phase the expression of tumor necrosis factor (TNF)-α cytokine was increased in the DI ($p<0.01$) and DFI ($p<0.001$) groups compared to the DP group (Fig. 11B). The DFI animals had an increase in the expression of this cytokine compared to the DI and DF groups ($p<0.001$). The DF group did not show any difference in the expression of this

protein compared to the DP animals. However, in the day 9, the expression of TNF-α during the proliferative phase had an increased in the DI, DF and DFI groups compared to the DP rats ($p<0.05$; Fig. 12B).

We observed a dramatically increase in the protein level of the stromal cell-derived factor (SDF)-1α chemokine in the DI ($p<0.05$) and DFI ($p<0.01$) animals compared to the DP group during the inflammatory phase. However, the DF group had a sharp decrease in the expression of SDF-1α compared to the DI ($p<0.01$) and DFI ($p<0.001$) rats (Fig. 11A). In the proliferative phase the expression of SDF-1α was increased in the DI rats compared to the other groups (DP and DF with $p<0.05$; DFI with $p<0.01$). The DFI group had a decreased of this chemokine expression compared to DF animals ($p<0.05$; Fig. 12A). These results suggest that the fenofibrate did not enhance the insulin action in the expression of SDF-1α in the inflammatory phase and neither the proliferative phase.

Next, we studied the vascular endothelial growth factor (VEGF) protein level in the inflammatory phase (Fig. 11C) and observed that it was increased in the DI and DFI groups compared to the DP animals ($p<0.05$). In the proliferative phase the DI group showed an increase in the VEGF level ($p<0.05$) compared to the other groups (Fig.12C). In the day 3, the transforming growth factor (TGF)-ß protein level showed an increase in the DI and DFI groups compared to the DP rats ($p<0.001$). Moreover, TGF-ß decreased in the DF group compared with DI and DFI rats ($p<0.001$), but showed no difference compared to the DP group. There was not a significant difference in regard to the expression of TGF-ß between DI and DFI groups (Fig. 11D). In the day 9, the DI ($p<0.05$), DF ($p<0.001$) and DFI ($p<0.001$) groups showed an increase in the expression of TGF-ß compared to the

DP rats. The DI animals had a decrease in the expression of this protein compared to DF and DFI groups ($p<0.05$; Fig. 12C).

DISCUSSION

A recent study showed that diabetic patients treated with fenofibrate had a reduced major-amputation risk probably through a non-lipid-way (26). However, in our study systemic application of fenofibrate by oral gavage in a therapeutic dose did not accelerate the wound healing, but did increase some important proteins in the wound healing process. Previous work has shown that insulin improves wound healing and in this study streptozotocin-diabetic-animals treated with insulin or with insulin plus fenofibrate had an improvement in the wound healing rates.

Insulin receptors are known to be present in keratinocytes of the epidermis, in fibroblasts and in hair follicles of intact skin. Insulin signaling pathways are activated in the skin after acute insulin stimulation (11). Unpublished data shows that topical insulin cream enhances the insulin signal transduction pathways to improve the wound healing and this process may be through the PI3-K and MAP-K pathways. Our data showed that insulin used alone activated both pathways in both phases. In the present study, in the inflammatory phase of healing, we observed that fenofibrate increases the phosphorylation of insulin receptor and also activates the mitogen-activated protein kinase, activating the MAP-K pathway. In addition, the fenofibrate plus insulin treatment group activated the PI3-pathway. Nevertheless, in the proliferative phase we observed in the fenofibrate group an increase in the phosphorylation of IR and GSK-3, activating now, the PI3-K pathway. The insulin plus fenofibrate continued the phosphorylation of the PI3-K pathway in the proliferative phase. Both pathways of the insulin signaling are involved in the wound healing process (21) and the GSK-3 is in the control of the tissue repair process (30).

Collagen is a key constituent of granulation tissue synthesized by fibroblasts and provides tensile strength, organization and integrity to the connective tissues (31). Experimental diabetes was shown to impair tissue repair by decreasing collagen concentration and the formation of granulation tissue (32) and also by increasing activities of protease and collagenase (33). Our data showed the only the group treated with insulin improved the mature collagen fibers in both day 3 and day 9.

Diabetic wounds in animals and humans are characterized by dysfunction of the normal wound healing phases, with a delay in cellular infiltration and formation of granulation tissue also the delay of the inflammatory phase (4, 34). Inflammatory cells, fibroblasts and keratinocytes are important cell types involved in the wound healing process, modulating the reconstruction of the wound healing area. In this study the insulin shows an increase in cell infiltration and new vessels in the inflammatory phase. It is well-know that VEGF, one of the most potent angiogenic cytokines, is produced by the keratinocytes. It initiates the signaling cascades leading to nitric oxide production and angiogenic activation of endothelial cells (35). The nitric oxide enhances VEGF production by augmenting its expression through activation of AKT kinase (36) and enhancing the mobilization of EPC, which is recruited to the cutaneous wound site by an increase in tissue levels of SDF-1α. Diabetic wounds have a lack of SDF1-α (37) and the treatment of this deficiency improves healing (38). According to this data, a recent study showed that alterations in the wound level of SDF1-α impair healing by decreasing cellular migration and angiogenesis (39). In the present study, in the inflammatory phase, we observed that the fenofibrate treatment attenuated the expression of SDF1-α and VEGF compared to both the insulin and the insulin plus fenofibrate treatment. Nevertheless, the insulin and the insulin plus fenofibrate improved the expression of these proteins compared to the control group. In the

proliferative phase just the insulin cream increased the expression of SDF1-α and VEGF.

Our results showed that the fenofibrate treatment attenuated the expression of TNF-α and TGF-ß in the inflammatory phase, but increased in the proliferative phase. However, insulin and the insulin plus fenofibrate enhanced these proteins in both phases. After skin injury, neutrophils are attracted to the wound site followed by macrophages (40). Neutrophils and macrophages release several growth factors and pro-inflammatory cytokines, i.e., TNF-α, which stimulate granulation tissue formation (41) and angiogenesis (42). TNF-α is a cytokine that regulates the inflammatory response to wounding. The role of TNF-α in wound healing is contradictory. Depending on the concentration, length of exposure, and presence of other cytokines the effect of TNF-α can be beneficial or deleterious to the skin repair (43). The increase in the expression of TNF-α did not impair the healing of animals receiving topical insulin or insulin plus fenofibrate.

The TGF-β is an ubiquitously expressed cytokine and plays an active role in the wound healing process (44) and in the synthesis of ECM molecules (45). The TGF-β isoforms are necessary in the inflammatory phase for proper hemoattraction of monocytes; in the proliferative phase, it stimulates extracellularmatrix production and affects angiogenesis and epithelialization, and, moreover, induces myofibroblast contraction and formation in skin wounds. The isoform TGF-ß1 can present contradictory effects on skin, but it is also well known that this mediator, released by cells that are localized at sites of tissue repair, such as platelets, activated macrophages and possibly fibroblasts, exerts beneficial effects in each phase of wound healing (46). Recently, TGF-β1 increased the rate of healing and the breaking strength of the repaired tissue in aged animals; it also

enhanced angiogenesis and consequent blood flow to dermal wounds in these animals, by stimulating, at least in part, local release of other growth factors (46). The TGF-β superfamily signaling pathways also crosstalk extensively with many signaling pathways including, i.e., the MAPK, PI(3)K/Akt, tyrosine kinase receptor-mediated pathways and the extracellular-signal-regulated kinase (47). We considered the possibility that the increase of wound healing in the diabetic-wound treated with insulin and insulin plus fenofibrate may be through this TGF-ß crosstalk with insulin signaling.

Therefore, the results of this study indicate that systemic treatment with oral fenofibrate in therapeutic dose does not accelerate the rate of wound healing in diabetic rats, however there is a greater activation of IR, ERK-1 in the inflammatory phase, and IR and GSK-3 in the proliferative phase, besides the improvement of the expression of TGF-ß and TNF-α. The combination of fenofibrate with insulin enriched cream showed an increased rate of wound healing by promoting the activation of the PI3-K, as well as increased expression of cytokines (TNF-α and SDF1-α) and growth factors (VEGF and TGF-ß) in the inflammatory phase, and enhanced TGF-ß and TNF-α during the proliferative phase. We can conclude that the use of the insulin enriched cream was more effective in accelerating the healing when used alone, demonstrated by an increased inflammatory infiltrate, the maturation of collagen fibers in the dermis and angiogenesis, as well as activation of insulin signaling and the enhanced expression of SDF-1α, VEGF, TGF-ß and TNF-α in both the proliferative and inflammatory phases.

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Figure 1. Insulin accelerates wound healing. Macroscopic evaluation of wounds. All the figures: diabetic control (DP); diabetic treated with insulin cream (DI); diabetic treated with fenofibrate (DF); diabetic treated with insulin plus fenofibrate (DFI), n=8. (A) Representative images of wounds on 0 (0d), 3 (3d) and 9 (9d) days after wounding. (B) Percentage of original wound area in the groups DP, DI, DF and DFI. Data are expressed as mean ± S.E.M. ($p<0.01$, *, DI vs. DP and DF; $, DFI vs. DP and DF).

Figure 2. Photomicrographs of cross-sections of skin obtained from the back of Wistar rats on day 3 and day 9 after surgically induced injury. The experimental groups were DP, DI, DF and DFI. The sections were stained with Toluidine blue. (►) – Dermis under repair; (*) – Epithelium; (→) – Blood vessels. Bar = 50μm (3 days) e 100μm (9 days).

Figure 3. Photomicrographs of cross-sections of skin obtained from the back of Wistar rats on day 3 after surgically induced injury. The experimental groups were DP, DI, DF and DFI. The sections were stained with Picrossirius-hematoxilin method and analysed in ligth (LM) and polarized light (PLM)

microscopes. (►) – Dermis under repair; (*) – Epithelium; (→) – Blood vessels. Bar = 50μm.

Figure 4. Photomicrographs of cross-sections of skin obtained from the back of Wistar rats on day 9 after surgically induced injury. The experimental groups were DP, DI, DF and DFI. The sections were stained with Picrossirius-hematoxilin method and analysed in ligth (LM) and polarized light (PLM) microscopes. (►) – Dermis under repair; (*) – Epithelium; (→) – Blood vessels. Bar = 100μm.

Figure 5. Total number of cells ($n/10^4$ μm^2) in the region of the experimental wound. The experimental groups were DP, DI, DF and DFI. Samples collected on day 3 (3d) and 9 (9d) after injury were analysed. The results are reported as the mean and standard deviation obtained for each group and were compared by ANOVA and Tukey post-test ($p<0,05$). (*) Significant differences between control and treated groups in the distinct times of sampling.

Figure 6. Total number of newly formed blood vessels ($n/10^4$ μm^2) in the region of the experimental wound. The experimental groups were DP, DI, DF and DFI. Samples collected on day 3 (3d) and 9 (9d) after injury were analysed. The results are reported as the mean and standard deviation obtained for each group and were compared by ANOVA and Tukey post-test ($p<0,05$). (*) Significant differences between control and treated groups in the distinct times of sampling.

Figure 7. Epithelial thickness (μm) in the region of the experimental wound. The experimental groups were DP, DI, DF and DFI. Samples collected on day 3 (3d) and 9 (9d) after injury were analysed. The results are reported as the mean and standard deviation obtained for each group and were compared by

ANOVA and Tukey post-test (p<0,05). No significant differences between control and treated groups in the distinct times of sampling were detected.

Figure 8. Percentage of occupied area by mature collagen fibers in relation to total repair region (%) in the experimental injury. The experimental groups were DP, DI, DF and DFI. Samples collected on day 3 (3d) and 9 (9d) after injury were analysed. The results are reported as the mean and standard deviation obtained for each group and were compared by ANOVA and Tukey post-test (p<0,05). (*), (**) Significant differences between control and treated groups in the distinct times of sampling.

Figure 9. Activation of insulin signaling. The experimental groups were DP, DI, DF and DFI. Wound extracts were extracted on day 3. Tissue extracts were immunoblotted with (A) anti-pIR, (B) anti-pAKT, (C) anti-pGSK3, (D) anti-pERK1/2. To determine the protein levels of IR, AKT, GSK3 and ERK1/2, the membranes were stripped and reprobed with anti-IR, -AKT, -GSK3 and -ERK1/2. Equal protein loading in the gel was confirmed by reblotting the membranes with anti-β-actin. These results are represented as the mean ± S.E.M. of scanning densitometry of 6 experiments, and the bar graphs represent the ratio of phosphorylation/protein. In all the experiments, p<0.001, §, vs. DP; €, vs. DF; ¥, vs. DFI; p<0.01, #, vs. DP; p<0.05, *, vs. DP; &, vs. DF; $, vs. DFI.

Figure 10. Activation of insulin signaling. The experimental groups were DP, DI, DF and DFI. Wound extracts were extracted on day 9. Tissue extracts were immunoblotted with (A) anti-pIR , (B) anti-pAKT, (C) anti-pGSK3, (D) anti-pERK1/2. To determine the protein levels of IR, AKT, GSK3 and ERK1/2, the membranes were stripped and reprobed with anti-IR, -AKT, -GSK3 and -ERK1/2. Equal protein loading in the gel was confirmed by reblotting the membranes with anti-β-actin. These results are represented as the mean ± S.E.M. of scanning

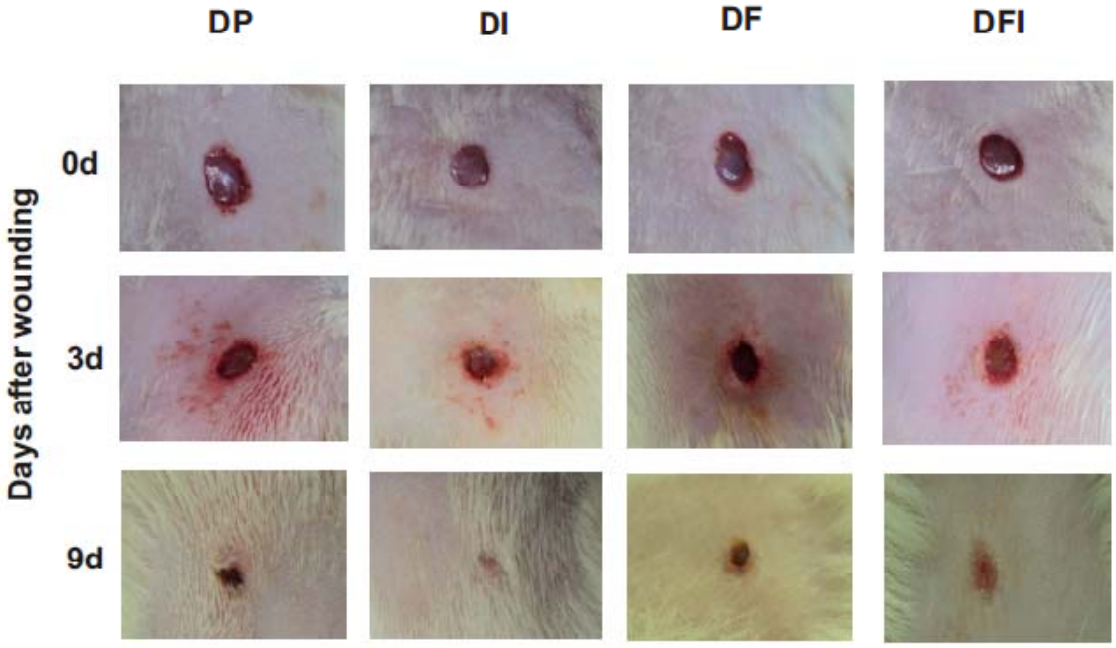
densitometry of 6 experiments, and the bar graphs represent the ratio of phosphorylation/protein. In all the experiments, p<0.001, §, vs. DP; €, vs. DF; ¥, vs. DFI; p<0.01, #, vs. DP; p<0.05, *, vs. DP; &, vs. DF; $, vs. DFI.

Figure 11. Effect of insulin and fenofibrate on cellular and molecular mechanisms of wound healing in diabetes. The experimental groups were DP, DI, DF and DFI. Wound extracts were extracted on day 3. Tissue extracts were immunoblotted with (A) anti-SDF1-α, (B) anti-TNF-α, (C) anti-VEGF, (D) anti-TGF-ß. Equal protein loading in the gel was confirmed by reblotting the membranes with anti-β-actin. These results are represented as the mean ± S.E.M. of scanning densitometry of 6 experiments, and the bar graphs represent the ratio of phosphorylation/protein. In all the experiments, p<0.001, §, vs. DP; Ψ, vs. DI; €, vs. DF; p<0.01, $, vs. DP; &, vs. DF; p<0.05, *, vs. DP.

Figure 12. Effect of insulin and fenofibrate on cellular and molecular mechanisms of wound healing in diabetes. The experimental groups were DP, DI, DF and DFI. Wound extracts were extracted on day 9. Tissue extracts were immunoblotted with (A) anti-SDF1-α, (B) anti-TNF-α, (C) anti-VEGF, (D) anti-TGF-ß. Equal protein loading in the gel was confirmed by reblotting the membranes with anti-β-actin. These results are represented as the mean ± S.E.M. of scanning densitometry of 6 experiments, and the bar graphs represent the ratio of phosphorylation/protein. In all the experiments, p<0.001, §, vs. DP; Ψ, vs. DI; €, vs. DF; p<0.01, $, vs. DP; &, vs. DF; Ω, vs. DFI; p<0.05, *, vs. DP; ¶, vs. DI; &, vs. DF.

Figure 1

(A)


DP
DI
DF
DFI
Days after wounding
0d
3d
9d

(B)

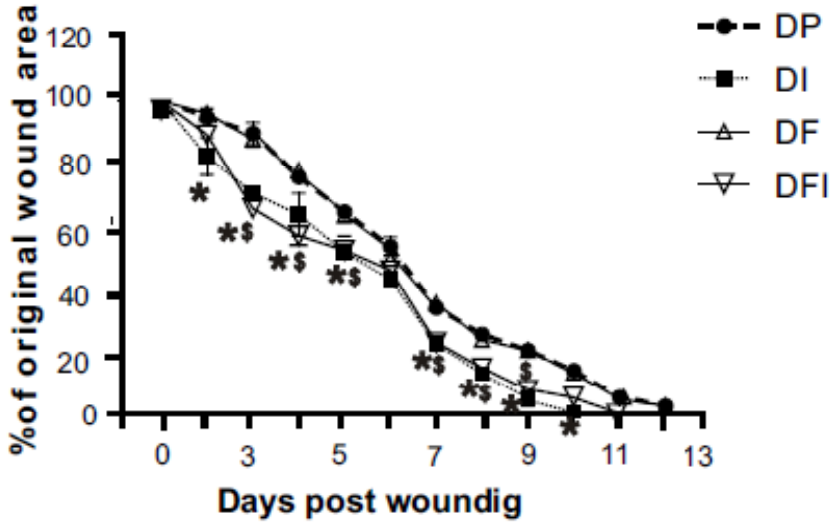

%of original wound area
120
100
80
60
40
20
0
0
3
5
7
9
11
13
Days post woundig
DP
DI
DF
DFI

Figure 2

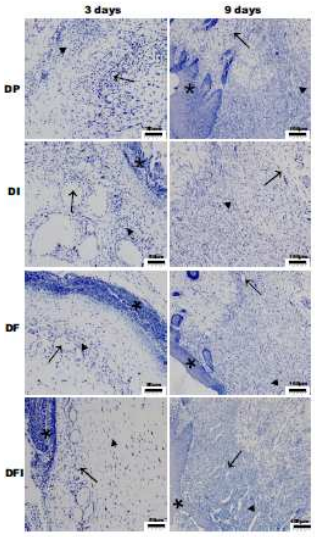


Figure 3

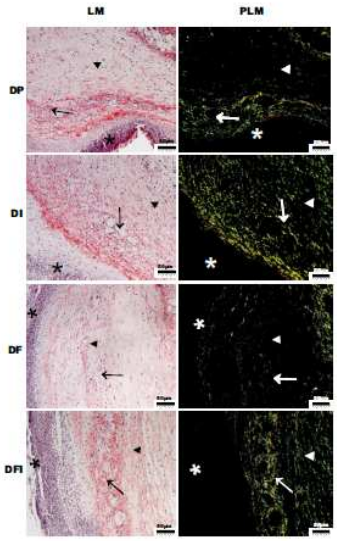


Figure 4

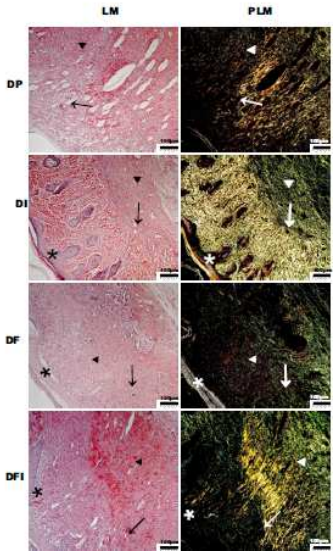


Figure 5

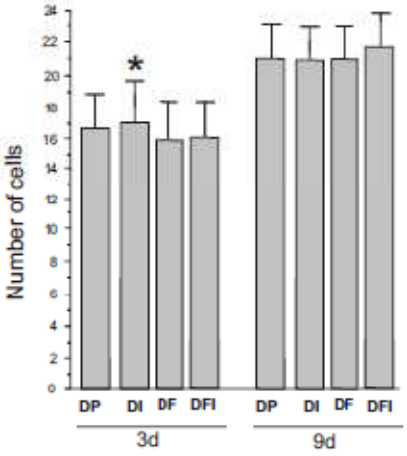


Figure 6

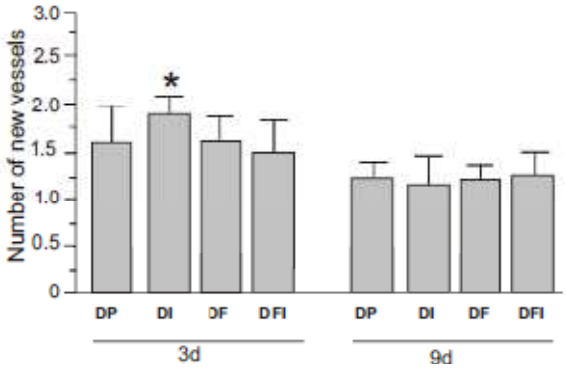


Figure 7

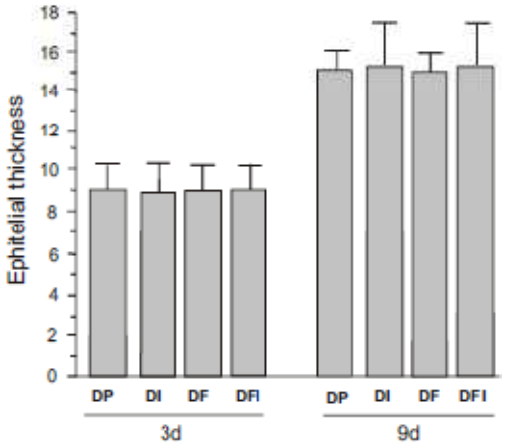


Figure 8

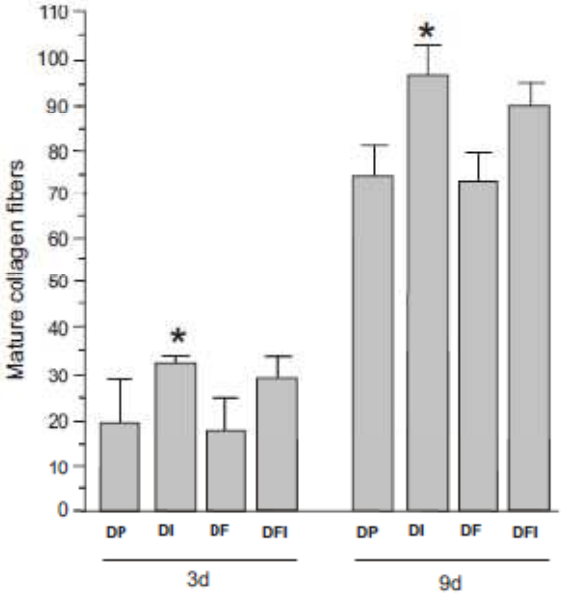


Figure 9

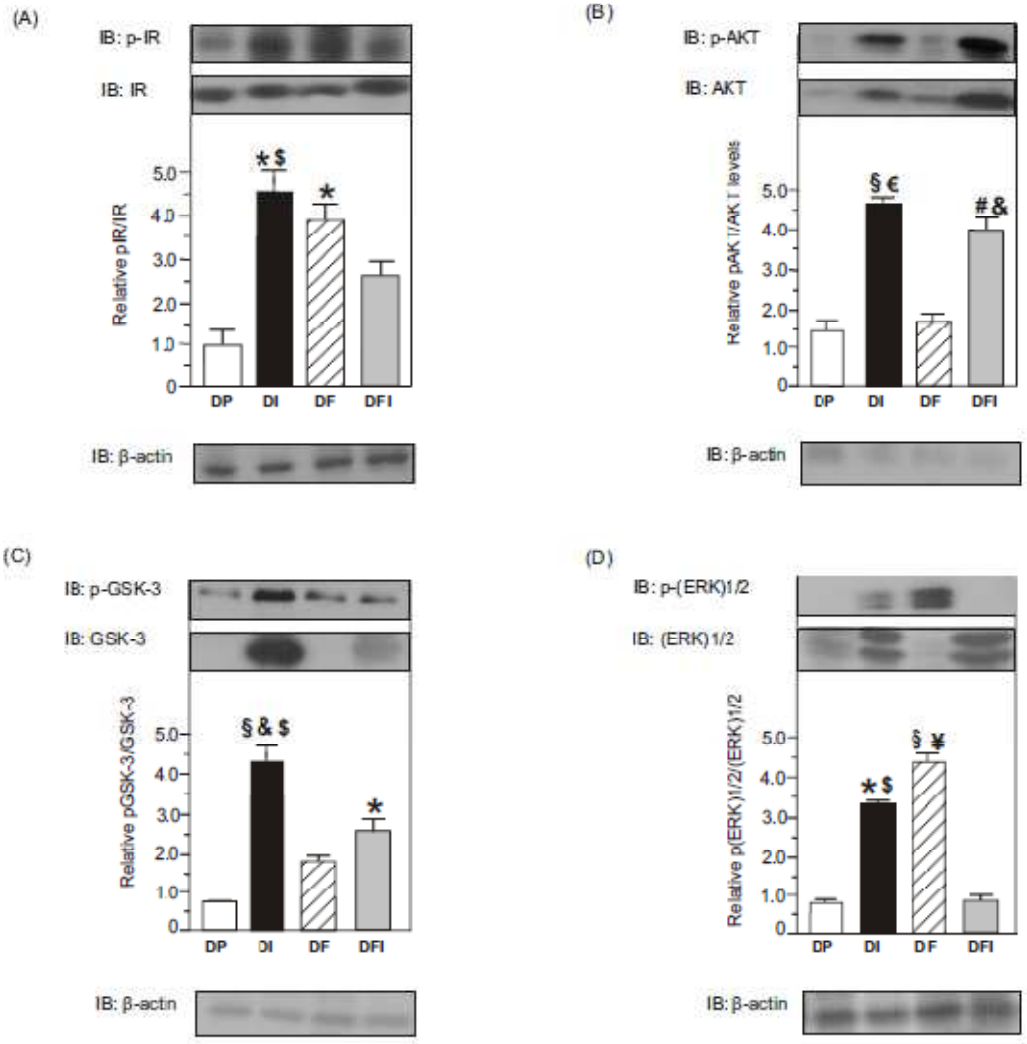

(A)
IB: p-IR
IB: IR
Relative pIR/IR
5.0
4.0
3.0
2.0
1.0
0
*$
*
DP
DI
DF
DFI
IB: β-actin
(B)
IB: p-AKT
IB: AKT
Relative pAKT/AKT levels
§ €
#&
IB: β-actin
(C)
IB: p-GSK-3
IB: GSK-3
Relative pGSK-3/GSK-3
§ & $
*
IB: β-actin
(D)
IB: p-(ERK)1/2
IB: (ERK) 1/2
Relative p(ERK)1/2/(ERK)1/2
*$
§ ¥
IB: β-actin

Figure 10

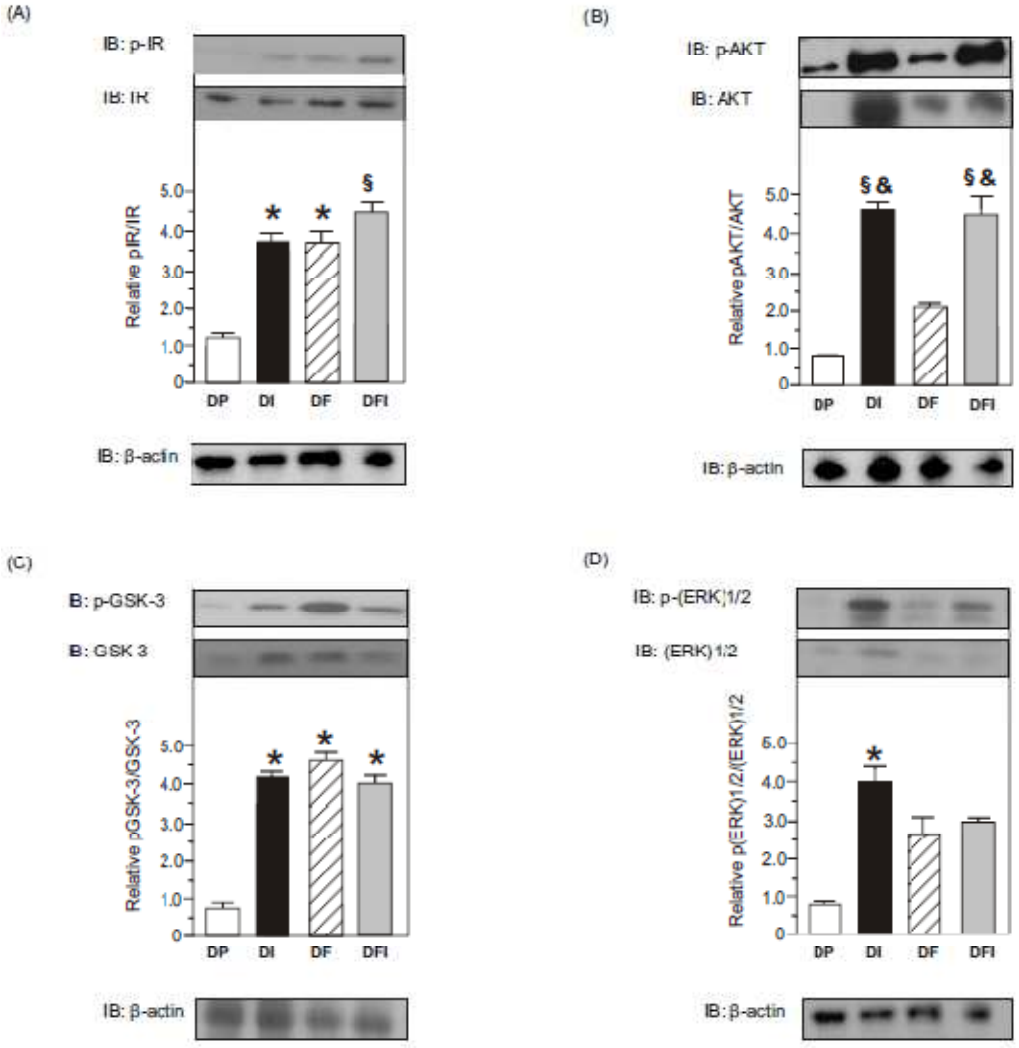

(A)
IB: p-IR
IB: IR
Relative pIR/IR
5.0
4.0
3.0
2.0
1.0
0
*
*
§
DP
DI
DF
DFI
IB: β-actin
(B)
IB: p-AKT
IB: AKT
Relative pAKT/AKT
§ &
§ &
DP
DI
DF
DFI
IB: β-actin
(C)
B: p-GSK-3
B: GSK 3
Relative pGSK-3/GSK-3
*
*
*
DP
DI
DF
DFI
IB: β-actin
(D)
IB: p-(ERK)1/2
IB: (ERK)1/2
Relative p(ERK)1/2/(ERK)1/2
6.0
4.0
3.0
2.0
1.0
0
*
DP
DI
DF
DFI
IB: β-actin

Figure 11

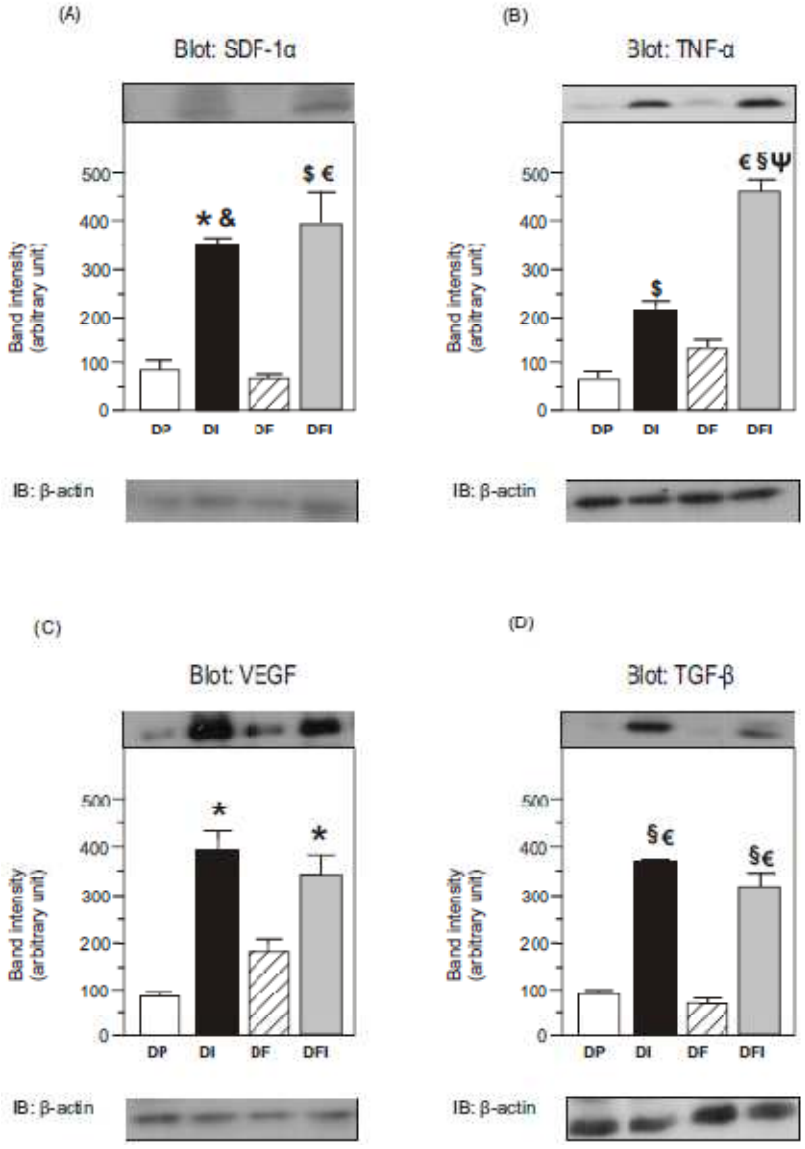
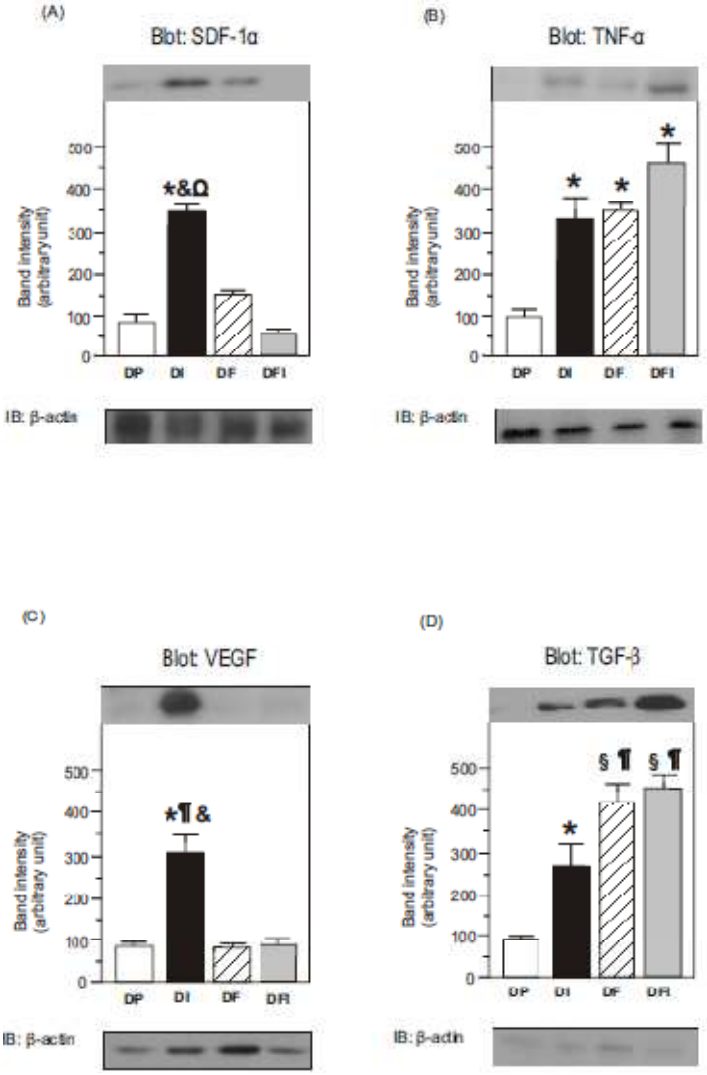

(A)
Blot: SDF-1α
Band intensity (arbitrary unit)
500
400
300
200
100
0
* &
$ €
DP
DI
DF
DFI
IB: β-actin
(B)
Blot: TNF-α
Band intensity (arbitrary unit)
$
€ § Ψ
IB: β-actin
(C)
Blot: VEGF
Band intensity (arbitrary unit)
*
*
IB: β-actin
(D)
Blot: TGF-β
Band intensity (arbitrary unit)
§ €
§ €
IB: β-actin

Figure 12


(A)
Blot: SDF-1α
Band intensity (arbitrary unit)
*&Ω
DP
DI
DF
DFI
IB: β-actin
(B)
Blot: TNF-α
Band intensity (arbitrary unit)
*
*
*
DP
DI
DF
DFI
IB: β-actin
(C)
Blot: VEGF
Band intensity (arbitrary unit)
*¶ &
DP
DI
DF
DFI
IB: β-actin
(D)
Blot: TGF-β
Band intensity (arbitrary unit)
*
§ ¶
§ ¶
DP
DI
DF
DFI
IB: β-actin

CONCLUSÃO

Portanto, os resultados deste estudo indicam que o tratamento sistêmico com fenofibrato por via oral em doses terapêuticas não acelera a taxa de cicatrização de feridas em ratos diabéticos, porém existe uma maior ativação do IR, ERK-1 na fase inflamatória; e do IR e GSK-3 na fase proliferativa, além da melhora da expressão de TGF-ß e TNF-α. O tratamento combinado do fenofibrato com o creme enriquecido de insulina apresentou uma aceleração da taxa de cicatrização, promovendo a ativação da via da PI3-K, além do aumento da expressão de citocinas (TNF-α e SDF1-α) e fatores de crescimento (VEGF e TGF-ß) na fase inflamatória; e melhora de TGF-ß e TNF-α durante a fase proliferativa. Neste sentido, podemos concluir que o uso do creme enriquecido de insulina mostrou-se mais eficaz na aceleração da cicatrização quando utilizada sozinha, pois houve um aumento do infiltrado inflamatório, da maturação das fibras de colágeno e da angiogênese na derme, bem como a ativação da sinalização de insulina e melhora da expressão de SDF-1α, VEGF, TGF-ß e TNF-α tanto na fase inflamatória quanto na proliferativa.

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