THE EFFECT OF FLAXSEED OIL ON WOUND HEALING OF STREPTOZOTOCIN-INDUCED DIABETIC RABBITS: A HISTOPATHOLOGICAL, IMMUNOHISTOCHEMICAL, GENE EXPRESSION AND BIOPHYSICAL STUDY

BY

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ABSTRACT

Patients with diabetes are susceptible to develop chronic, nonhealing wounds which cause pain, suffering, and poor quality of life. This, together with high prevalence of delayed wound (15%), increases necessity to find new and more efficient approaches for diabetic wound treatment. Researchers have explored flaxseed oil to expedite *in vivo* wound healing. Flaxseed oil is known for its anti-inflammatory and antioxidative effects that improve wound healing because the inflammatory process and oxidative damage are implicated in the pathogenesis of diabetic wounds. However, studies utilising flaxseed oil on diabetic animal models are scarce. This study investigates the therapeutic effect of flaxseed oil on wound healing in diabetic animals in 4^{th}, 7^{th}, and 14^{th} day intervals. The study has two phases: the streptozotocin (STZ) diabetes induction phase consisting of 27 male rabbits and the flaxseed oil treatment phase applied to diabetic and nondiabetic animals consisting of 54 male rabbits, in which a full-thickness skin incisional wound (15–17 mm length) were created. They were divided into flaxseed group for diabetic (n=9) and nondiabetic animals (n=9). Positive control (Fucidin cream 2%) group for diabetic (n=9) and nondiabetic animals (n=9), and negative control (nontreated) group for diabetic (n=9) and nondiabetic animals (n=9). The gross wound was monitored using a digital camera and J image software to measure wound length. Flaxseed group diabetic animals group showed regular and approximate smooth edges of the skin wound with organised brightly coloured eschar tissue. All groups have the same days of complete wound closure. However, the wound healing efficiency was higher for flaxseed group diabetic animals ($p<0.05$) than the control group. The assessment of skin elasticity for flaxseed group nondiabetic animals had the highest viscoelasticity (VE) values with significant differences for three-week intervals. Histological analyses of H&E and Mallory-Trichrome were used to study wound healing. Immunohistochemical evaluations (*VEGF* and *TGF-β*) with biochemical analysis (ELISA) of (*MMP-2*, *PDGF-A*, and *VEGF*) protein expression was performed on day 4, day 7, and day 14 of wound healing. The wound healing of the flaxseed group accelerated initially by increasing cellular proliferation (keratinocytes, fibroblast, and endothelial cell) and reducing inflammation via modulation of the protein signalling pathway. In diabetic animals, flaxseed oil enhanced healing by reducing oxidative damage through increased activities of endogenous antioxidants as the flaxseed antioxidant activity was accompanied by up-regulation of pro-fibrotic (*TGF-β*) gene expression, which triggers fibrogenesis and angiogenesis of wound healing. These mechanisms were more pronounced in flaxseed groups. This study proved that flaxseed oil is a good product for treating diabetic wounds, either alone or combined with biocompatible and biodegradable wound dressing. In conclusion, the results justified that flaxseed oil can be further developed to obtain new and more efficient dressing agent to treat diabetic wounds and other types of skin wounds.

خلاصة البحث

مرض السكري من الأمراض المزمنة التي تصيب الإنسان في جميع أنحاء العالم ، ومرضى السكري معرضون للإصابة بجروح مزمنة غير قابلة للشفاء تسبب الألم والمعاناة وتؤثر على حياتهم اليومية .هذه الحقيقة وبالإضافة إلى انتشار الجرح الغير الملتئم بين مرضى السكري بنسبه (15)٪ مما يزيد من الحاجة إلى إيجاد طرق جديدة وأكثر كفاءة لعلاج الجرح السكري. في السنوات الأخيرة، اكتشف الباحثون أن استخدام مستخلص بذور الكتان لتسريع التئام الجروح .ان هذا المستخلص معروف بتأثيراته المضادة للالتهابات والأكسدة قد يحسن التئام الجروح في مريض السكري، و للضغط التأكسدي دور في التسبب في جروح السكري المزمن بشكل خاص. وبالرغم من ذلك فإن الدراسات التي تستخدم نماذج حيوانية مستحثة بداء السكري ومعالجة بمستخلص بذور الكتان نادرة .ان الهدف من هذه الدراسة هو معرفة التأثير العلاجي لبذور الكتان على التئام الجروح في الحيوانات المصابة بالسكري في فترات اليوم الرابع والسابع والرابع عشر .تنقسم هذه الدراسة إلى مرحلتين :مرحلة تحريض مرض السكري باستخدام مواد ستربتوزوتوسين ومرحلة العلاج بمستخلص بذور الكتان .تضمنت هذه الدراسة 54 من ذكور الأرانب النيوزيلندية البيضاء مقسمة إلى(مجموعة مصابة بمرض السكري)عدد = 27 و(مجموعات غير مصابة بالسكري)عدد = 27. كانت المرحلة الأولى من الدراسة هي تحريض مرض السكري بواسطة ستربتوزوتوسين لمجموعات مرضى السكري في 27 من ذكور الأرانب النيوزيلندية البيضاء .ثم المرحلة الثانية التي تنطبق على كل من الحيوانات المصابة بالسكري وغير المصابة بالسكري هي كما يلي ، تم تحريض 54 من ذكور الأرانب النيوزيلندية البيضاء بجرح بعمق لكل طبقات الجلد) بطول 2±18 مم (ثم تقسيمها إلى مجموعات معالجة ببذور الكتان)عدد=9 (حيوانات مصابة بداء السكري)عدد=9 (حيوانات غير مصابة بداء السكري .مجموعة التحكم الإيجابي) كريم فيوسدين2 ٪ عدد=9 (مجموعات مصابة بداء السكري)عدد=9 (مجموعات غير مصابة بالسكري وسلبية)غير معالجة عدد=9 (مصابة بداء السكري)عدد=9 (حيوانات غير مصابة بالسكري). تم رصد نتائج اكتشاف الجرح الإجمالي باستخدام كاميرا رقمية وبرنامج صور لقياس طول الجرح .أظهرت حيوانات المجموعة المصابة بمرض السكري التي عولجت ببذورالكتان حوافًا ناعمة متقاربة ومنتظمة للجرح مع أنسجة منظمة ذات ألوان زاهية موضوعة بالضبط فوق الجروح .على الرغم من أن جميع المجموعات المدروسة استغرقت نفس الأيام لإغلاق الجرح بالكامل، إلا أن كفاءة التئام الجروح المحسوبة من الوقت المستغرق لإغلاق الجرح الكامل كانت أعلى معنوياً ($p < 0.05$) للحيوانات المصابة بداء السكري المعالجة ببذور الكتان مقارنة بمجموعة التحكم .أظهر تقييم مرونة الجلد في المجموعة المعالجة ببذور الكتان غير المصابة بالسكري أعلى قيم لزوجة (VE) مع وجود اختلافات معنوية لمدة ثلاثة أسابيع . تم استخدام التحليل النسيجي لصبغة هيماتوكسيلين و ايوسين و مالوري ترايكروم لفحص آليات التئام الجروح .تم إجراء تقييم كيميائي مناعي *(VEGF)* و *(TGF-β)* مع التحليل الكيميائي الحيوي (ELISA) لتعبير البروتين *(MMP-2)* و *(PDGF-A)* و *(VEGF)* على عينات الدم وأنسجة الجرح المستأصلة في نقطة زمنية مختلفة تقابل في يوم الرابع، اليوم السابع، وبعد ذلك اليوم الرابع عشر من مرحلة التئام الجروح . تسارع التئام جروح المجموعة المعرضة لمستخلص بذور الكتان في البداية عن طريق(زيادة التكاثر الخلوي لخلايا الكيراتينية ، والأرومة الليفية ، والخلية البطانية) وتقليل الالتهاب عن طريق تعديل مسار إشارات البروتين في الحيوانات المصابة بداء السكري، عزز مستخلص بذور الكتان الشفاء عن طريق تقليل الضغط التأكسدي من خلال زيادة أنشطة مضادات الأكسدة الذاتية مثل نشاط مضادات الأكسدة لبذور الكتان، مصحوبًا بتنظيم أكبر للتعبير الجيني المؤيد للتليف *(TGF-β)* الذي يؤدي إلى تكوين الألياف وتكوين الأوعية الدموية في التئام الجروح وضوحا في مجموعات مكشوفة بذور الكتان . تُظهر هذه الدراسة أن مستخلص بذور الكتان منتج واعد ويمكن استخدامه لعلاج جروح مرضى السكري ، إما بمفرده أو مع ضمادات الجروح المتوافقة حيويًا والقابلة للتحلل . في الختام ، أثبتت النتائج التي تم الحصول عليها بنجاح أنه يمكن تطوير مستخلص بذور الكتان بشكل أكبر من أجل الحصول على ضمادات جروح جديدة وأكثر كفاءة لعلاج جروح مرضى السكري وحتى أنواع أخرى من الجروح .

APPROVAL PAGE

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Omar Abdul Jabbar

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LIST OF ABBREVIATIONS

-	Hyphen-minus
+	Plus sign
=	Equal sign
%	Percent sign
&	Ampersand
(	Left parenthesis
)	Right parenthesis
,	Comma
.	Full stop
/	Solidus
:	Colon
;	Semicolon
[	left square bracket
]	Right square bracket
<	Less-than sign
>	Greater-than sign
±	Plus-minus sign
°	Degree sign
µm	Micrometre
γδ.	Gamma delta T cells
αβ	Alfa-Beta
µl	Microliter
A.U.	Arbitrary unit
ADP	Adenosine 5’-diphosphate
AGEs	Advanced Glycation End Products
ALA	Alpha-linolenic acid
ATP	Adenosine triphosphate
BCAA	Branched-chain amino acids
bp	Base pairs
COX-2	Enzyme cyclooxygenase-2
CRP	C-reactive protein
CSF-1	Colony-stimulating factor 1
CTGF	Connective tissue growth factor
DNA	deoxyribonucleic acid
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECM	Extracellular matrix
EGF	Epidermal growth factors
ESA	α-eleostearic acid
FGF	Fibroblast growth factor
FTU	Fingertip Unit Measurement
GAGs	Glycosaminoglycans
GC-MS	Gas Chromatographic-Mass Spectrometry
GLUT2	Glucose transporter 2

GNG	Gluconeogenesis
α	Greek small letter alpha
β	Greek small letter beta
γ	Greek small letter gamma
HbA 1C	Hemoglobin A1c
HLA	Human Leukocyte Antigen
HPA	Hypothalamic-pituitary-adrenal
HPF	High power field
HUVEC	human umbilical vein endothelial cells
IACUC	Institutional Animal care and Use Committee
IDDM	Insulin-dependent diabetes mellitus
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry
IIUM	International University Islamic Malaysia
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IR	Insulin resistance
IREC	IIUM Research Ethics Committee
JNK	c-jun N-terminal kinase
K1-14	Keratin Proteins
Kg	Kilogram
LA	Linoleic acid
LDL	Low-density lipoprotein
LDL-c	Low density lipoprotein cholesterol
LPO	lipid peroxidation
LSO	Linseed oil
MAPK	Mitogen-activated protein kinase
MCP-1	Chemoattractant protein 1
MDA	Malondialdehyde
MMP2	Matrix metalloproteinase-2
MPa	Mega Pascal
MPO	Myeloperoxidase
MRSA	Methicillin-resistance Staphylococcus aureus
MUFA	Mono unsaturated fatty acids
n-3 FAED	n-3 fatty acid enriched diet
n-3 FAED	n-3 fatty acids enriched diet
NAD	Nicotinamide Adenine Dinucleotide
NAD+	Nicotinamide dinucleotide hydride
NAFLD	Non-alcoholic fatty liver disease
ND	NanoDrop
Ng	Nano gram
NIDDM	Non-insulin-dependent diabetes mellitus
nm	Nanometre
NMFs	natural moisturizing factors
NO	Nitric oxide
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGF-A	Platelet-derived growth factor

pg\ml	Picogram/millilitre
PI3K	Phosphoinositide-3-kinase
PMNL	Polymorphonuclear leucocyte
PPAR γ	peroxisome proliferator-activated receptor
PUFA	Poly unsaturated fatty acids
qRT PCR	Real-time quantitative RT-PCR
ROS	Reactive oxygen species
ROSs	Reactive oxygen species
rpm	Revolutions per minute
SCP	Single-camera computerized photogrammetry
SDG	Secoisolariciresinol diglycoside
SECO	Secoisolariciresinol
STZ	Streptozotocin
TCR	T Cell receptor
TG	Triglyceride
TGF-β	*Transforming growth factor beta*
TGF-β	Transforming growth factor-β
TIMPs	Tissue inhibitor of metalloproteinases
TNF-α	Tumor necrosis factor alpha
TZD	Thiazolidinedione
™	Trade mark sign
VE.	Viscoelasticity
VEGF	*Vascular endothelial growth factor*
VLDL	Very low density lipoprotein
w/v	Weight per 100 mL

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Cutaneous or skin wounds are injuries to the outermost protective barrier in which partial or full-thickness skin tissue is lost. This disruption of tissue integrity arising from various causes such as surgeries, traumas, burns, or arterial diseases and can result in either acute or chronic wounds. Wounds can compromise an individual's independence, working capacity, and self-image, which may eventually affect one's quality of life. Therefore, appropriate wound management is critical to achieve optimum healing of a wound.

Wound healing process is essentially a series of events that attempts to restore the injured tissue to a normal state, thus avoiding serious complications. It is one of the most complex biological processes in multicellular organisms and can be subdivided into four stages: haemostasis, inflammation, proliferation, and remodelling. Growth factors, cytokines, and chemokines play a key role in the signalling mechanisms to coordinate the healing process. The activation of cellular proliferation is crucial in the tissue repair and regeneration stage. This wound healing process is not only complex with diverse cellular and biochemical responses, but it is also fragile and susceptible to interruption or failure leading to the formation of chronic nonhealing wounds.

Globally, the incidence of wounds with different aetiologies was reported to be more than 149.5 million annually. Surgical wounds are the most common type of wound (73.6%), followed by burn wound (2.3%), and traumatic wound (1.1%) (Antonic, Mittermayr, Schaden, & Stojadinovic, 2011). Generally, most of the wounds have good outcomes. Nonetheless, some wounds fail to promptly progress through the expected