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HEALING PROPERTIES OF EPIDERMAL GROWTH FACTOR AND TOCOTRIENOL-RICH FRACTION FORMULATION IN DEEP PARTIAL-THICKNESS BURN WOUND MODEL

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By

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November 2017

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Burns are one of the most devastating injuries in the world, divided into superficial, partial-thickness (superficial or deep partial-thickness) and full-thickness burns. The current treatment of deep partial-thickness burns is mainly focused on preventing wound infections and less attention to the burn healing process. Therefore, it is imperative to develop medications aimed at promoting the stage of wound healing. It has been shown that epidermal growth factor (EGF) can promote the proliferation of various cells and it is known that tocotrienol-rich fraction (TRF) from palm oil has a strong antioxidant activity. Thus, a combination of EGF and TRF is included in the present formulation (EGF+TRF cream), which is expected to accelerate wound healing and inhibit oxidative stress in the burn. Deep partial-thickness burn wounds were produced on the dorsal part of Sprague-Dawley rats. Animals were then randomly divided into six groups: untreated, treated with Silverdin® cream, base cream, base cream with c% EGF, base cream with 3 % TRF or base cream with c% EGF and 3% TRF, respectively. Creams were applied once daily for 21 consecutive days. Digital images were captured daily for macroscopic evaluation. Six animals from each group were sacrificed on the 3rd, 7th, 11th, 14th and 21st day post-burn, harvesting skin tissues with the wound for histological, cellular, biochemical and gene expression analysis. Prior to the research of the EGF+TRF formulation in wound healing, a new apparatus was designed to create burn wounds. With this device, uniform deep partial-thickness burn was achieved with a contact temperature of 70°C, a fixed pressure of 300 g, and duration of 10 seconds. In addition, a novel histological scoring system was developed to make the evaluation process more systematic and reproducible. The chronological events in burn healing were initiated by the recruitment of neutrophils to the wound site on the 3rd

day post-burn followed by the up-regulation of tumor necrosis factor-α (*TNF-α*) expression and rapid synthesis of collagens to facilitate leukocytes adhesion and crust attachment. The EGF+TRF treatment preceded this process and on the 7th day post-burn, wound contraction accelerated and the dermal healing was sped up by the proliferation of myofibroblasts and augmented expression of *Collagen-1*. By down regulation of interleukin 6 (*IL-6*) and *TNF-α* expression on Day 11 post-burn, the EGF+TRF treatment expedited the wound contraction and entering the re-modelling phase and arriving epithelialization on Day 14. This formulation was capable of promoting the recovery of the dermis and attenuate the oxidative stress by down regulating the expression of *IL-6, TNF-α,* inducible nitric oxide synthase (*iNOS*), matrix metalloproteinase-2 (*MMP-2*), tissue inhibitor of metalloproteinase-2 (*TIMP-2*), transforming growth factor-β1 (*TGF-β1*), vascular endothelial growth factor-A (*VEGF-A*) and *Collagen-1*. Finally, by Day 21 post-burn, the treatment of EGF+TRF formulation could advance the restoration of the epidermis and dermis and cut down the oxidative stress by down regulating the expression of *IL-6* and *iNOS*. In conclusion, all results from the present study fully supported the beneficial application of EGF+TRF formulation in the treatment of deep-partial thickness burn, adhered to the mechanisms of burn healing process.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

CIRI PENYEMBUHAN FORMULASI FAKTOR PERTUMBUTHAN EPIDERMAL DAN FRAKSI KAYA TOKOTRIENOL DALAM MODEL LUKA KETEBALAN SEPARA TERBAKAR

Oleh

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Luka terbakar adalah satu kecederaan yang dahsyat di dunia terbahagi kepada kebakaran superfisial, ketebalan separa dan ketebalan penuh. Rawatan terkini untuk luka terbakar ketebalan separa lebih mengutamakan pencegahan jangkitan luka berbanding proses penyembuhan. Oleh itu, penemuan rawatan yang berfokus kepada penggalakan proses penyembuhan luka adalah sangat penting. Faktor pertumbuhan epidermis (EGF) telah terbukti dapat membantu proses percambahan sel serta, fraksi kaya tokotrienol (TRF) daripada sawit mengandungi 25% α-tokoferol dan 75 % tokotrienol yang mempunyai aktiviti antioksidan yang tinggi. Oleh itu, kombinasi EGF dan TRF yang digunakan dalam formulasi (krim EGF+TRF) dijangka dapat mempercepatkan penyembuhan luka dan menghalang tekanan oksidatif dalam luka terbakar. Luka terbakar ketebalan separa telah diwujudkan di bahagian dorsal tikus *Sprague-Dawley*. Tikus dibahagikan secara rawak kepada enam kumpulan: tanpa rawatan, rawatan dengan krim *Silverdin,* krim asas, krim asas dengan c% EGF, krim asas dengan 3% TRF atau krim asas dengan c% EGF dan 3% TRF. Krim diaplikasi setiap hari selama 21 hari berturut. Imej digital diambil setiap hari untuk penilaian makroskopik. Enam tikus dari setiap kumpulan dikorbankan pada hari ke-3, ke-7, ke-11, ke-14 dan ke-21 setelah dirangsang luka terbakar, lalu tisu diambil dari luka untuk analisis histologi, selular, biokimia dan ekspresi gen. Satu radas baru untuk mewujudkan luka terbakar juga telah direka. Arus yang stabil dan terkawal, tekanan tetap, suhu yang tepat dan kemudahan operasi yang selamat dan tepat sebagai teknik untuk mendapatkan luka ketebalan separa yang sekata dengan suhu 70°C, tekanan 300g selama 10 saat. Tambahan lagi, sistem penskoran histologi yang unik telah dicipta. Sistem ini memudahkan untuk memadankan skor histologi dengan paras

kesembuhan epidermis dan dermis, maka proses penilaian adalah lebih sistematik dan *boleh direproduksi.* Kronologi penyembuhan luka terbakar bermula dengan pengumpulan neutrofil di tempat luka pada hari ke-3 selepas induksi luka, kemudian *peningkatan* ekspresi *TNF-α* dan penghasilan kolagen yang pantas untuk membantu infiltrasi leukosit dan *lekatan kuping luka.* Rawatan EGF +TRF mendahului proses ini dan pengecutan luka terlihat lebih cepat pada hari ke-7 selepas induksi luka, diikuti oleh penyembuhan dermis yang lebih pantas disebabkan oleh percambahan miofibroblas dan peningkatan ekspresi *kolagen-1.* Dengan penurunan ekspresi *IL-6* dan *TNF-α* pada hari ke-11 selepas induksi luka, didapati rawatan EGF+TRF meningkatkan pengecutan luka dan memasuki proses pembentukkan semula lalu mencapai *epitialisasi* pada hari ke-14. Formulasi ini mampu meningkatkan penyembuhan dermis dan mengurangkan tekanan oksidatif dengan menurunkan ekspresi *IL-6, TNF-α*, *iNOS*, *MMP-2*, *TIMP-2*, *TGF-β1*, *VEGF-A* and *Collagen-1*. Akhir sekali, selepas hari ke-21 luka, rawatan formulasi EGF+TRF mampu mempercepatkan proses pemulihan epidermis dan dermis serta menurunkan tekanan oksidatif melalui *penurunan IL-6* dan *iNOS*. Sebagai kesimpulan, kesan formulasi EGF+TRF pada ketebalan separa luka terbakar yang dinilai di peringkat makroskopik, histologi, selular, biokimia dan ekspresi gen, hasil dapatan kajian menyokong manfaat aplikasi dalam penyembuhan luka terbakar seiring dengan mekanisma proses penyembuhan luka.

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Life is a journey; thank all of the people who have ever helped me in my life. You are the ones that make this journey wonderful.

I certify that a Thesis Examination Committee has met on 10 November 2017 to conduct the final examination of Guo Huifang on her thesis entitled "Healing Properties of Epidermal Growth Factor and Tocotrienol-Rich Fraction Formulation in Deep Partial-Thickness Burn Wound Model" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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Name of Member of Supervisory Committee: Dr. Roslida Binti Abd Hamid

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LIST OF ABBREVIATIONS

bFGF	Basic fibroblast growth factor
CC	Cysteine-cysteine
cDNA	Complementary deoxyribonucleic acid
CVIU	Chronic venous insufficiency ulcer
CXC	Cysteine-X amino acid-cysteine
DAG	Diacylglycerol
ECM	Extra cellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDP	Guanosine diphosphate
Grb-2	Growth factor receptor-bound protein 2
GTP	Guanosine-5'-triphosphate
H&E	Hematoxylin and eosin
H_2O_2	Hydroxyl peroxide
HER1	Human epidermal growth factor 1
HICs	High-income countries
IGF-1	Insulin-like growth factor-1
IL-10	Interleukin-10
IL-1α	Interleukin-1α

IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
IP3	Inositol-1，4，5-triphosphate
LDL	Low-density lipoprotein
LMICs	Low-to-middle-income countries
LMWP	Low-molecular-weight protamine
MAP	Mitogen-activated protein
MDA	Malondialdehyde
MMP	Matrix metalloproteinase
MMPIs	Matrix metalloproteinase inhibitors
MT-MMP	Membrane-type matrix metalloproteinase
NF-ĸB	Nuclear factor kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2	Nitrite
NO_3	Nitrate
NOS	Nitric oxide synthase
O2.-	Superoxide anion radical
OH.	Hydroxyl radical
ONOO-	Peroxynitrite
PBO-TRF	TRF from rice bran oil

PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PGK1	Phosphoglycerate kinase 1
PI-3	Phosphatidylinositol-3
PLC-γ	Phospholipase C-γ
PLGF	Placenta growth factor
PO-TRF	TRF from palm oil
Q-PCR	Quantitative real time polymerase chain reaction
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S.D.	Standard error
SOD	Superoxide dismultase
SOS	Son of sevenless
SSD	Silver sulfadiazine
T-cell	Thymus-derived cell
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor-α
TRF	Tocotrienol-rich fraction
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Burns are defined as skin lesions caused mainly by heat or other acute trauma including scalds, contact burns and flame burns. In the world, approximately 300,000 people die each year from burns (Peck, 2012). The incidence of burns in low- and middle- income countries is higher than in the developed countries (Atiyeh et al., 2009). Depending on the burn depth, burn patients may experience many mental and physical complications such as shock, trauma, infection, electrolyte imbalance and respiratory failure (Evers et al., 2010). In addition to physical complications, the patient may be physically and emotionally suffering from surgery and long-term hospitalization, and some may even have to endure physical scars for a lifetime (Grivna et al., 2014).

Burn injuries lead to different degrees of vascular damage (Shakespeare, 2001). Some blood vessels can be completely coagulated while others may be moderately restricted, leading to different tissue change. The local changes in burn wounds are divided into three zones. They are: i) zone of coagulation—the central point with maximum destruction; ii) zone of hyperemia—the outermost zone without any structural changes; iii) zone of stasis—the area between the zone of coagulation and hyperemia (Evers et al., 2010).

According to the depth of the injured tissue, burns are classified as superficial, partial-thickness and full-thickness burn (Frantz & Byers, 2011). As evidenced by histologic studies, burns are dynamic. In other words, the depth of the burn may further deepen over time. But usually it stabilizes within about 3 days post-burn (Pereira et al., 2012). Necrosis of the stasis area may be responsible for this progress.

Burn wound healing process is similar to the general wound healing, which is the interaction of several cellular and biochemical components of synchronized and macroscopic coordination including hemostasis, inflammation, proliferation and tissue re-modelling (Enoch & Leaper, 2008). In the hemostatic phase, the formation of blood clots prevents further blood loss and helps to attract the inflammatory cells into the wound area (Velnar et al., 2009). In the inflammatory phase, a variety of inflammatory cells accumulate in the wound site and remove bacteria, foreign particles and damaged tissue to protect the tissue from infection (Shakespeare, 2001). Then the wound

moves to the third stage, the proliferative phase, where new blood vessels are growing, fibroblasts are migrating into the wound site and producing matrix proteins and keratinocytes are proliferating, migrating and covering the wound. When the wound is closed, it is usually considered to have achieved maximum healing. At the same time, the re-modelling phase begins and may last from months to years. At this stage, the fibrous tissue laid in the wound site is modified. Any aberration in the process can lead to poor healing processes such as chronic wounds, or excessive healing including hypertrophic scars and keloids (Tuan & Nichter, 1998).

Many growth factors are involved in the wound healing process. Epidermal growth factor (EGF) is a small peptide with 53 amino acids (Değim et al., 2011). Since 1989, topical administration of EGF has been used to increase the wound healing process of various wounds and proved to be safe and beneficial (Berlanga-Acosta et al., 2009). EGF achieves its biological function by binding to specific receptors on the cell membrane (Süzük et al., 2011)

The vitamin E extracted from crude palm oil is mainly composed of a mixture of tocotrienols and some tocopherols and referred as tocotrienol-rich fraction (TRF) (Wu et al., 2008). It has been reported that palm oil TRF has a strong activity in anti-oxidation (Minhajuddin et al., 2005), prevention and treatment of cancer (Srivastava & Gupta, 2006) and reduction in serum cholesterol levels (Qureshi et al., 2002). In addition, it is reported that TRF is more effective than α-tocopherol in reducing oxidative damage in brain mitochondria (Kamat & Devasagayam, 1995) and inhibiting protein oxidation and lipid peroxidation in liver microsomes (Kamat et al., 1997). Although the effect of TRF has been shown in cellular biologic experiments, to the best of our knowledge, TRF studies in burn wound healing is limited, therefore we take the advantage of exploring the combination of EGF and TRF formulation for the treatment of deep partial-thickness burns.

1.2 Problem statement

Many topical medications have been used in the treatment of burns, such as antiseptic agents, antimicrobial agents and enzymatic debriding agents (Leon-Villapalos et al., 2008). Silver sulfadiazine (SSD) is an antibacterial agent and acts on the bacterial wall (Lee et al., 2005). Soon after its discovery, it became a standard local treatment for patients with partial-thickness burns (Jurjus et al., 2007). However, it has been suggested that the local application of the SSD is either no better or worse than the control dressing in preventing wound infection and promoting burn healing (Aziz et al., 2012). Moreover, the benefits of SSD treatment have gone beyond the risk (Miller et al., 2012). Therefore, it is necessary to explore more efficient treatments for burn wounds.

EGF with a small polypeptide of 53 amino acids is of interest in the present study. The use of topical application of growth factor therapy has assisted in minimizing the incidence of burn wound scar and long-term healing. In this study, the combination of EGF and TRF with potent antioxidant activity was used for the treatment of deep partial-thickness burn wounds. The mechanism for understanding these two elements involved in the healing process was elucidated.

1.3 Research objectives

1.3.1 General objective

To elucidate the effect and mechanism of EGF+TRF formulation in the healing process of deep partial-thickness burn in rat model

1.3.2 Specific objective

- To evaluate the effect of EGF+TRF formulation on the macroscopic and microscopic changes involved in the deep partial-thickness burn wound healing process
- To determine the effect of EGF+TRF formulation on oxidative stress involved in the deep partial-thickness burn wound healing process
- To elucidate the effect of EGF+TRF formulation on the wound healing-related genes involved in the deep partial-thickness burn wound healing process

1.4 Hypothesis

The EGF+TRF formulation has synergistic effects in treating the deep partial-thickness burn.

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LIST OF PUBLICATIONS

Hui-Fang Guo, Razana, M.L., Roslida, A.H., Asma, A.Z., & Huzwah, B. K. (2017). A new model for studying deep partial-thickness burns in rats. *Internal Journal of Burns and Trauma*, 7(6): 107-114.

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