SYNTHESIS AND CHARACTERIZATION OF NOVEL PALMITIC ACID CONJUGATED TETRAPEPTIDE AS A NEW ACTIVE MOLECULE FOR WOUND TREATMENT

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By

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Therapeutic drugs have generated a great interest in pharmaceutical field for their beneficial effects towards wound treatment. However, clinical applications of drugs as therapeutic agent such as antiseptic are limited due to high toxicity and side effects including irritation and itching caused by the drug host. Therefore, a new sequence of peptide was designed, characterized and explored to find out its potential application in wound treatment. A novel fatty acid conjugated tetrapeptide known as palmitic acid conjugated glycine-aspartic acid-proline-histidine (Palmitoyl-GDPH) was synthesized using Solid Phase Peptide Synthesis (SPPS) method. It was determined by High Performance Liquid Chromatography (HPLC) with high percentage purity of 98.6%. Screenings of its biological activities were done by *in vitro* studies using enzymatic and cell based assays. From the results, it was evident that Palmitoyl-GDPH gave higher percentage activity of collagen enzyme (80.00 ± 2.22%). Besides, the EC_{50} of Palmitoyl-GDPH towards nitric oxide (NO) scavenging effect was low (1.05 ± 0.10 mg/mL) which shows a good anti-inflammatory compound. This peptide was further evaluated on normal human dermal fibroblast (NHDF) cell. Accordingly, the percentage of cell viability remained above 90% throughout the Palmitoyl-GDPH treatment ($p<0.01$) and the cells did not showed cytotoxicity up to concentration of 100 µg/mL. Meanwhile, the cells were grown and showed proliferation in time-dependent manner (72 h). From the mimic wound *in vitro* study, the Palmitoyl-GDPH treated cells were significantly promoted with high percentage of NHDF migration from 32.10 ± 2.74% to 98.39 ± 2.79% and reached full gap closure faster at 100 µg/mL during 48 h treatment ($p<0.01$) compared to standard drug. Through a combination of cell proliferation and cell migration, Palmitoyl-GDPH was comparable to be a therapeutic agent for skin wound healing. The results from *in vivo* study revealed that Palmitoyl-GDPH treated wound displayed significantly 100% wound closure at day 18 with smooth and flat appearance

compared to non-treated and standard drug (tetracycline) groups as evident by macroscopical analysis. Histological examination of the wound treated by Palmitoyl-GDPH presented no scar, fewer inflammatory cells, more hair follicles, fibroblast and blood vessels, and also extensive collagen deposition that were equivalent to normal skin tissue group. The epidermis and dermis thickness of Palmitoyl-GDPH treated wound were 46.99 ± 3.49 μm and 983.52 ± 8.41 μm, respectively which were comparable to skin layer of intact skin tissue and thus represented good epithelialization progress. At the end of the experimental time, rats were sacrificed and blood samples were collected and tested for hematology and biochemistry changes. The results indicated that Palmitoyl-GDPH treated group had intermediate levels of red blood cells and platelet counts while the values showed no significant difference compared to normal rats ($p>0.05$). Overall from the blood tests, there were no significant difference ($p>0.05$) and no systemic adverse effects on the animals following wound treatment with Palmitoyl-GDPH. These findings revealed the potential of Palmitoyl-GDPH to be used as an effective therapeutic agent for wound treatment through reduced wound area, increased re-epithelialization, enhanced collagen deposition and diminished scar formation.

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

SINTESIS DAN PENCIRIAN ASID PALMITIK BERKONJUGAT TETRAPEPTIDA BAHARU SEBAGAI MOLEKUL AKTIF YANG BARU UNTUK RAWATAN LUKA

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Ubat-ubatan terapeutik telah menarik minat besar dalam bidang farmaseutikal untuk kesannya kepada rawatan luka. Walau bagaimanapun, aplikasi klinikal ubat-ubatan sebagai ejen terapeutik contohnya antiseptik adalah terhad kerana ketoksikan yang tinggi dan terdapat kesan sampingan termasuk keradangan dan kegatalan yang disebabkan oleh dadah tersebut. Oleh itu, satu rangkaian urutan peptida yang baru telah direka bentuk, disintesis dan diterokai untuk mengetahui potensi penggunaanya dalam rawatan luka. Satu asid lemak berkonjugat tetrapeptida yang dikenali sebagai asid palmitik berkonjugat asid glisin-asid aspartik-prolin-histidin (Palmitol-GDPH) telah disintesis menggunakan kaedah Sintesis Peptida Fasa Pepejal (SPSS). Ketulenan ditentukan dengan kromatografi cecair prestasi tinggi (HPLC) dan didapati peratusan ketulenan yang tinggi sebanyak 98.6%. Saringan kajian *in vitro* telah dilakukan atas beberapa aktiviti biologi menggunakan enzimatik dan sel. Keputusannya menunjukkan bahawa Palmitol-GDPH memberikan aktiviti peratusan kolagen yang tinggi iaitu 80.00 ± 2.22%. Di samping itu, kepekatan ubat yang memberi respon separuh maksimal bagi Palmitol-GDPH terhadap kesan pengoksidaan nitrik oksida (NO) lebih rendah (1.05 ± 0.10 mg/mL), yang menunjukkan ia adalah sebatian anti-radang yang lebih baik. Peptida ini dinilai lebih lanjut pada daya hidup sel normal fibroblast manusia (NHDF). Keputusannya menunjukkan peratusan daya hidup sel-sel kekal melebihi 90% sepanjang rawatan Palmitol-GDPH ($p<0.01$) dan sel-sel tidak menunjukkan ketoksikan sehingga kepekatan 100 µg/mL. Sementara itu, sel-sel tersebut hidup and menunjukkan percambahan dalam masa 72 jam. Dari kajian mimik *in vitro* luka, sel-sel yang dirawat Palmitol-GDPH telah menggalakkan penghijrahan sel-sel daripada 32.10 ± 2.74% kepada 98.39 ± 2.79% dan mencapai penutupan jurang luka lebih cepat pada 100 µg/mL selama 48 jam rawatan ($p<0.01$) berbanding dengan ubat standard. Melalui kombinasi percambahan dan penghijrahan sel, Palmitol-GDPH adalah setanding untuk menjadi agen terapeutik bagi penyembuhan luka kulit. Hasil dari kajian *in vivo*

menunjukkan bahawa luka yang dirawat Palmitol-GDPH menutup luka 100% secara ketara pada hari ke 18 dengan penampilan lancar dan rata berbanding dengan kumpulan tidak dirawat dan piawaian (tetracyclin) seperti yang terbukti dengan analisis makroskopik. Pemeriksaan histologi luka yang dirawat oleh Palmitol-GDPH tidak menunjukkan parut, sedikit sel radang, lebih banyak folikel rambut, fibroblast dan saluran darah, juga banyak pemendapan kolagen yang sama dengan kumpulan tisu kulit biasa. Ketebalan epidermis dan dermis bagi luka yang dirawat Palmitol-GDPH adalah masing-masing 46.99 ± 3.49 μm dan 983.52 ± 8.41 μm, yang boleh dibandingkan dengan lapisan kulit tisu yang sihat dan dengan ini mencatatkan kemajuan epitelialisasi yang baik. Pada akhir eksperimen, tikus telah dimatikan dan sampel darah dikumpulkan seterusnya dianalisis untuk perubahan hematologi dan biokimia. Keputusan menunjukkan bahawa kumpulan yang dirawat Palmitol-GDPH mempunyai tahap perantaraan sel-sel darah merah dan bilangan platelet manakala nilai menunjukkan tiada perbezaan yang ketara berbanding dengan tikus normal ($p>0.05$). Keseluruhannya dari ujian darah, tidak terdapat perbezaan yang ketara ($p>0.05$) dan tiada kesan buruk sistemik pada haiwan berikut rawatan luka dengan Palmitol-GDPH. Penemuan ini mendedahkan potensi Palmitol-GDPH yang mempunyai keupayaan untuk digunakan sebagai agen terapeutik bagi rawatan luka dengan pengurangan saiz luka, peningkatan epitelialisasi, pemendapan kolagen yang lebih tinggi dan juga pembentukan parut yang berkurang.

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LIST OF ABBREVIATIONS

AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
AU	Absorbance unit
AuNPs	Gold nanoparticles
AUP	Animal utilization protocol
ARF	Animal research facility
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
cm	Centimeter
CD	Circular dichroism
CFU	Colony-forming unit
CO_2	Carbon dioxide
Da	Dalton
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
DCM	Dichloromethane
DMF	Dimethylformamide
DIEA	N,N-Diisopropylethylamine
D_2O	Deuterated water
DPPH	2,2-diphenyl-1-picrylhydrazyl
DMEM	Dulbecco's Modified Eagle's MediuM
EGF	Epidermal growth factor
ECM	Extracellular matrix
EC_{50}	Half maximal effective concentration
EDTA	Ethylene diamine tetra-acetic acid
ESI-MS	Electrospray ionization mass spectrometry
FGF	Fibroblast growth factor
FTIR	Fourier transform infrared spectroscopy
FBS	Fetal bovine serum
h	hour
HPLC	High performance liquid chromatography
HCl	Hydrochloric acid
HPF	High power fields
Hb	Haemoglobin
HCTU	O-(6-chloro-1-hydrocibenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluoro phosphate
IC_{50}	Half maximal inhibitory concentration
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IACUC	Institutional animal care and use committee
IP	Intraperitoneal
m^2	Meter square
mm	Millimeter
mL	Millimeter
mg	Milligram
min	Minutes
MMP	Matrix metalloproteinase

MS	Mass spectrometry
MW	Molecular weight
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MCV	Mean red blood cell volume
MCHC	Mean corpuscular Hb concentration
NMR	Nuclear magnetic resonance
NHDF	Normal human dermal fibroblast
NMP	N-methyl-2-pyrrolidone
NO	Nitric oxide
nm	Nanometer
PBS	Phosphate-buffer saline
ppm	Parts per million
µg	Microgram
µm	Micrometer
µL	Microlitre
%	Percentage
KGF	Keratinocyte growth factor
PDGF	Platelet derived growth factor
ROS	Reactive oxygen spesies
RBC	Red blood cells
SPPS	Solid phase peptide synthesis
SOD	Superoxide dismutase
SD	Sprague dawley
SD	Standard deviation
sec	Second
TFA	Trifluoroacetic
TGF	Transforming growth factor
TIMP	Tissue inhibitors of metalloproteinase
TMS	Tetramethylsilane
TOCSY	Total correlation spectroscopy
TB	Total bilirubin
UV	Ultraviolet
v/v	Volume per volume
WHO	World Health Organization
WBC	White blood cells

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Globally, delayed wound healing is among the main problem worldwide and it represents a significant clinical and economic. Every year, thousands of patients suffered from different classes of epidermal skin damage commonly caused by burn, hot water or oil, flames and accidents. According to the World Health Organization (WHO), it is stated that over 30,000 deaths occurred per year due to burn forms and scalds. The wound healing process is either cutaneous or chronic, where it might indicate that there are treatments associated with significant limitations in the effectiveness of treating the diseases (Kamoun *et al.,* 2017).

In addition, it is estimated that 2.6 million Malaysian are diabetic, where 15.2% of our total population and these numbers have been projected to increase sharply over the years. The statistics of diabetes mellitus disease are of great concern because Malaysia is the country with the highest number of people with diabetes in Southeast Asia and ranked 10th in the world. The diabetic foot ulcer could affect 15 to 25% of diabetic patients and become one of the major problems for those chronic wounds (Singh *et al.,* 2005; Lam *et al.,* 2014). Therefore, it shows an important need to identify new and more effective therapeutic agents as a drug to treat wounds.

There are several current therapeutics approaches to support wound healing such as metal nanoparticles (Rajendran *et al*., 2018), scaffolds (Waghmare *et al.,* 2018), bioactive dressings (Schoukens, 2019), hydrogels (Zheng *et al*., 2018), polymeric nanostructures, growth factors (Park *et al*., 2017) and membranes (Miguel *et al.,* 2019). However, the efficacy of these therapeutics are limited by constrains of side effects, inefficient and costly (Li *et al.,* 2015). The new therapeutic agent that is applied to the wound is an interesting strategy and absolutely necessary to improve the efficacy of wound treatment.

Bioactive peptide with good stability, high activity and specificity has promising in the field of medicinal chemistry especially for wound treatment. It offers many advantages over other therapeutic drugs which includes multiple biological actions that appear to be health positive, building blocks of proteins therefore, it is no surprise that it produces collagen and cost effective (Pickart and Margolina, 2018; Veith *et al.,* 2018; Felician *et al.,* 2019). These advantages of little protein have the power to boost the skin cells and make them to produce more collagen, thus it is beneficial in wound healing. A short peptide also showed an increase in treatment efficacy such as peptide AES16-2M, which

comprises of REGRT sequence possess wound healing effect via promoting keratinocyte migration (Lee *et al.,* 2018).

Fatty acid conjugated peptide, along with its conjugation, is a compound from the reaction of amino acid residues with fatty acid. Conjugation is a new and popular concept in order to minimize long sequence and enhance the properties of peptide drug candidates (Lau and Dunn, 2018). These therapeutic agents are widely shown to be effective in wound healing in which fatty acids as chemical enhancers increase topical delivery throughout the epidermis (Kanikkannan *et al.,* 2000; Robinson *et al.,* 2005). With the advantages of fatty acid conjugation of peptides, a new short sequence can be designed and explored for its biological properties for wound treatment. Up to date, the therapeutic small peptide with less than five amino acid residues for wound treatment is still in its infancy and have not yet been fully exploited and published in clinical development.

1.2 New Sequence Designation of Peptide

1.2.1 Origin of Peptide

A new sequence of peptide was designed based on an active ingredient used in skin care products developed by Sederma which marketed as RIGIN. It is a tetrapeptide of four amino acids with sequence Gly-Glu-Pro-Arg and conjugated to palmitic acid at the *N*-terminal become Palmitoyl-GQPR. The ability of RIGIN to down-regulate interleukins-6 (ILs) thus can increased skin firmness, smoothness and elasticity (Zhang *et al.,* 2009). The previous *in vitro* study revealed that RIGIN was comparable to dehydroepiandrosterone (DHEA), a secretary product of the human adrenal gland which had proven to have therapeutics benefits on anti-aging, acceleration on wound healing and also reduction of ILs levels in inflamed cells (Allolio and Arlt, 2002; Mills *et al.,* 2005; Kim *et al.,* 2006). Therefore, RIGIN can be said to be bio-mimic DHEA since it has similar properties and both two actives were comparable.

1.2.2 Fatty Acid Conjugated Tetrapeptide Design

To begin with, designation a new tetrapeptide has been made based on the sequence of tetrapeptide in RIGIN (Palmitoyl-GQPR) with expected similar effects or even more potent than RIGIN. A novel sequence of palmitic acid conjugated Gly-Asp-Pro-His (Palmitoyl-GDPH) has been designed, synthesized and characterized. In the sequence of tetrapeptide, amino acid residues of glutamine (Q) and arginine (R) were replaced with aspartic acid (D) and histidine (H), respectively. Amino acid residue of D was reported to have a contribution in the formation of keratin in human skin since it is an important protein in epidermis to adhere cells to each other while amino acid residue of H helps in protecting skin from infection. Besides, both amino acid residues of

glycine (G) and proline (P) were stayed as previous study had reported that they played a special role for regeneration by enhancing collagen synthesis (De Paz-Lugo *et al.,* 2018).

Fatty acid-like molecule was selected because it containes both active amino and carboxyl groups. For the design of a shorter peptide with excellent activities, fatty acid was conjugated to the tetrapeptide while also enhancing peptide stability. In this study, palmitic acid was chosen to conjugate with GDPH tetrapeptide since it is the most potent chemical penetration enhancer for peptide derivative (Wang *et al.,* 2002; William and Barry, 2012). Conjugation of palmitic acid to tetrapeptide has the potential of being effective in term of delivery across the skin specifically in improving the skin penetration property (Robinson *et al.,* 2005). Therefore, the palmitic acid conjugated Gly-Asp-Pro-His tetrapeptide (Palmitoyl-GDPH) was then synthesized, analyzed and characterized. It is a novel approach long-acting strategy for the development of therapeutics peptides.

1.3 Problem Statement

In developing countries, the treatment of wounds such as skin ulcers and burns represent a major health burden and clinical challenges. Wound healing is a complex and interactive process that involves four stages which are hemostasis, inflammation, proliferation and remodeling (Goh *et al.,* 2016). Any complications and disturbance in these phases will affect the normal wound repair process, leading to chronic non-healing wounds. If the infected wound failed to be healed, the patient will suffer more trauma thus invoke high cost for therapy (Fonder *et al.,* 2008). Therefore, it is necessary to develop more efficacious and active compound to heal wounds as can improve healing process and to reduce of total treatment costs as well.

Current therapeutic drugs and delivery systems have been extensively investigated for wound healing (Li *et al.*, 2015; Ain *et al.,* 2018; Chin *et al.,* 2018). Nevertheless, common drugs such as antibiotic creams and antiseptics were limited in terms of healing time, itching, irritation, dryness and scar formation (Murphy and Evans, 2012). Therefore, it is generally preferred new therapeutic drug that aims to increase the efficacy while minimizing the side effects.

Peptides are considered to be an important category of active ingredient of new drugs. The studies of peptides with therapeutic efficacy have attracted their use in medicinal chemistry field (Del Gaudio *et al.,* 2015). The major challenge is to design peptide sequence with a better stability for appropriate applications. Hence, a biodegradable and biocompatible peptide should be proposed to enhance wound healing activity. It is important to find the right amino acid residues in designing peptide sequence with appropriate characteristics for wound treatment.

1.4 Scope of Study

This study concentrated on the conjugation of fatty acid to tetrapeptide for application of wound healing. The early stage was the synthesis of four amino acids sequence. The tetrapeptide was then conjugated with fatty acid at the *N*-terminal of the sequence. The characteristic and biological properties of the fatty acid conjugated tetrapeptide were determined by *in vitro* based assay. Thereafter, wound healing activity treated topically by fatty acid conjugated tetrapeptide was evaluated using Sprague Dawley rats. The biochemical molecules in blood after treatment were examined with respect to organs function (liver, kidney and pancreas).

1.5 Objectives of Study

The main objective of this research is to propose a novel peptide therapeutic agent for wound healing. Hence, the following objectives were targeted to assist in achieving main objective:

i. To synthesize and characterize the fatty acid conjugated tetrapeptide
ii. To determine its biological activities and cytotoxicity by *in vitro* based assay
iii. To evaluate wound healing activity of microstructures and histological analysis by *in vivo* study on rats
iv. To assess the toxicity effect of fatty acid conjugated tetrapeptide towards biochemistry profile of organs function in blood

1.6 Hypothesis of Study

Fatty acid conjugated tetrapeptide, Palmitoyl-GDPH demonstrates high purity and good stability at different temperatures. There are null hypothesis (H_o) and alternative hypothesis (H_a) as follows:

H_o : As results from *in vitro* studies, the Palmitoyl-GDPH has no significant different towards cell viability proliferation and migration of cells compared to untreated control.
H_a : As results from *in vitro* studies, the Palmitoyl-GDPH significantly supports cell viability proliferation and promotes the migration of cells compared to untreated control.
H_o : By conducting animal studies, the topical application of Palmitoyl-GDPH has no effect on wound healing properties.
H_a : By conducting animal studies, the topical application of Palmitoyl-GDPH reveal good wound healing properties.
H_o : The hematological parameters and biochemical markers have no adverse systemic effect on organs function after Palmitoyl-GDPH treatment.
H_a : The hematological parameters and biochemical markers have an adverse systemic effect on organs function after Palmitoyl-GDPH treatment.

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