Research proposal for Doctoral Degree Program

Research Title:

The effect of hydroxyproline in the accelerating healing of type 2 diabetic wounds in rats

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Major Applying for: Pharmacology/Pharmacognosy

Background

1-Theoretical significance

Diabetes mellitus (DM) is a complicated disease, which seriously affects the normal function of patients, including significantly delayed wound healing process [1]. Diabetic foot ulcer is an essential source of disease, with the rapid rise of the cost of health care system [2]. The incidence of foot ulcers is 4%-10% in the population diagnosed with diabetes, and the incidence rate is 1.0%-4.1% in the annual population. These ulcers are often infected, causing serious incidence rates, resulting in considerable economic cost, and the first step in lower limb amputation [3]. With the growing worldwide prevalence of diabetes, there has been a resultant increase in the incidence of diabetic foot ulcerations (DFUs), which frequently become infected, cause great morbidity, mortality, engender considerable financial costs, and are the usual first step to lower extremity amputation [4]. Diabetic foot ulcers represent a significant morbidity source, with rapidly intensifying costs to the health care system [5, 6].

Among the various complications of diabetes, neuropathy and vascular disease are the main two determinants of delayed wound healing. DFUs is the most notorious health problem in clinical practice, because most diabetic patients develop early diabetic neuropathy and loss of lower limb pain, which leads to an unknown wound until serious infection occurs [5, 7]. The poor prognosis is partly related to microvascular diseases, peripheral neuropathy, hemorheology changes, glycosylation and impaired angiogenesis. Among them, angiogenesis plays a crucial role in wound repair [7]. Although poor wound healing is a major complication in patients with diabetes, which may lead to morbidity or death, leukocyte-mediated inflammation and tissue ischemia caused by inadequate neovascularization are considered to be the main factors leading to delay wound healing [7]. Once diabetic wound healing is very difficult, it may be due to vascular ischemia and various metabolic abnormalities [8].

The biological process of wound healing goes through different stages, which are determined by their functions, such as hemostasis, inflammation, proliferation (angiogenesis), repair and epithelization. Angiogenesis is a complex, multi-step process, including endothelial cells from the parent vessel development, and then migration, proliferation and anastomosis with other vessels, leading to regeneration, development and homeostasis. The disorder of angiogenesis leads to many diseases, including neuropathy, retinopathy, chronic lung disease and arthritis[9].

Hyperglycemia is one of the most important pathogenic factors leading to delayed wound healing, which directly affects the function of neutrophils, such as chemotaxis, adhesion, phagocytosis, and bactericidal activity [10]. Contraction at the early stage of wound healing is necessary for the formation and re-epithelization of granulation tissue. Fibroblasts play an important role in wound contraction by activating collagen production. The appearance of fibroblasts in the wound is a characteristic of tissue contraction [10].

Deficiency of protein during wound healing may diminish new capillary development, fibroblast proliferation, collagen and proteoglycans synthesis, and remodeling and contraction of the wound and sometimes it may result as suppression of the immune system [69].

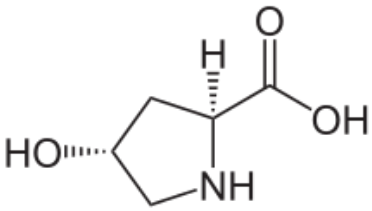
Collagen plays a crucial role in wound healing. Its biosynthesis is complex and involves many successive and interrelated steps. Fibroblasts are responsible for collagen synthesis in the wound region. The level of fibroblasts in the wound tissue can be taken as a gauge for wound healing [11]. Wound strength depends on collagen reinstallation and deposition of newly synthesized collagen fibers at the wound site. Both proline and hydroxyproline are essential for collagen biosynthesis, structure, and strength. Their cyclic structure restricts the rotation of the polypeptide collagen chain and creates and strengthens the helical characteristic of the molecule [12]. Hydroxyproline is an important constituent of the major structural protein, collagen, and play a key role in the synthesis and stability of the collagen.

Hydroxyproline is a nonproteinogenic and nonessential amino acid having two isomeric forms, i.e., trans-4-hydroxyL-proline and trans-3-hydroxy-L-proline.

Since the collagen is very important for wound healing, and hydroxyproline is essential for synthesis of collagen, we expected that hydroxyproline as a topical application may be a promise medicine for diabetic wound healing.

Breakdown of collagen releases free hydroxyproline and its peptides. Thus, hydroxyproline as a biochemical marker during wound healing is extensively used to evaluate the tissue collagen content and as an indicator for collagen turnover after wound-healing [70]. Increased hydroxyproline content in granulation tissue is the indicator of increased collagen turnover, which indicates better maturation and proliferation of collagen during wound healing [71]. There are numerous reasons to support the use of hydroxyproline as a biomarker of the collagen

content within tissues after the wound-healing process as it is abundantly found in collagen and play a vital role in wound healing[13].



Literature review and research progress

Impaired wound healing is a major complication of diabetes. Despite the associated risks, treatment strategies for diabetic wounds are still limited. This is partly due to the incomplete understanding of the underlying pathological mechanism [14]. Previous studies have reported various treatments, which have produced controversial results in wound healing in patients with diabetes [15]. Diabetes has been studied to inhibit angiogenesis [16].

On the other hand, systemic microvascular disease can reduce the nutritional supply of ulcer tissue, resulting in impaired healing of diabetic foot ulcers [16]. Therefore, angiogenesis is very important in diabetic patients. In 2014, a study found that diabetes is the main cause of wound healing due to long-term inflammation, angiogenesis defects, and decreased fibroblast proliferation[6].

Previous studies have shown that diabetic wound healing is related to many factors, such as hyperglycemia, angiogenesis, inflammation, etc. Previous studies have demonstrated whether C-peptide can activate the migration, proliferation, and tube formation of endothelial cells [17]. One study suggests that VEGF therapy may help to treat diabetic complications characterized by impaired neovascularization [18][. A study has shown that MLL1 plays an important role in regulating macrophage-mediated inflammation in wound healing and identifies a potential target for treatment of chronic inflammation in diabetic wounds [[19].

Hydroxyproline, the main constituent of collagen, serves as a marker of collagen biosynthesis at the wound site. Collagen not only provides strength and integrity to the tissue matrix, but also plays a vital role in homeostasis and in epithelialization at the later phase of healing. Augmented levels of hexosamine and uronic acid support the stabilization of collagen molecules by

accelerating electrostatic and ionic interactions (Siegel 1976). Collagen is a major protein in the extracellular matrix and is the essential component that ultimately contributes to wound tensile strength[20]. The synthesized collagen is laid down at the wound site and cross-linked to form fibers. Wound strength is acquired from both remodeling of collagen and the formation of stable intra and intermolecular cross-links [21][22].

The present investigation shows that the administration of pro leads to increased levels of enzymatic and nonenzymatic antioxidants in the granulation tissues, which might be due to the free radical scavenging capacity of proline. Proline plays a major role in protein synthesis and structure, metabolism, nutrition, wound healing, antioxidative reactions, and immune responses. Proline acts in accordance with arginine, glutamine, and leucine to enhance protein synthesis in cells and tissues [22].

The present investigation shows that the administration of pro leads to increased levels of enzymatic and non-enzymatic antioxidants in the granulation tissues, which might be due to the free radical scavenging capacity of proline. This antioxidant property of pro may explain why its concentrations increase markedly in response to cellular oxidative stress [23]. As L-glutamic acid (LG) is a direct precursor for proline synthesis, which is crucial for collagen synthesis, a previous study have prepared chitosan CS + LG hydrogels to accelerate diabetic wound healing. The results demonstrate that incorporation of LG can improve collagen deposition, and vascularization, and aid in faster tissue regeneration. Therefore, CS + LG hydrogels could be an effective wound dressing used to treat diabetic wounds [24].

The objective

The diabetic wound is a complicated disease and is difficult to be treated. There is still no specific drug for that. So in this study, we aim to use hydroxyproline to treat the diabetic wound in rats, we expected to have a good therapeutic effect.

Research methodology

1-Animals

Thirty healthy SD rats weighing (200±20g) will be purchased. All the animals will treat humanely according to the guidelines provided in the Guide for the Care and Use of Laboratory Animals, published by the National Institute of Health. All animals will house under standard conditions and will feed with a normal diet and water ad libitum.

2-Induction of type 2 diabetes mellitus (T2DM)

To induce T2DM, rats will feed a high-fat diet (HFD, 60% calories from fat and 70% animal fat) initially for five weeks followed by a single intraperitoneal (i.p.) injection of a low dose of streptozotocin (STZ, 30mg/kg, in citrate buffer pH 4.5). Five days later, fasting blood glucose and serum insulin levels will be measured. Hyperglycemic rats will be considered diabetic and will be used in the study after being acclimatized for one week. Blood samples will take from the tail of these rats to determine fasting blood glucose level. Plasma glucose levels more than 220 g/dl will be accepted diabetic and were included for the experiment.

3-Wound creation

The animals will be anesthetized by an intraperitoneal injection of chloral hydrate 10% (0.3/100g), or pentobarbital the dorsal surface of the rat will shave, and the underlying skin was cleaned with 70% ethanol. A 3x3 cm^2 open excision-type wound will be created using a scalpel blade to the depth of loose subcutaneous tissues. Incisions will be made under sterile conditions. Animals will be allowed to recover from anesthesia and will be housed individually in sterile cages.

4-Experimental design

Eighty rats will randomly be divided into four groups (n=10 per group) as the following:

Group I: is a normal control. The rats will be treated with phosphate-buffered saline (PBS), once daily.

Group II: is a diabetic control. The rats will be treated with phosphate-buffered saline (PBS), once daily.

Group III: Treated diabetic. The rats will be treated with PBS + Hydroxyproline orally, once daily, until complete healing.

Group IV: Treated diabetic. The rats will be treated with PBS + Hydroxyproline, topically once daily, until complete healing.

5-Measuring blood sugar

We will measure the blood sugar for diabetic rats every five days as the following table:

Day / Rat NO.	0 day	3rd day	7th day	14th day	21th day
I. Normal control					
1					
2					
3…….20					
II. Diabetic control					
1					
2					
3…….20					
III.Receive PBS+HYP orally					
1					
2					
3					
IV.Receive PBS+HYP topically					
1					

2					
3…….20					

6. Photographing, weighing, and measuring the wound area of the rats

Weighing the rats, photographing the wounds, and measuring the length and the width of the rats' wounds will be taken on 0, 4, 8, 12, 16, and 21 post-wounding days. The wound area will be determined planimetrically on these days, and percent wound contraction will be calculated by the formula as follows:

$$\% \text{ Wound contraction} = \frac{\text{0 day wound area - wound area on particular day}}{\text{0 day wound area}}$$

Day / Rat NO.	0 day				3rd day				7th day				14thday				21st day			
	Height	Width	Area	Wound	Height	Width	Area	Wound	Height	Width	Area	Wound	Height	Width	Area	Wound	Height	Width	Area	Wound
I.Normal control																				
1																				
2																				
3…….20																				
II.Diabetic control																				
1																				
2																				
3…….20																				

III.Receive PBS+HYP orally																				
1																				
2																				
3…….20																				
IV.Receive PBS+HYP topically																				
1																				
2																				
3…….20																				

The graph of percent wound closure versus time in days from wound creation will be plotted using the software GraphPad Prism v, and linear regression analysis will be performed.

7-Tissue harvesting

On days 0, 3rd, 7th, 14^{th,} and 21th the skin tissues are taken from the scar around the wound. The rats will be killed on the 21st day. Firstly, the rats will be anesthetic with ketamine (75mg/kg, i.p) and xylazine (10mg/kg i.p.), and the blood will be collected from the dorsal aortic vein. We will collect the liver, kidneys, spleen, pancreas, and granulation/healing skin tissue. The entire wound with a margin of approximately 5mm^2 of surrounding unwounded skin was excised. We will divide the skin tissue into three portions. One portion preserves in 10% neutral buffer formalin for histopathological evaluation. The second portion will put in an EP tube and store at - 20C until RNA extraction. The third portion will also put in EP tube and store at - 80c for Western blotting and enzyme-linked immunosorbent assay (ELISA).

8. Biochemical analysis

At the end of the experiment and fasting for one night, rats were anesthetized by chloral hydrate, and blood was collected from the dorsal aorta. After standing for 1 hour, it was centrifuged at 3000 r•min-1 for 15 min. The supernatant was stored at -20 °C. Rats were then sacrificed, and their liver and pancreas were excised immediately and perfused with ice-cold saline.

Serum biochemical parameters

8.1. Measurement of FBG

Fasting blood glucose was measured by the glucose oxidase method using a commercial diagnostic kit.

8.2. Determination of serum insulin content

Serum insulin content was determined by enzyme-linked immunosorbent assay (ELISA) kit using rat insulin as standard (Ultra Sensitive Rat Insulin ELISA kit).

8.3. Determination of serum c-peptide content

Serum c-peptide content was determined by enzyme-linked immunosorbent assay (ELISA) kit

8.4. Detection of blood lipids

Detection of blood lipids (including triglycerides, total cholesterol, low-density lipoprotein LDL, high-density lipoprotein HDL)

8.6 Assessment of serum TNF-α and IL-1β and IL-6

Serum tumor necrosis factor-alpha (TNF-α) and interleukin IL-1β levels were measured by commercially available rat ELISA kits.

8.7 Measurement of NF-_kB (p65) activity by (ELISA)
Nuclear factor NF-Kb (NF-_kB-p65) will be performed by ELISA. We will follow the instruction of the ELISA kit, which we will use.

9. Quantitative polymerase chain reaction (qPCR)
We will use the real-time RT-PCR to determine the mRNA expressions of VEGF, EGF, TGF-B1, SDF-1, IL-10，IL-1b, TNF-α，NF-kb, MMP-9，eNOS, and heme oxygenase-1 (HO-1).

The mRNA expressions of VEGF, EGF, TGF-B1, SDF-1, IL-10，IL-1b, TNF-α，NF-kb, MMP-9，eNOS, and heme oxygenase-1 (HO-1). RNA will be extracted from wound tissues and cDNA will be synthesized using cDNA synthesis kit (Takara, Japan). cDNA will be used as a template for the subsequent real time RT-PCR. The real time PCR assay will be performed by using SYBR Green Master Mix in 7300 Real Time PCR System. The qPCR experiment will be carried out according to the manufacturer's instruction.

9. Western blotting

The protein expression levels of VEGF, TGF- β 1, PDGF, MMP-9, collagen IV, HYP, EGF, and β-actin will be determined by Western analysis. Proteins will be extracted by grinding the tissues using mortar and pestle in liquid nitrogen. Proteins will be separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels, and then will be transferred to polyvinylidene difluoride (PVDF) membranes. The membranes will be then incubated with rabbit polyclonal antibodies against VEGF (1:1000), TGF- β1 (1:1000), PDGF, collagen IV, HYP, EGF, and MMP-9 for 12 h at 4°**C on shaker**. After washing, membranes, they will be incubated with fluorescence-labeled anti-rabbit immunoglobulin G (1:10000) for 1.5 h on the shaker and at room temperature. The blots will be subsequently scanned, and the band intensity was quantified by densitometry ImageJ software.

10.Haematoxylin and Eosin (H&E) staining

The paraffin-embedded skin tissues will be cut 5-lm-thick sections, deparaffinized in xylene, and rehydrated in graded concentrations of ethanol. We will place the sections on glass slides and stain them with hematoxylin-eosin (HE) according to the standard procedure. A laser scanning confocal microscope will be used to examine the sections, and images were recorded.

11. Immunohistochemical examination

The paraffin-embedded skin tissues will be cut 5 μm-thick sections, deparaffinized in xylene and rehydrated in graded concentrations of ethanol. Hydrogen peroxide (0.1%) will be used to inactivate the endogenous peroxidase activity in the skin sections. After blocking the sections with bovine serum for 30 min, we will incubate them with anti-EGF polyclonal antibody PCNA, eNOS, and CD31 (1:2000) for 12 h, then will be incubated with a secondary antibody for 1 h. Finally, we

counterstained the sections with hematoxylin. We will perform the microscopic observations using a laser scanning confocal microscope.

12. Picrosirius red staining

Estimation of collagen in wound sections will be done by staining with picrosirius red by modified picrosirius procedure[25]. Stained sections will be observed under polarized light (Leica DM2500P) and quantitative study of images for total collagen fraction will be done in eight random images (20x) from each group by using ImageJ software.

13. Statistical analysis

All data will be expressed as mean ± standard error. Data were analyzed by two-way analysis of variance (ANOVA), and then the posttest will be performed by Graphpad prism software. The difference between the two groups will be statistically significant ($P < 0.05$).

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