

EFFECT OF TCM *YINHUANGSAN* ON RAT'S DIABETIC ULCER HEALING MORPHOLOGY AND RECOVERY FACTORS

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Abstract: The research was done to study the role of the application of Traditional Chinese Medicines (TCM) external treatment, "*Yinhuangsan*", in the process of the wound tissue healing when it is used to intervene in the diabetic foot ulcers, and then to explore the mechanism of the rat diabetic ulcer healing. For the establishment of diabetic ulcer model, rats were randomly divided into control group, western medicine group, and TCM group, each consisted of 10 rats, respectively, corresponding to no drug, metronidazole and *Yinhuangsan* treatment. After 10 days of modeling, analyses were made to detect the contents of serum AGEs, inflammatory factor and growth factor, using proliferating cell nuclear antigen (PCNA) staining to detect the number of fibroblast in the wound granulation tissue, using CD34 staining to detect the number of new capillaries in granulation tissue and hematoxylin and eosin (HE) staining was used to observe morphological changes. Compared with the model group, in serum of rats, advanced glycosylation end products (AGEs) and tissue necrosis factor (TNF- α), interleukin-1 (IL-1), and C-reactive protein (CRP) were reduced after drug intervention, newborn capillaries, basic fibroblast growth factor (bFGF), endothelial growth factor (EGF), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF) were improved after drug intervention, newborn capillaries, fibroblasts were increased ($p < 0.05$) in granulation tissue, and TCM treatment group was more pronounced ($p < 0.05$) than western medicine treatment group. *Yinhuangsan* can significantly promote the ulcer healing in diabetes mellitus rats, because it can reduce inflammatory factor and improve growth factor in rat serum. Besides, *Yinhuangsan* can reduce AGEs in rat serum and promote new capillaries and fibroblasts proliferation in the granulation tissue.

Keywords: *Yinhuangsan*, diabetic ulcer healing, inflammatory factor, growth factor, AGEs

Diabetic foot is a complicated disease with a few theories of mechanism. In recent years, the lipid and glucose toxicity has become the research interest, especially the accumulation of AGEs (advanced glycosylation end products) on skin tissue. It has resulted in the draw of intense attention of researchers to histocytology (1). Research shows that high blood sugar and the accumulation of AGEs not only impair the capillaries and peripheral vessel, but also reduce the expression of multiple blood vessels and neurotrophic factors, which could be the important reason for the difficulty in diabetic foot ulcer. The healing process of diabetic foot ulcer is dynamic involving three continuous and overlapping stages during reparative process: inflammatory phase, granulation tissue forming phase and cicatrix remodeling phase (2).

In Traditional Chinese Medicines (TCM), the diabetic foot ulcer belongs to the field of gangrene and consumptive thirst. Most TCM practitioners believe that the pathophysiological mechanisms of this disease involve depletion of Qi and Yin, blood stasis blockage of the collaterals and exogenous damp and hot (3). In our opinion, the main causes of diabetic foot ulcer are damp, hot and toxicity stasis, and the TCM external treatment emphasis on clearing heat, detoxification and eliminating dampness.

Yinhuangsan is made up of golden cypress, lumbricus, dragon's blood and other traditional Chinese medicines (4, 5). Following the way of clearing heat, detoxification and eliminating dampness, together with importance of removing necrotic tissue and promoting granulation princi-

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ple, this medicine could be considered to promote wound healing and reduce the rate of amputation (6).

The aim of this study involves the assessment of cytobiological activity changes of AGEs, inflammatory factors (TNF- α , IL-1, hs-CRP) and growth factors (bFGF, EGF, VEGF, PDGF) in the wound healing process after applying *Yinhuangsan* to explore the related mechanisms of diabetic foot ulcer healing.

EXPERIMENTAL

Reagents and drugs

Streptozotocin (SZT, lot number: 20120728, CAS number: 18883-66-4) was purchased from Sigma-Aldrich, USA. Citric acid and sodium citrate were acquired from Beijing Chemical Reagent Factory (lot number 051024). Elisa kit (Shanghai Lianshuo Biotechnology Ltd.), IHC kit (Beijing Zhongshanjinqiao Biological Product Ltd.), PCNA detection kit (ZM-0213, lot number: 13132A10), and CD34 detection kit (ZM-0046, lot number: 12201110) were purchased through commercial sources.

Experimental animal and fodder

SPF grade, SD male rats having 80-100 g weight were purchased from CAMS, China and

were kept in iron cages (5 animals per cage) in controlled temperature conditions, i.e., approximately 37°C. Fodder (high fat and sugar) formula consisted of basal feed 67.5%, sucrose 20%, lard 10%, and yolk 2.5%. Water was provided *ad libitum*.

STZ solution preparation

Solutions A and B comprising of citric acid and sodium citrate, respectively, were made. Both solutions were mixed with each other to make buffer solution with A : B = 1 : 1.32 proportion. The STZ was precisely weighed and dissolved to yield 1% concentration in the buffer solution. Attention: STZ should be compounded in dark place and in ice-bath, and it is required to be consumed in 15 min. The formula of STZ injected dose: weight \times STZ presupposed dosage of administration/1000 = STZ dosage (mg) (7).

Drug preparation

Yinhuangsan was produced by Beijing Kangrentang pharmacy Ltd., with golden cypress, lumbricus, dragon's blood and other medicine smashed to fine powder (went beyond No. 6 grid-dle). The contrast drug was metronidazole glucose injection (Beijing Shuanghe pharmacy Ltd., lot number: listed product 1102161), used for comparative analysis of data obtained from Chinese herbal drug.

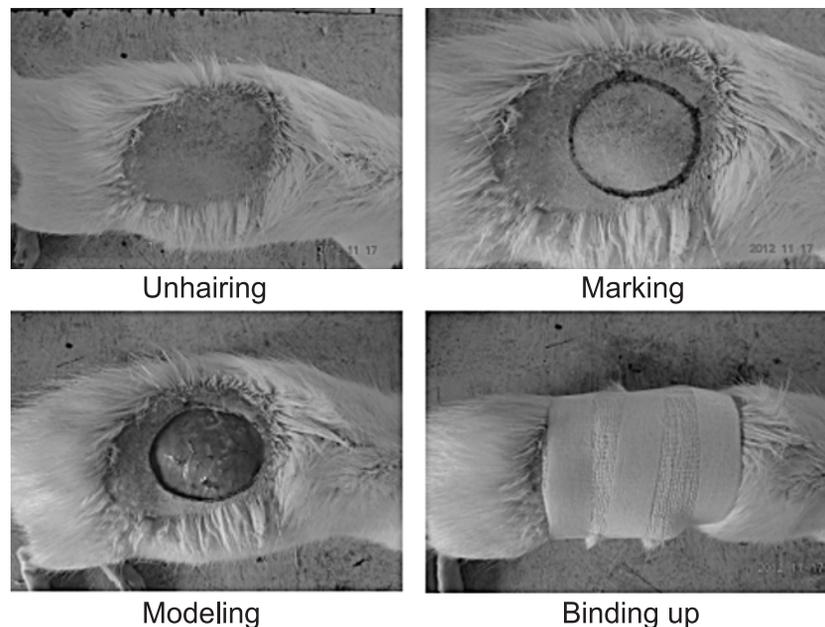


Figure 1. Diabetic ulcer rat model preparation

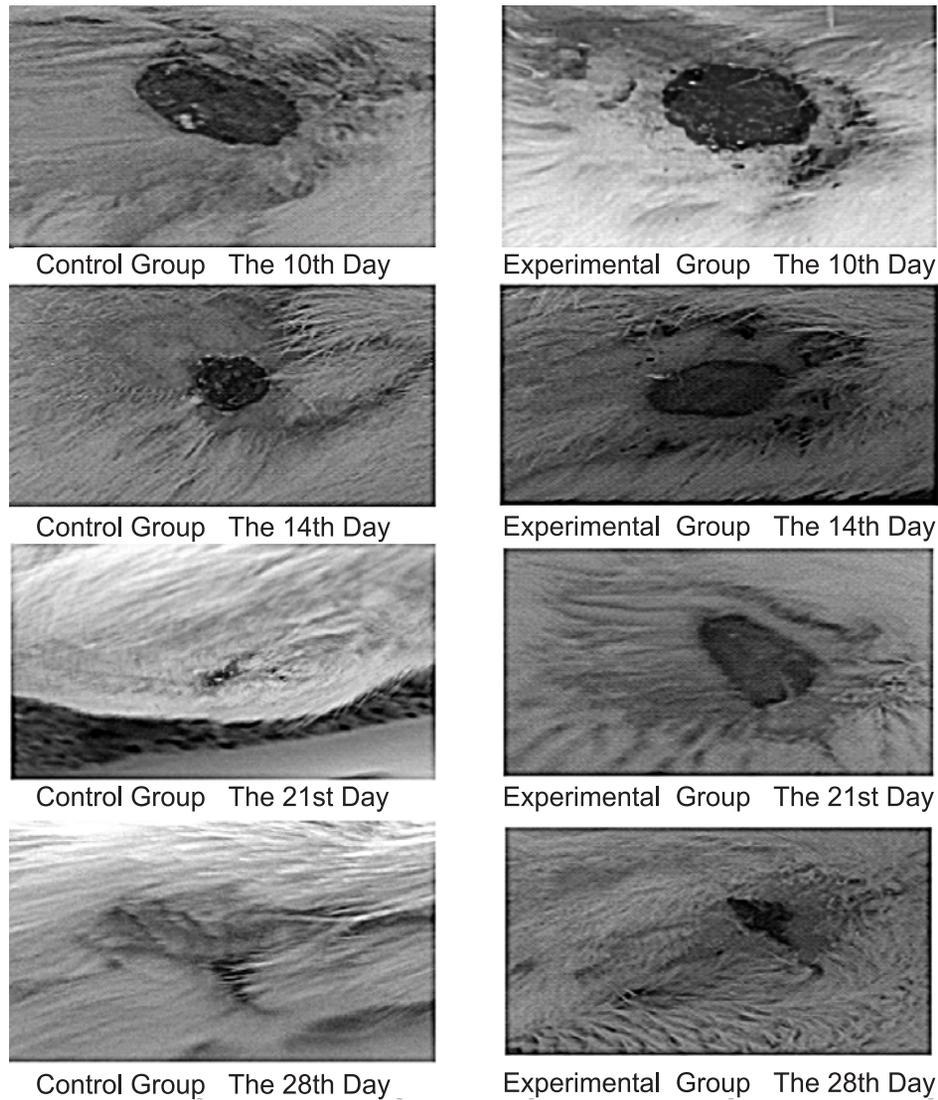


Figure 2. The wound condition of control and experimental groups

Method

Model preparation of diabetic ulcer rat

Sixty rats were chosen and fed with high fat and sugar fodder for a month. Then, STZ was injected in a quantity of 40 mg/kg body weight to their abdominal cavity to prepare diabetic rat model. To 30 model rats and 10 ordinary rats, 3.5% chloral hydrate was injected in a quantity of 1 mL/100 g to their abdominal cavity to induce narcosis. After removing long hair on their back with an electric razor, the molding area was marked with gentian violet and cut out the skin in the molding area deep

into the fascia under the aseptic condition (8). The wound area needs to be covered by 6 layers of medical gauze and be banded up and fixed with medical paper tape (Fig. 1).

Method of administration

Rats with diabetic ulcer were randomly divided into 3 groups: 10 rats in model control group, 10 in western medicine group, and 10 in Chinese medicine group. In addition, 10 normal SD rats were kept in blank group. Rats with wounds in the model control group and blank group were not treated since they act as positive and negative control group,

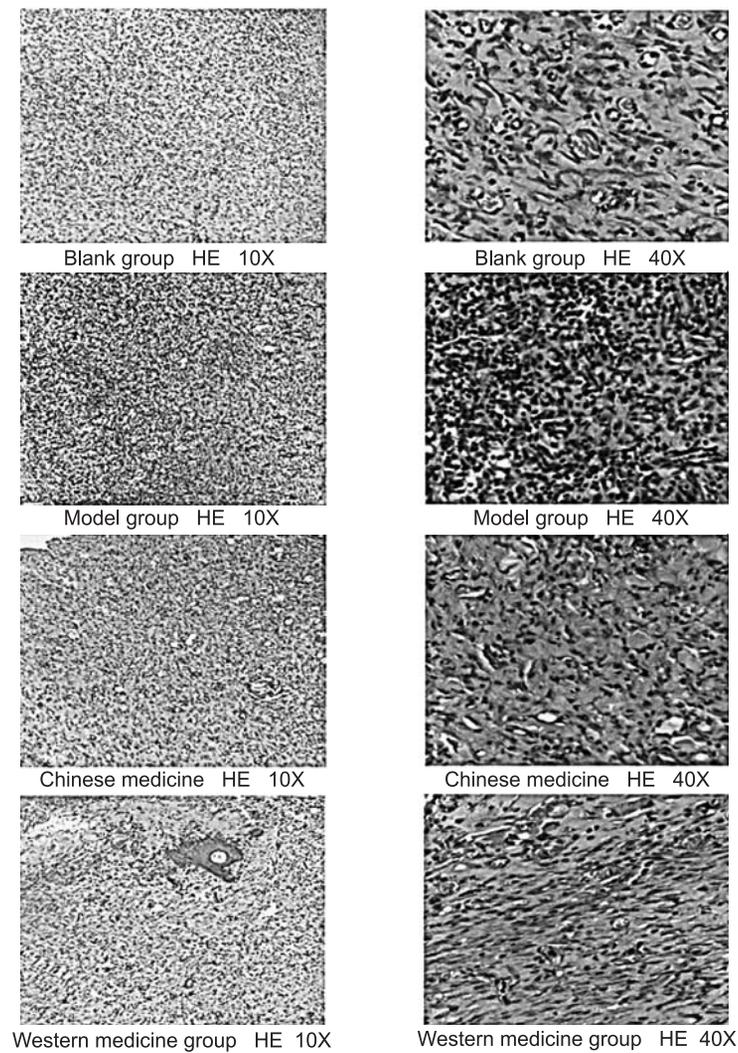


Figure 3. HE staining of paraffin sections of rat's granulation tissue

Table 1. Test of inflammation and growth factors.

Items	Blank group	Model control group	Western medicine group	Chinese medicine group
n→	10	10	10	10
TNF- α (ng/L)	17.51 \pm 0.39	21.71 \pm 1.17*	18.06 \pm 0.59 [#]	17.66 \pm 0.49 [#]
hs-CRP (ng/L)	0.33 \pm 0.03	0.56 \pm 0.08*	0.42 \pm 0.05 [#]	0.35 \pm 0.08 ^{#s}
IL-1 (ng/L)	4.99 \pm 0.23	5.68 \pm 0.43*	4.56 \pm 0.40 [#]	4.23 \pm 0.40 ^{#s}
bFGF (ng/mL)	3.04 \pm 0.35	2.20 \pm 0.33*	3.5 \pm 0.37 [#]	4.47 \pm 0.32 ^{#s}
EGF (ng/mL)	903.52 \pm 25.60	661.34 \pm 30.50*	862.35 \pm 33.94 [#]	968.15 \pm 30.02 ^{#s}
VEGF (pg/mL)	84.38 \pm 6.26	65.36 \pm 2.80*	84.76 \pm 2.86 [#]	95.74 \pm 3.02 ^{#s}
PDGF (ng/mL)	1.43 \pm 0.10	1.25 \pm 0.02*	1.47 \pm 0.09 [#]	1.64 \pm 0.04 ^{#s}
AGEs (ng/mL)	55.09 \pm 5.82	72.16 \pm 5.97*	54.84 \pm 6.01 [#]	46.96 \pm 2.16 ^{#s}

* Compared with blank group, $p < 0.01$, [#] compared with model control group, $p < 0.01$ and ^s compared with western medicine group, $p < 0.01$.

respectively. Rats in western medicine groups were applied externally gauze (4 × 4 cm) with metronidazole and glucose injection and bound up with non-woven medical tap once a day. Rats in Chinese medicine group were applied externally “Yinghuang Powder”, then covered with 6 layers of gauze (4 × 4 cm) and bound up with non-woven medical tap once a day (9). All those last 10 days to observe (Fig. 2).

Collection and test of serum sample

Blood from rats' abdominal aorta was collected and centrifuged for 10 min at the speed of 3000 rpm. Serum separated and extracted is moved into EP tube and preserved in fridge at -20°C. The contents of TNF- α , IL-1, hs-CRP, EGF, bFGF, VEGF, PDGF, and AGEs were tested by ELISA (enzyme-linked immunosorbent assay) (10).

Collection of granulation tissue and preparation of paraffin sections

Granulation tissue (about 5 g) was took from the center of wound, instilled neutral PA (paraformaldehyde) and shaped, washed in clear water, soaked, dehydrated, cleared, immersed in wax and sliced into 5 μ m sections (11).

HE staining

Paraffin sections were dewaxed by xylene, cleaned by deionized water, stained by hematoxylin, color separated by hydrochloric acid and alcohol, cleaned in water, stained by hematoxylin and eosin, dehydrated by alcohol, cleared by xylene and sealed with gum. Then, observation and comparing around the units of skin structure and functions (units of revived structure) started and multi-functional fluo-

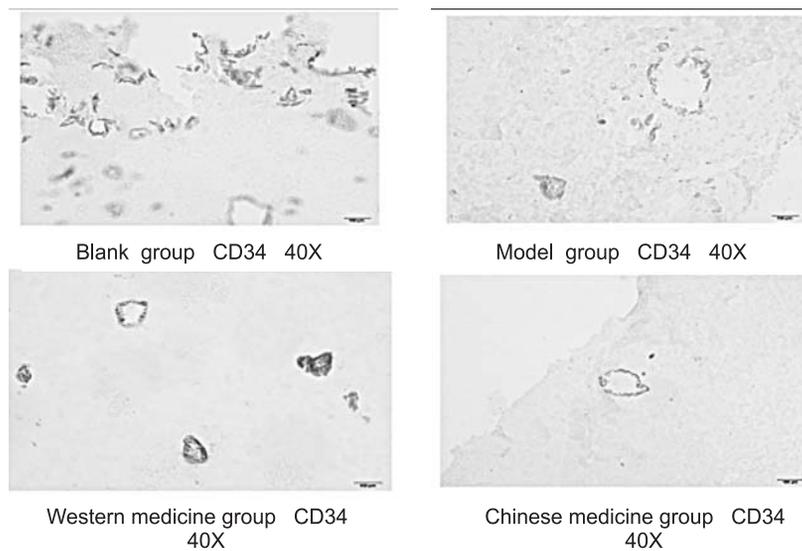


Figure 4. Immunohistochemical staining results of new blood capillaries of rat's granulation tissue

Table 2. Test of capillary density and fibroblast number.

Items	Blank group	Model control group	Western medicine group	Chinese medicine group
n→	10	10	10	10
New capillary density /10 ⁻⁵ / μ m ²	8.02 ± 0.48	4.47 ± 0.32*	6.56 ± 0.27 [#]	7.13 ± 0.17 ^{\$}
Fibroblast number per visual field	348.24 ± 6.12	157.19 ± 5.23*	259.46 ± 6.7 [#]	302.25 ± 4.15 ^{\$}

* Compared with blank group, $p < 0.01$, [#] compared with model control group, $p < 0.01$ and ^{\$} compared with western medicine group, $p < 0.01$.

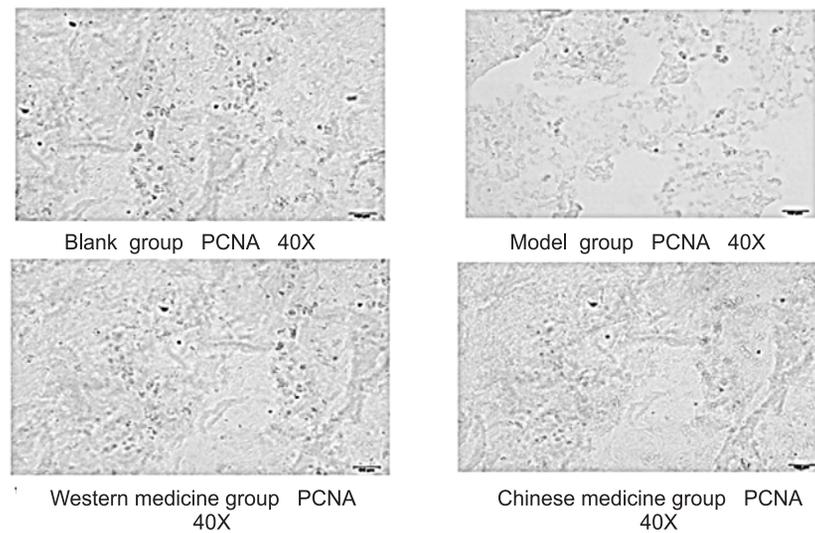


Figure 5. Immunohistochemical staining results of fibroblasts of rat's granulation tissue

rescence microscope (BX60, made by OLYMPUS, JAPAN) was used to observe the wound of tissue and pathomorphological characteristics of the surrounding tissue (12).

Number of fibroblasts in granulation tissue and capillary expression test with PCNA and CD34 staining

SP method of PCNA and CD34 immunohistochemistry staining include avoiding sections falling from glass slide by chemical reagent, dewaxing, inactivating endogenous enzymes, thermal remediation of antigen, dropwise adding PCNA (CD34) monoclonal antibody, DAB color developing, staining with hematoxylin, dehydrating, clearing, sealing sections and last microscopic observation (13).

Statistical method

SPSS version 19.0 is used to conduct ANOVA analysis of serological tests and pathological image analysis. Least significant difference (LSD) and Dunnett t-test were used for pairwise comparisons. In addition, $p < 0.05$ was set as significant difference.

RESULTS

Inflammation factors and growth factors

Compared with the blank group, TNF- α , IL-1, hs-CRP in the serum of rats in the model controlled group go up significantly, and bFGF, EGF, VEGF

and PDGF went down. During the advanced stage, AGES rises markedly and the difference is significant ($p < 0.01$). Compared with model control group, TNF- α , hs-CRP, IL-1 in the serum of rats in the western medicine and Chinese medicine groups went down markedly, bFGF, EGF, VEGF and PDGF went up markedly. During the advanced stage, AGES declines markedly and the difference is significant ($p < 0.01$). Compared with the western medicine group, TNF- α , IL-1 in the serum of rats in the Chinese medicine group went down clearly, the difference is significant ($p < 0.01$), FGF, EGF, PDGF, VEGF went up clearly, during the advanced stage, AGES declines clearly, while hs-CRP has no significant difference ($p > 0.05$) (Table 1).

Microscopic observation of paraffin sections of granulation tissue of rats and hematoxylin and eosin (HE) staining

There are a few of inflammatory cells, lots of fibroblasts, which are bigger cells with much cytoplasm, markedly colored nucleolus in the blank group, while the model group has more such inflammatory cells as neutrophils, lymphocytes and so on, less fibroblasts, some new capillary. In Chinese medicine group, there are a few of inflammatory cells, lots of fibroblasts, which are bigger cells with much cytoplasm, markedly colored nucleolus. The western medicine group has a few of inflammatory cells, more than the Chinese medicine group, less

fibroblasts than it, which are smaller cells, whose cytoplasm's protuberance was not obvious (Fig. 3).

Immunohistochemistry

Compared with the blank group, capillary density and fibroblast number in the model group are significantly ($p < 0.05$) decreased. Compared with the model group, the capillary density and fibroblast number in the Chinese and western group are significantly ($p < 0.05$) increased. Compared with the western medicine group, the capillary density and fibroblast number in the Chinese medicine group are significantly ($p < 0.05$) increased (Table 2 and Figs. 4, 5).

DISCUSSION

Influence of inflammatory factors on wound healing after diabetic ulcer

Hyper-sensitive C-reactive protein (hs-CRP) is a type of non-specific inflammatory marker, produced by liver induced by proinflammatory cytokines (including IL-1, TNF- α) derived from endotheliocytes, macrophagocytes and adipocytes (6). Hs-CRP is a type of acute inflammatory factor, which is sensitive to inflammation reaction. When the wound surface appears, the increased level of hs-CRP facilitates leukocyte chemotaxis and platelet aggregation, contributing to the wound's anti-infection and hemostasis. However, in this disease the continuous high level of hs-CRP would be one of the factors leading to wound ischemia, not good for wound healing. Therefore, reducing hs-CRP content would put inflammation under effective control, and lower the rates of wound ischemia (7). Tumor necrosis factor- α (TNF- α) is produced mostly by monocytes and macrophagocytes, also can be secreted by some inflammation cells. It has various biological effects, such as strengthening phagocytic ability of neutrophils, inhibiting production of tumor cell and acting on liver, increasing the production of hs-CRP. The increase of TNF- α content would also lead to such side effects as infectious shock and cachexy. Interleukin 1 (IL-1) is mainly produced by mastocytes and monocytes. It could also be generated by neutrophils, epithelial cells, endothelial cells, B/T lymphocytes and vascular smooth muscle cells. IL-1 is involved in all kinds of inflammation phenomenon, metabolism and cytothesis. Besides, as endocrine hormone, IL-1 could stimulate whole body's inflammation reaction, making engine body into the stress state, and regulating the cells in immune system (8), closely related to wound healing. A large number of IL-1 could induce liver acute phase protein synthesis,

triggering fever and cachexy, not good for wound healing.

Local inflammatory reaction would do certain harm to organization production, reducing the excessive synthesis and release of inflammation mediators such as hs-CRP/IL-1/TNF- α , helping to control inflammation reaction degree, and further helping wound healing.

According to this experiment's result, hs-CRP, TNF- α and IL-1 in rats' serum could decrease after medicine intervention, and in the Chinese medicine group the effect on decreasing TNF- α , IL-1 is obvious. Considering yinhuang powder, it could improve the inflammation condition of rats, which may be related to wound healing facilitation.

Impact of growth factors on wound healing after diabetic ulcer

Basic fibroblast growth factor (bFGF) is the major regulatory factor of tissue vascularization, which can promote the formation of new capillary. It is also the chemokinetic agent of fibroblast and a powerful growth stimulant. bFGF participates in several links during the process of wound healing. Previous studies have demonstrated that the expression of bFGF in local tissue of diabetic foot did not decrease (10). However, plenty of the bFGF lost their biological activity because of glycosylation. Therefore, wide glycosylation of bFGF protein without adequate new-born bFGF may be one of the essential causes of the difficult-to-heal diabetic foot ulcer wounds (10). Epidermal growth factor (EGF) is a polypeptide which can promote or restrain the growth of multiple kinds of cells. The combination of EGF receptor and EGF inside body produces receptor EGF compound. The compound regulates and controls important gene related to cell proliferation through a series of biochemical reactions, activates the process of wound healing and promotes the development of epithelial cells (11). In addition, EGF is beneficial to the proliferation and transportation of the vascular endothelial cell, as well as the formation of capillary. Vascular endothelial growth factor (VEGF) is a kind of active peptide which is secreted by macrophage, keratinocyte. It helps the proliferation of vascular endothelial cells and the combining of capillaries into bigger vessels. Moreover, VEGF can regulate the vascular endothelial cells proliferation and transport and promote the formation of new capillaries (11-15). During wound healing, together with FGF, VEGF stimulates endothelial cell growing and boosts the wound vascularization progress. Platelet derived growth factor (PDGF) is the serum growth factor which is released

by macrophage, keratinocyte, and stored in platelets. PDGF affects tissue repair cells, accelerates cells proliferation and boosts wound healing. In the advanced stage of wound healing, PDGF promotes the shrink of collagen matrix and condenses the wound. Besides, PDGF plays an important role in the remodeling stage.

In different processes of wound healing, growth factors play a role in various biological effects like chemotaxis, synthesis and secretion, proliferation and differentiation, apoptosis-inducing and angiogenesis, stimulating them in order to accelerate the wound healing. The imbalance of the types and content of endogenous growth factors of the wound, as well as the decline of the receptor activity, can lead to difficulty in wound healing (16).

According to this experiment's result, the level of bFGF, EGF, VEGF, PDGF in the rat serum can be improved after drug intervention. Yinhuang powder can remarkably enhance the level of bFGF, EGF, VEGF, PDGF in the rat serum, thus promoting the healing of diabetic ulcers.

Impact of AGEs on wound healing after diabetic ulcer

AGEs are irreversible end products. In normal bodies, amino acid and reducing sugar generate Amadori products that can produce highly active carbonyl compound through dehydration, oxidation and chemical rearrangement. Then, the compound reacts with free amino group and condenses into AGEs (16). Under the circumstances of continuous high sugar content, the aldehyde group of sugar and the amino of protein can generate AGEs. During glycosylation, the functional activity of growth factor declines, which influence the proliferation and transportation of repair cell. Glycosylation can lead to protein breakage. Glycosylation of sphingomyelin can cause the phagocyte to secrete protease, which results in demyelination disorders (14). Therefore, the accumulation and high sugar condition of AGEs can cause damage to capillaries and peripheral vessels, and cut down the expression of neurotrophic factor (2). It can be seen from the result of this experiment that drug intervention can reduce the amount of AGEs in the rat serum. Yinhuang powder plays a significant role in lowering the level of AGEs in the rat serum, thus promoting the healing of diabetic ulcers.

Angiogenesis and fibroblast

In the process of wound healing, the formation of granulation tissue, which has direct impact on wound healing and its prognosis, is very important.

The essence of granulation tissue is abundant fibroblast and new-born capillaries. New-born capillaries improve the microcirculation in the wound, accelerate the metabolism, provide rich nutrient and oxygen which is necessary in the process of tissue repairing, and promote the wound healing (17, 18). After skin injury, fibroblasts around the wound will be activated to start metaplasia. Stress fiber with contractility appears in cytoplasm and facilitates the process of wound healing

The result of this experiment shows that both metronidazole-glucose injection and yinhuang powder can promote the growth and proliferation of new-born capillaries. Yinhuang powder has the most significant effect on improving the number density of new-born granulation tissue. However, in terms of fibroblasts, both metronidazole-glucose injection and yinhuang powder can increase the amount of fibroblasts. Yinhuang powder also has the most remarkable influence on improving the number of fibroblasts. Yinhuang powder is beneficial to the proliferation of fibroblast and new-born capillaries and the growth of granulation tissue around the wound, therefore, accelerates the wound healing.

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