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Original Article

"Simmer pus and grow flesh" method promotes chronic wound healing in rats via bFGF-Wnt β-catenin signaling pathway

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Abstract

The purpose of this study was to explore the mechanism of "simmer pus and grow meat" method based on bFGF regulating WNT / β-Catenin signaling pathway. Of 100 SPF rats, 25 were randomly selected as blank group, and 75 rats were established chronic infectious wound model and divided into blank group, model group (normal saline treatment, n = 25), experimental group (purple and white ointment treatment, n = 25), and wet burn ointment group (wet burn treatment, n = 25). The wound healing rate of rats was compared. The protein expressions of PCAN, VEGF, bFGF, β-Catenin, GSK-3β and C-Myc in granulation tissues were detected. On the 7th day, the wound healing rate of the model group was lower than that of the other 3 groups (P<0.05), and the wound healing rate of the positive control group was higher than that of the experimental group and the control group (P<0.05). The expressions of bFGF, GSK-3β and C-MyC in model group were higher than those in control group (P<0.05). The β-catenin protein expression in the model group was lower than that in the control group (P<0.05), and the β-catenin protein expression in the experimental group and the positive control group was higher than that in the model group (P<0.05). The expressions of PCAN and VEGF in model group were lower than those in model group (P<0.05). We found that Zibai ointment promotes chronic wound healing by modulating the bFGF/Wnt/β-Catenin signaling pathway.

Keywords: bFGF/WNT/β-Catenin signaling pathway, Zibai ointment, Simmering pus and growing flesh, Wound healing.

1. Introduction

Chronic wounds refer to wounds that are difficult to heal or have not been completely healed for a long time due to various reasons [1]. The treatment of chronic wounds is an elaborate process, which usually requires comprehensive consideration of multiple factors and various treatment strategies. An unhealed wound may cause discomfort such as pain, infection, odor and exudation, which limits the patient's mobility and daily life function. Chronic wounds can also easily lead to various complications, such as infection, deep tissue injury, osteomyelitis and limited joint function [2]. In addition, chronic wounds not only have a negative impact on the body but also may have a negative influence on the mental health of invalid. Long-term wound treatment may make patients feel depressed, anxious and inferior. Therefore, it is of great meaning to study and apply the methods and strategies of chronic wound treatment to protect human health. Stewing pus and growing flesh are traditional Chinese medicine external treatment methods, which are often used to treat chronic wounds, ulcers and skin lesions that are difficult to heal [3]. It stimulates the wound, and promotes inflammatory reaction and wound repair, thus accelerating the healing process [4]. It is an ointment prepared from a variety of Chinese herbal medicines, which has the functions of diminishing inflammation, resisting bacteria, stopping bleeding and promoting wound healing [5, 6]. As a traditional Chinese medicine preparation, Zibai ointment has many mechanisms in wound healing: antibacterial and anti-inflammatory effects. Some components in Zibai ointment have antibacterial and anti-inflammatory effects, such as Arnebia euchroma and Angelica dahurica. They can restrain the growth of bacteria on the wound, reduce the risk of infection, and reduce the inflammatory reaction, which is helpful to the cleaning and repair of the wound [7]. The components of Zibai plaster include Radix Lithospermi, rhubarb, bletilla striata, and calcined gypsum, which can promote angiogenesis. Angiogenesis provides new blood vessels to supply the wound, increases the supply of oxygen and nutrients, and contributes to wound healing. Some components in Zibai ointment, such as Arnebia euchroma, are considered to promote the multiplication of epithelial cells [8]. This is very important for the epithelial metaplasia of wound surface and the contraction of wound edge, which is helpful for the closure and repair of wound surface. The use of Zibai ointment can form a protective covering and improve the microenvironment of the wound. It can keep the wound moist, provide a sui-

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table environment to promote cell growth and wound healing, and prevent dehydration and infection of the wound. Although Zibai ointment has some clear mechanisms in wound healing, its specific mechanisms need further study and verification. In addition, wound healing is a complex course, involving the interaction of multiple cell types and molecular signaling pathways. The role of Zibai ointment may be multifaceted, and further research is needed to fully understand its mechanism.

bFGF is a multifunctional growth element, which has been extensively studied and applied in the field of wound healing [9, 10]. bFGF can take part in the wound healing course by regulating Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling pathway plays a momentous role in the process of tissue development and regeneration [11]. The activation of this signal pathway involves the binding of Wnt protein and the activation of Frizzled receptor, which leads to the stabilization and nuclear translocation of β-catenin under normal circumstances, Wnt/β-catenin signaling pathway is maintained in a balanced state, which is involved in cell proliferation, differentiation and tissue repair [12]. However, when this signaling pathway is abnormally activated or inhibited, it will have a negative impact on wound healing. Studies have found that bFGF can regulate the Wnt/β-catenin signaling pathway, thus having a positive impact on wound healing. The target of this study was to explore the effect of bFGF in regulating Wnt/β-catenin signaling pathway in wound healing, and to evaluate the mechanism of Zibai ointment and simmering pus and growing flesh method in promoting chronic wound healing in rats.

2. Materials and methods

2.1. Experimental rats

100 four-month-old SPF rats (120-160 g) were obtained from the Institute of Zoology of the local Academy of Agricultural Sciences. SPF rats should be kept in SPF feeding room, with ambient temperature of 25±3°C and humidity of 40-70%. The 12-hour day and 12-hour night illumination period was realized by the automatic control system. During this period, rats can freely get food and clean drinking water and regularly monitor the health of SPF rats. Raising SPF rats needed to strictly abide by the ethical standards for raising and using experimental animals, and operate according to relevant regulations and guidelines.

2.2. Establishment of chronic infectious wound in rats

The rats were placed in SPF experimental animal facilities, and the rats were anesthetized by inhalation of isoflurane to ensure that the rats were in a painless state. Using a scalpel, the wounds were made in appropriate positions. Carefully remove a certain area (about 1.5 cm radius) of skin and soft tissue, and control the size and depth of the wound to simulate the chronic infectious wound. Prepare an appropriate infection solution (such as Staphylococcus aureus), and apply the infection solution directly to the wound surface to guarantee that the infection is in full get in touch with the wound surface. After inoculation, the pathological reaction and healing of the wound after treatment were observed and recorded. On the 7th and 14th day after infection, the wound surface was examined regularly, and the wound size was tested, and the swelling, exudation and inflammatory reaction of the wound surface were observed, and the healing situation of the wound surface was recorded, including the healing time, healing degree and histopathological characteristics.

2.3. Experimental grouping

According to the research plan, rats were separated into the following groups: the healthy cultured cells were separated into blank group (the rats in this group only made skin wound models, without other treatment, n=25), model group (the chronic infectious wound models on both sides of the back spine were established as mentioned above, and physiological saline was used as simulation control, n=25), experimental group (Zibai ointment was prepared in our hospital, and the drug quality was controlled by professionals in the preparation room, n=25), and positive control group (treated with moist burn ointment on rat wounds, n=25).

2.4. Methods

2.4.1. Statistics of wound healing rate in rats

On the 7th and 14th day of wound healing in rats, the length and width of the wound were tested with a ruler measuring tool. Ensure consistent methods and techniques for measuring each group of rats to reduce errors. Record the initial wound size and current wound size, and calculate the wound healing rate.

2.4.2. Western blotting

The homogenate of rat wound granulation tissue was placed in RIPA lysis buffer including protease inhibitor cocktail (Sigma-Al-Drich, St. Louis, MO, USA). Total protein was measured by BCA. The same amount of albumen was loaded on 12% SDS-PAGE. After electrophoresis, protein was imprinted on the polyethylene difluoride film. After blocking with a limit of 2 h, the blots were compared with those for bFGF, β-catenin, c-Myc and GAPDH. After incubating with primary antibodies, goat anti-rabbit was added. Imprint was developed with ECL Plus Kit (Amersham Biosciences) and visualized with Image Lab 5.1 software. Image J was used to obtain the gray value of the imprint for statistical analysis.

2.4.3. Immunohistochemical analysis

According to the experimental design and needs, tissue samples of rat wounds were collected through tissue sections. 4% paraformaldehyde and wax block were used for embedding treatment. The fixed wound tissue samples were dehydrated and buried to prepare wound tissue slices (thickness 5 microns). The wound tissue slices were dewaxed, and the paraffin was removed by soaking in xylene, an anti-wax agent. The dewaxed sections were treated with antigen repair to restore antigen expression in tissues. Antigen repair was carried out by heat treatment. Adding appropriate nonspecific binding inhibitor (bovine serum albumin) to block nonspecific binding. The diluted specific primary antibody (anti-PCAN or anti-VEGF antibody) was added to the wound tissue section, and the incubation time was appropriate to make the primary antibody specifically bind to the target protein. A suitable chromogenic substrate (such as DAB) was used for chromogenic reaction to form a visualized dyed product. The stained wound tissue sections were placed under a microscope, and the staining of PCAN and VEGF in the tissues was observed with appropriate magnification. Using image analysis

software or calculation methods, the stained wound tissue image was quantitatively analyzed to obtain the expression level data of PCAN and VEGF.

2.4.4. HE staining of wound tissue buds

Samples of rat wound tissue buds were collected, embedded with 10% buffered formalin and wax block, and the wound tissue sections were stained with HE to visualize the tissue cells and structure. Firstly, the wound tissue slices embedded in wax blocks were dewaxed to remove wax. Wash the dewaxed wound tissue slices with buffer solution to remove the dewaxing agent. The wound tissue sections were soaked in alkaline heme solution (Hematoxylin) to stain the nucleus and acidic Eosin solution (Eosin) to stain the cytoplasm. The stained wound tissue sections were dehydrated and sealed in alcohol solution with increasing concentration in turn. The stained and sealed wound tissue sections were placed under a microscope, and the morphological characteristics of the tissues were observed with 400 magnification. Through light microscope observation, we can evaluate the cell type, cell distribution, inflammation degree and angiogenesis of wound tissue.

2.5. Statistical analysis

All data in this study were processed using Statistic Package for Social Science (SPSS) 20.0 statistical analysis software (IBM, Armonk, NY, USA). The measurement data is represented by "mean ± standard deviation" ($\bar{x}\pm s$), inter-group comparisons are performed using one-way ANOVA or repeated measurement ANOVA, and inter-group pairwise comparisons are performed using LSD-t-test. The counting data is expressed as a percentage (%), and inter-group comparisons are made using χ^2 Analysis. $P<0.05$ represents a statistically significant difference.

3. Results

3.1. Analysis of wound healing rate

By linear measurement, the wound healing proportion of rats was observed on the 7th and 14th day after modeling. On the 7th day, the wound healing proportion of rats in the model group was lower than other three groups ($P<0.05$), while that of rats in the positive control group was higher than experimental and control group ($P<0.05$). On the 14th day, the wound healing rate of rats in the model group was lower than other three groups ($P<0.05$), and there was no distinction between the experimental group and the positive control group ($P>0.05$) (Figures 1 & 2 and Table)

3.2. HE staining

By HE staining and observing the pathological changes of the wound tissue under light microscope, the epidermis construction in the blank group was clear and flat, with sebaceous glands and other skin appendages visible, and no obvious inflammatory cell infiltration was found. The epidermal structure of the model group disappeared, a large number of inflammatory cells infiltrated, red blood cells extravasated in the dermis, and the collagen fiber structure was disordered and looser than control group ($P<0.05$). In the experimental group, the epidermal keratinization was incomplete, and the basal cell gap was loosely arranged. The epidermal layer was thickened, containing tissue fluid and blood sacs than model group ($P<0.05$), while in the positive group, the epidermal layer was thickened, and the collagen fibers were arranged in disorder. (Figure 3)

Table 1. Statistics of wound healing rate in rats (x±s).

Groups	7 d	14 d
Blank group	51.99±4.75	84.34±5.01
Model group	39.96±7.76	74.63±4.48
Experimental group	50.12±4.28	88.48±3.01
Positive control group	55.21±2.96	88.54±3.33
variance ratio	19.302	16.719
P value	0.014	0.002

3.3. Western blotting detection

After 7 days and 14 days of modeling, the albumen ex-

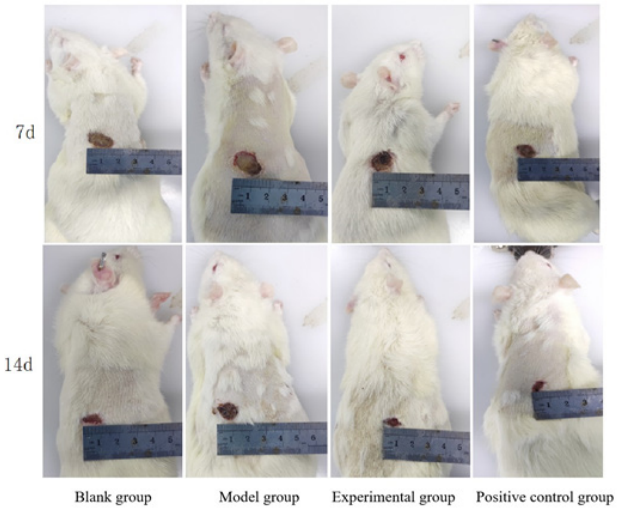

Fig. 1. Wound proportion in rats

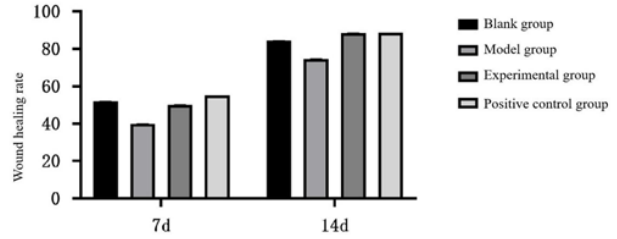

Fig. 2. Statistics of wound healing proportion in rats.

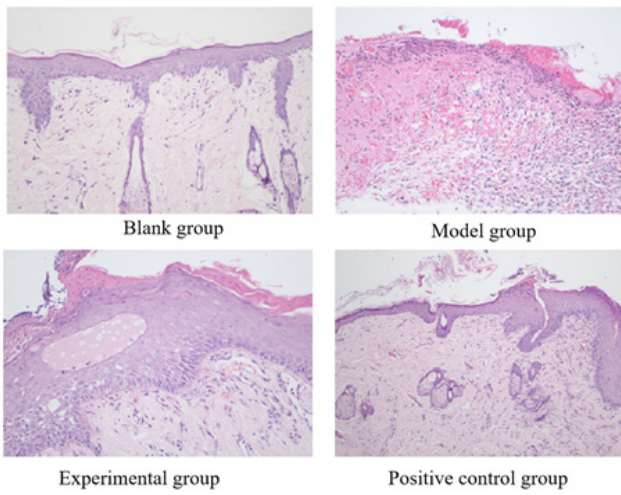

Fig. 3. HE staining analysis.

pressions of bFGF, β-catenin, GSK-3β and c-Myc in granulation tissue of wounds were tested by Western blotting. The expressions of bFGF, GSK-3β and c-Myc in the model group were higher than control group (P<0.05), while the expressions of bFGF, GSK-3β and c-Myc in the experimental group and positive control group were lower than model group (P<0.05). The expression of β-catenin in the model group was lower than control group (P<0.05), while the expression of β-catenin in the experimental group and the positive control group was higher than model group (P<0.05). (Figure 4 and 5)

3.4. Immunohistochemical analysis

The expression of PCAN and VEGF in granulation tissue of wounds in each group was analyzed by immunohistochemistry. The expression of PCAN and VEGF in the model group was lower than control group (P<0.05), while the expression of PCAN and VEGF in the experimental group and positive control group was higher than model group (P<0.05). (Figure 6)

4. Discussion

Zibai ointment is a traditional Chinese medicine preparation widely used for hemorrhoids or wound treatment after hemorrhoid surgery. Its mechanism of action involves many aspects. First of all, the components in Zibai ointment have antibacterial and anti-inflammatory effects, such as Radix Arnebiae and Radix Angelicae Dahuricae, which can reduce the infection and inflammatory reaction of the wound. Secondly, the components in Zibai ointment can promote angiogenesis, such as Gleditsia sinensis, which is very important for blood supply and nutrition supply of wounds. In addition, Zibai ointment can also advance the multiplication and migration of epithelial cells, which is helpful in the closure and repair of wounds. In addition, Zibai ointment can improve the microenvironment of the wound, keep the wound moist and provide an environment conducive to cell growth and wound healing. And bFGF, as an important growth factor, plays a momentous effect in wound healing [13-15]. The wnt/β-catenin signaling pathway has multiple functions in wound healing, including promoting epithelial cell proliferation and migration, promoting angiogenesis, and regulating inflammatory response [16, 17]. Specifically, bFGF may advance the multiplication of epithelial cells and accelerate wound closure by activating Wnt/β-catenin signaling pathway [18]. At the same time, bFGF may regulate Wnt/β-catenin signal pathway, promote angiogenesis and improve blood supply of wound surface, which is beneficial to nutrient supply and oxygen delivery during wound healing. In addition, bFGF regulating Wnt/β-catenin signaling pathway may adjust inflammatory reactions, reduce the degree of wound inflammation and contribute to the smooth progress of wound healing [18,19]. In addition, the method of simmering pus and growing meat is a traditional Chinese drug treatment method, which is widely used in chronic wound healing. Its basic principle is to stimulate the wound by simmering pus, and thus speed up the wound healing. The system of simmering pus and growing meat may promote wound healing through the following mechanisms. First of all, pus-simmering stimulation can raise the blood flow of the wound, and raise the nutrient supply and oxygen delivery of the wound, which is conducive to the healing of the wound. Secondly, the stimulation of simmering pus may promote the shape of new granulation tissue and the closure of the wound by activating the cell proliferation of the wound. In addition, the stimulation of simmering pus may advance the inflammatory reaction of the wound, remove necrotic tissue and pathogenic microorganisms,

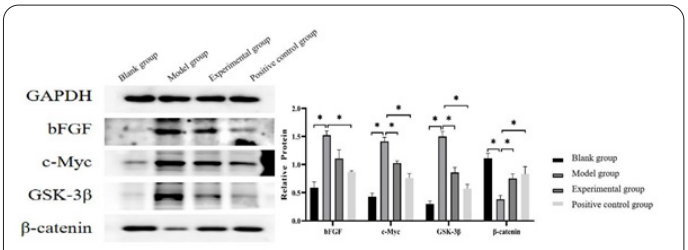

Fig. 4. 7 d Western blotting detection of granulation tissue in rat wound.

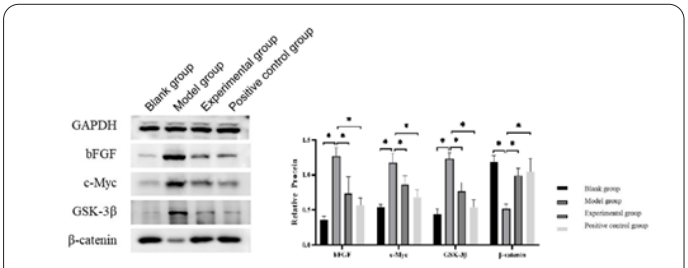

Fig. 5. Western blotting detection of granulation tissue in rat wound on 14th day.

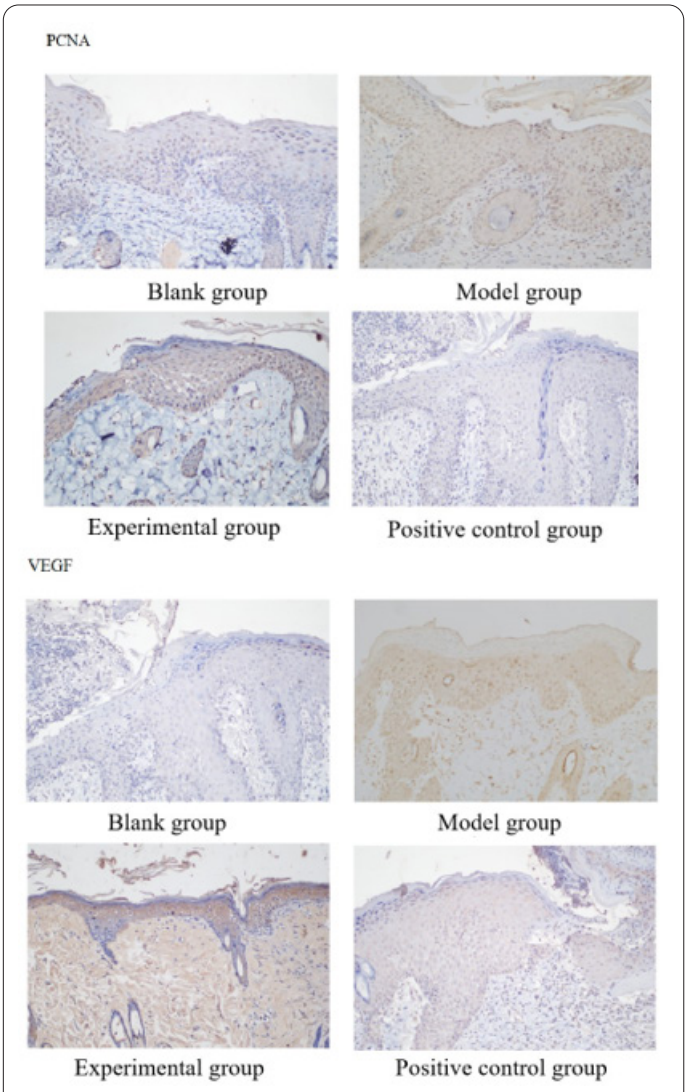

Fig. 6. Immunohistochemical analysis of PCAN and VEGF expression.

and provide a clean environment for wound healing. These mechanisms work together, which may make the method of simmering pus and growing meat an effective treatment to advance the healing of chronic wounds.

This study aimed to research the mechanism of Zibai Ointment on chronic wound healing in rats by regulating Wnt/β-catenin signaling pathway based on bFGF. Rats with chronic infectious wounds were treated differently, and the wound healing proportion and the expression of related signal pathways and biomarkers were evaluated by linear measurement, histopathological observation, Western blotting and immunohistochemical analysis. The results found that the wound healing proportion of the model group was lower than other three groups, indicating that the chronic infectious wound surface had an inhibitory effect on the healing process. However, in the positive control group, Zibai ointment was used, and the wound healing rate was higher than experimental group and the control group, which indicated that Zibai ointment might have the effect of promoting wound healing. The results of histopathological observation showed that the epidermis structure disappeared, inflammatory cells infiltrated, dermal red blood cells extravasated and collagen fiber structure was loose in the model group, which was obviously abnormal in control group. However, in the experimental group, the epidermis of the wound was thickened, containing tissue fluid and blood sac, which indicated that the method of "simmering pus and growing meat" based on bFGF regulating Wnt/β-catenin signaling pathway may advance epithelial cell multiplication in the process of wound repair. In addition, the positive control group also showed the disorder of collagen fiber arrangement and the formation of skin appendage structure, which further supported the positive role of Zibai ointment in wound repair.

At the molecular level, the expressions of bFGF, GSK-3β and c-Myc protein in wound granulation tissue in model group were higher than control group, while the expression of β-catenin was lower. This indicated that chronic infectious wounds may lead to abnormal activation of Wnt/β-catenin signaling pathway and affect the healing process of wounds [19]. However, the expressions of bFGF, GSK-3β and c-Myc protein in experimental group and positive control group were lower than model group, while the expression of β-catenin was significantly higher. This suggested that the method of "simmering pus and growing flesh" based on bFGF regulating Wnt/β-catenin signaling pathway may promote wound healing by inhibiting the abnormal activation of Wnt/β-catenin signaling pathway. PCAN and VEGF play a momentous effect in wound healing in rats. PCAN is a kind of proteoglycan, that exists widely in extracellular matrix and participates in the regulation of the function of extracellular matrix. PCAN plays a momentous effect in the process of wound healing. It can provide support and structural support, and provide a platform for cells to adhere and migrate. In addition, PCAN can also interact with growth factors and cytokines to regulate their activity and release. VEGF is a kind of protein promoting angiogenesis, which plays a momentous role in wound healing. VEGF can advance the proliferation and migration of vascular endothelial cells. In wound healing, the formation of new blood vessels is very important to offer oxygen and nutrients for the wound. VEGF can also increase vascular permeability, enable immune cells and other healing-related cells to enter the wound surface and advance the process of inflammation and repair. The interaction between PCAN and VEGF goes hand in hand the wound healing in rats. PCAN can bind VEGF and regulate its activity, thus affecting the angiogenesis process. In addition, PCAN can also provide a matrix for cell adhesion and promote the signal transduction and function of VEGF. Therefore, the synergistic effect of PCAN and VEGF is very momentous for the normal progress of wound healing. In a word, PCAN and VEGF play a momentous effect in wound healing in rats. PCAN provides a structural scaffold for extracellular matrix and regulates the activity of growth factors, while VEGF promotes angiogenesis and cell migration. Their interaction is very important for the success and recovery of wound healing. In this study, the expression of PCAN and VEGF in experimental group and positive control group increased, while the expression in model group decreased. This indicated that Zibai Ointment's "simmering pus and growing flesh" method based on bFGF regulating Wnt/β-catenin signaling pathway may improve local blood supply and wound structure, and then advance wound healing by promoting angiogenesis.

Firstly, bFGF is an important growth factor, which can advance wound healing. bFGF can stimulate cell multiplication and migration, promote angiogenesis, increase collagen deposition and promote epithelial cell regeneration [20]. These processes are very important for the healing of chronic wounds. bFGF may further enhance its effect of promoting wound healing by activating Wnt/β-catenin signaling pathway [21, 22]. By regulating the activity of Wnt/β-catenin signaling pathway, bFGF may enhance the vitality of cell proliferation and migration and promote the regeneration and angiogenesis of wound epithelial cells [23, 24]. Secondly, the active ingredients in Zibai ointment may directly or indirectly regulate the signal pathway of bFGF/Wnt/β-catenin, thus promoting the healing of chronic wounds. Zibai ointment contains a variety of medicinal components, such as Arnebia euchroma and Fraxinus chinensis, which have anti-inflammatory, antibacterial, repercussive and tissue regeneration-promoting effects. These active components may affect the bFGF and Wnt/β-catenin signaling pathways through many ways. For example, some components in Zibai ointment may promote the expression and release of bFGF, or directly act on the key molecules of Wnt/β-catenin signaling pathway, thus enhancing the activity of signaling pathway. These effects may be achieved by influencing cell signal transduction, gene expression and protein activity. Finally, Zibai ointment can accelerate the repair process of chronic wounds by promoting wound healing in many aspects. Zibai ointment can advance the multiplication of wound epithelial cells, which is helpful in the sealing and epithelization of wounds. It can also advance the formation of new blood vessels and nutrients needed by the wound. In addition, Zibai ointment also has anti-inflammatory and antibacterial effects, which can reduce the inflammatory reaction of the wound and prevent the occurrence of infection. These comprehensive mechanisms make Zibai ointment an effective drug for treating chronic wounds.

To sum up, this study proves that Zibai ointment has an important mechanism for advancing the healing of chronic wounds by regulating the signal pathway of bFGF/Wnt/β-catenin. It can accelerate the process of wound repair by increasing cell proliferation and migration, promoting

epithelial cell regeneration, enhancing angiogenesis and anti-inflammatory and antibacterial effects. As traditional Chinese medicine treatment methods, Zibai ointment and the method of simmering pus and growing meat promote wound healing through various mechanisms, which have potential clinical application value. However, further research is needed to verify these mechanisms and explore more possible therapeutic approaches and drug targets to improve the effect and speed of wound healing.

Conflict of interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
This study was approved by the Animal Ethics Committee of Fujian University of Chinese Medicine Animal Center.

Informed consent
The authors declare not to use any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions
Zhenpeng Xu, Erwei Cai: Conceptualization, methodology, writing original draft preparation. Suyuan Shan, Cheng Zhao, Feng Lin: Investigation, software, statistical analysis. Yanyan Wu: Reviewing and editing, funding acquisition, supervision. All authors read and approved the final manuscript.

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