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Study on the Effect and Mechanism of Dan Huang San on the Healing of Ulcer Wounds in Diabetic Rats

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Abstract

Aims of the study: To investigate the possible mechanism of action of Dan Huang San in promoting the healing of diabetic infected ulcer wounds using network pharmacology and animal experiments.
Materials and methods: 60 male Wistar rats were randomly divided into 5 groups: control group, model group, antibiotic group, growth factor group, and Danhuangsan group by using random number table method. The rats in each group were given the corresponding drug intervention once a day for 21 consecutive days. After the intervention, the healing rate of traumatic ulcers and pathological changes of traumas were observed in each group, colony counting was performed on rat traumatic tissues, the expression levels of hs-CRP, IL-1, and IL-6 were detected by immunohistochemistry in rat serum tissues, the expression level of CD31 in traumatic tissues by immunofluorescence, and LC3-II was detected by protein western blotting in traumatic tissues in the detection of LC3-II in rat traumatic tissues, PINK1 and Parkin in rat trauma tissue.

Results: Dan Huang San significantly increased the wound healing rate, improved the wound healing environment, reduced the number of wound colonies, and favored the expression of CD31. Compared with the blank group, the model group showed decreased wound healing rate, increased colony number, increased expression levels of serum inflammatory factors hs-CRP, IL-1, and IL-6, and decreased expression of LC3-II, PINK1, and Parkin in wounds (all $P<0.05$); compared with the model group, the antibiotic group, the growth factor group, and the Danhuangsan group were able to reduce hs-CRP, IL-1, IL-6 levels ($P<0.05$), increased the expression of LC3-II, PINK1 and Parkin in the wound ($P<0.05$), inhibited the expression of inflammatory factors, and promoted ulcer healing, and the Dan Huang San group was more effective in reducing the number of colonies and anti-inflammation.

Conclusion: In conclusion, Danhuangsan can effectively remove wound bacteria and accelerate the healing of diabetic infected ulcers, and its mechanism may be related to the activation of PINK1/Parkin pathway.

Keywords: Danhuang San; Diabetes; Ulcer; Anti-Inflammatory; PINK1/Parkin

Introduction

The latest data from the International Diabetes Federation (IDF) for the year 2021 shows that worldwide, 537 million people aged 20-79 have diabetes, the vast majority of whom have type 2 diabetes, and that this number is expected to increase to nearly 783 million by 2045, according to the IDF.[1] The number is expected to increase to nearly 783 million by 2045. It is estimated that 15% to 25% of people with diabetes will develop diabetic foot ulcers (DFUs), and 50% to 70% of DFUs may eventually lead to amputation.[2] The 5-year mortality rate after amputation (including major and minor amputations) is as high as 40%.[3] DFU has become a major public health problem worldwide because of the serious physiological and psychological burdens as well as the economic and productivity losses caused to patients.

The disease state of diabetes mellitus severely disrupts the delicate balance between promoting angiogenesis and vascular maturation in normal wound healing, delaying wound healing, tissue regeneration, and recovery of a healthy vascular system.[4,5] The growth factors and inflammatory factors in the wound are altered to varying degrees, leaving the wound in a state of chronic inflammation and damaging repair cells such as vascular endothelial cells and fibroblasts, resulting in a difficult and long-lasting wound repair.[6,7] The wound is difficult to be repaired, and it is difficult to be healed for a long time. In addition, open wounds are easily infected with pathogenic bacteria, especially staphylococci, streptococci and gram-negative bacilli.[8] With the discovery of antimicrobial drugs and in-depth research, this has led to a high degree of resistance of pathogenic bacteria, resulting in the aggravation of the patient's condition, which has brought great difficulties in clinical treatment.[9] The current basic treatment of DFU is mainly focused on the treatment of Gram-negative bacteria. Currently, the basic treatment of DFU is mainly symptomatic, including debridement, infection control, and revascularization, etc. In addition, new strategies such as topical dressings offer the possibility of treating diabetic foot ulcers, which can alleviate symptoms, provide wound protection, and promote healing, based on timely examination and evaluation of wounds.[10] .

The research group found that the compound Chinese herbal preparation Dan Huang San (mainly composed of Salvia miltiorrhiza, Rhubarb, Angelica sinensis, Sedum, rosin, myrrh and other traditional Chinese medicines) used for the treatment of diabetic foot ulcers has the efficacy of clearing heat and detoxifying toxins, activating blood circulation and removing blood stasis, and removing dampness and regenerating muscle, etc. It can be used externally on diabetic foot ulcers, which can promote the growth of granulation tissue of the ulcers, and shorten the time of healing.[11,12] It can promote the growth of granulation tissue and shorten the healing time of ulcer wounds. Salvia divinorum is a commonly used traditional Chinese medicine, and its root is rich in many active ingredients, among which Dan Huang San is one of the important water-soluble ingredients in Salvia divinorum. Danhuangsan is condensed from compounds such as salvinorin and caffeic acid, and has various pharmacological effects, such as antioxidant, anti-inflammatory, cardioprotective, inhibition of platelet aggregation and thrombosis. These effects make Danhuangsan have a wide range of application prospects in the field of traditional Chinese medicine, and at the same time, it has also received attention from modern medical research.[13,14] The study was carried out to investigate the effects of Danhuang San on the heart. Based on this, the present study investigated the effects and mechanisms of Dan Huang San on the healing of infected ulcers in diabetic rats by observing the effects of Dan Huang San on the healing of infected ulcers in diabetic rats, with the aim of providing a theoretical basis for the future use of Dan Huang San in the treatment of DFU.

Materials and Methods

Network Pharmacology Studies

Acquisition and Screening of Active Ingredients and Action Targets of Danhuangsan

Danhuangsan is composed of six herbs, including rhubarb, danshen, angelica, sedum, rosin, and myrrh, etc. The information of active ingredients and action targets of danshen, angelica, sedum, rosin, and myrrh were screened on the basis of pharma-

cokinetic parameters (ADME) in TCMSP https://old.tcmsp-e.com/tcmsp.php. As Danhuangsan is a topical drug, refer to similar studies to obtain and screen the active ingredients and action targets of Danhuangsan. Since Danhuangsan is a topical drug, the oral utilization (OB) was not included as a screening condition, and the screening was based on the drug-like properties (DL) ≥ 0.18, with reference to the literature of similar studies. For drugs that could not be searched in the TCMSP database, the ETCM database (http://www.tcmip.cn/ETCM/ index.php/Home/Index/) was used. Subsequently, this target information was normalized by the UniProt database (https://www.uniprot.org/) to obtain normalized component target data.

Diabetic Ulcer Target Acquisition

Apply OMIM(https://www.omim.org/), TTD(http://db.idrblab.net/ttd/),

DrugBank (https://go.drugbank.com/) and GeneCards (https://www.genecards.org/) databases were searched for disease targets with the keyword "diabetic ulcer", and the targets related to diabetic ulcer disease were obtained after merging and de-emphasizing. The targets related to diabetic ulcer were obtained after combining and de-emphasizing the keywords "diabetic ulcer".

Access to Iron Death-Related Targets

Iron death-related gene targets were obtained by searching "Ferroptosis" in OMIM (https://www.omim.org/), TTD (http://db.idrblab.net/ttd/), DrugBank (https://go.drugbank.com/), GeneCards (https://www. GeneCards)(, and GeneCards (genecards.org/) were searched for "Ferroptosis" to obtain relevant targets, which were merged and de-emphasized.

Construction of Protein-Protein Interaction Networks (PPI) and Screening Of Key Core Targets

The intersections of the obtained targets of Danhuangsan, diabetic ulcer and iron death were obtained with the help of Venny2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/) online mapping tool. The PPI was constructed using the STRING database (https://string-db.org/), and the species "Home sapiens" was selected to hide the disconnected nodes in the network to construct the PPI network diagram. The corresponding tsv files were downloaded and imported into Cytoscape 3.9.1 software for visualization, and the MCC algorithm was used to filter out the core targets (hub genes).

Constructing A "Drug-Active Ingredient-Target-Disease" Network: Cytoscape 3.9.1 software was used to construct the relationship network of "Danhuangsan-targets-diabetic ulcers", and the topological structure of the network was analyzed by using the cyto-NCA plug-in, and the core components and targets were screened according to the topological parameters.

GO Functional Analysis and KEGG Pathway Enrichment Analysis: The analysis was performed by importing the obtained intersection target core targets into the DAVID (https://david.ncifcrf.gov/home.jsp) database, setting the threshold $P<0.05$ for GO function enrichment analysis and KEGG pathway enrichment analysis, and visualizing the data results by using the Microbiotics cloud platform, and the enrichment analysis results were presented in bubble diagrams. The results of enrichment analysis were presented in bubble diagrams, in which different color nodes represent different types of enrichment results, and their sizes are positively correlated with the degree of significance. The results were imported into "bioinformatics online tool" (http://www.bioinformatics.com.cn/) and presented in the form of bubble plots.

Experimental Animals: Healthy male Wistar rats, 6 months old, clean grade (CL grade), body mass (180±20g), were purchased from Changsha Tianqin Biological Co. Ltd, Changsha City, Hunan Province, China (Quality Certificate No. SCXK (Xiang) 2019-0013). Feeding conditions: room temperature 23-25℃, relative humidity 40%-70%, 12h alternating light and dark. The control rats were fed continuously with basal feed, and the remaining rats were fed uninterruptedly for 4 weeks with a high-sugar and high-fat diet of 59% basal feed, 18% lard, 20% white sugar, and 3% egg yolk.

Reagents and Instruments: Streptozotocin (STZ, No. 572201); Hematoxylin-Eosin (HE) HD Constant Staining Solution (Batch No. BA4232), Masson Staining Solution (Batch No. BA4079A), Bicinchoninic Acid (BCA) Protein Quantification Kit (Batch No. 33267) were purchased from Zhuhai Beso Bio-technology Co. Immunosorbent assay (ELISA) test kits: tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1 (lot numbers: KE10002, KE10007, KE10003), Wuhan Three Eagles Bio-Technology Co. Ltd.; recombinant CD31 antibody (lot number: ab18298) was purchased from abcam (UK); LC3-II antibody, PINK1 antibody, Parkin antibody, β-actin primary and secondary antibodies (Item No.: 3868, 6946, 32833, 4970, 4412), purchased from CollSignaling, USA; 30B pulverizer (Changzhou Jiu Chuan Drying Equipment Co., Ltd.); frozen sectioning machine, rotary slicer, fluorescence microscope (Ltd., Germany); JT-12S automatic dehydrator (Wuhan Junjie Electronics Co., Ltd.); BMJ-A tissue embedding machine (Changzhou suburb Zhongwei Electronic Instrument Factory); high-speed freezing centrifuge (Hunan Kaida Scientific Instrument Co., Ltd.); transmission electron microscope (FEI Company, U.S.A.); enzyme labeling instrument (Fermentas Company, U.S.A.); gel imaging system (Beckma Company, U.S.A.); statistical analysis system (FEI); and gel imaging system (FEI). Beckma Corporation); statistical graphing software (GraphPad Prism 8.0.), Graph-Pad Software, USA.

Preparation of Diabetic Foot Ulcer Rat Model: Before model preparation, rats were fasted for 12 hours. Except for the control group, the remaining rats were given 0.1% streptozotocin 45 mg/kg by intraperitoneal injection once a day for 7 consecutive days, and the rats with blood glucose higher than 16.7 mmol/L measured after 7 days were diabetic models[15, 16] The rats were then used as diabetic models. After successful preparation, all rats were weighed and anesthetized according to 40 mg/kg concentration of 1% pentobarbital sodium, and the hair on the back of the rats was shaved under aseptic technical operation to make a 1.5 cm × 1.5 cm trauma, which was deep to the fascia [17] . In addition to the control group, the rest of the rats were hemostatically injected with 100 μL of 2.4×1012 Staphylococcus aureus solution (China General Microbial Strain Conservation and Management Center, ATCC6538) at 5 points randomly under the trauma's fascia using the puncture method, and then the trauma was covered with a disposable medical transparent film [18].The wound was then covered with a disposable transparent patch.

Grouping of Experimental Animals and Drug Administration: This experiment was approved by the Ethics Committee for Animal Experiments of the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine (No. 20200005). Sixty experimental rats were randomly divided into five groups of 12 animals each using the random number table method, control group, model group, antibiotic group, growth factor group, and Danhuangxuan group. In the control and model groups, the wounds were cleaned with saline without any interventions; in the Danhuangsan group, the wounds were coated with the corresponding drugs using sterile cotton swabs, the coated area was at least 2.0 mm beyond the wound margins, and the wounds were covered with sterile gauze and then fixed properly; the drugs were changed once a day in all the rats, and the drugs were administered for 21 consecutive days. All animal experimental protocols and procedures strictly followed the internationally recognized principles for the use and care of laboratory animals, i.e., the guidelines revised by the National Institutes of Health (NIH) in 1985 (NIH publication no. 85-23).

Sampling: The healing rate of the ulcerated surface was measured at the 7th, 14th and 21st d of the rat intervention. Three rats were randomly selected from each group at 7d, 14d and 21d, anesthetized and executed, and under aseptic surgical operation, the granulation tissue of the ulcer wound was excised and immersed in paraformaldehyde solution, and then prepared for staining (hematoxylin and eosin (HE)), Masson staining and colony counting. Finally, the remaining rats were anesthetized and killed, and blood was collected from the abdominal aorta of the rats under aseptic operation for enzyme-linked immunosorbent assay (ELISA) analysis of serum inflammatory factors; the granulation tissues of the ulcerated wounds were cut out, frozen in liquid nitrogen and then stored in the refrigerator at -80℃, and then prepared for immunofluorescence staining and Western blot detection.

Detection Indicators

Ulcer Healing Assay: The healing rate was calculated by taking photographs of the wound area using a camera at a fixed height perpendicular to the wound on d 7, 14 and 21 of the intervention, and tracing the outline of the wound using Image J software[19].The healing rate was calculated using Image J software. Healing rate = healed area/original wound area × 100%; healed area is the difference between the original wound area and the unhealed wound area.

HE staining to observe the histopathological and morphological changes of the wound tissue: On the 7th, 14th and 21st day of intervention, the wound tissue was collected and fixed in 4% paraformaldehyde, embedded in paraffin, and sliced in 4 μm sections. 100%, 90%, 80%, and 70% ethanol were placed in each of them for 5 min, and distilled water was placed in each of them for 5 min. hematoxylin staining was done for 5 min, and then the wound tissue was rinsed twice with distilled water, and the stained tissue was rinsed off with running water. After 5 min of hematoxylin staining, rinse twice with distilled water, rinse off the hematoxylin staining solution with running water, and observe the degree of staining under microscope. Alcohol differentiation with 1% hydrochloric acid for a few seconds, washed twice with water. HE staining, rinsing of sections for 10 min, dehydration with ethanol and xylene, sealing, and subsequent microscopic observation.

Masson staining to observe the collagen formation of traumatic tissue: Rat traumatic tissue was fixed with 4% paraformaldehyde for 24 h. Tissue sections were stained in the same way as HE staining. Xylene Ⅰ for 5 min, xylene Ⅱ for 5 min, xylene Ⅲ for 5 min, anhydrous ethanol for 1 min, 95% ethanol for 1 min, 75% ethanol for 1 min, and rinsed with tap water for a few seconds; stained with Vigot Ferric Hematoxylin for 5 min, and washed with 1% hydrochloric acid in ethanol for a few seconds after differentiation; Masson Blueing Solution returned to blue for 5 min, and washed with water. Distilled water wash for 1 min; Lichun red magenta staining solution staining for 5 min; weak acid working solution wash for 1 min; phosphomolybdic acid solution wash for 1 min, weak acid working solution wash for 1 min; aniline blue staining solution staining for 2 min, weak acid wash for 1 min; 95% ethanol rapid dehydration for 2~3s, anhydrous ethanol dehydration for 3 times, each time for 5-10s, xylene transparent for 3 times, each time for 1~2 min, neutral tree glue sealing[13] Sealing.

Wound colony counting: On the 7th, 14th and 21st days of administration, take wound tissue and grind it by adding 0.9% sodium chloride injection at 1:10, inoculate the plate for culture of pathogenic bacteria, and then count the number of colonies = (the number of colonies×dilution times/100)/mass of diluted tissue (CFU/g).The number of colonies = (number of colonies × number of dilutions / 100 / mass of diluted tissue (CFU/g).

ELISA analysis of serum inflammatory factors: After the intervention, rats were anesthetized with 1% sodium pentobarbital 40 mg/kg, and 5 mL of blood was taken from the abdominal aorta, and the supernatant was separated by centrifugation at 3,000 r/min, 5 cm radius, for 10 min. The supernatants were separated from the blood by centrifugation at 3,000 r/ min, radius of 3,000 cm, and the serum was analyzed according to the instructions of the ELISA kits for the detection of hs-CRP, IL-1 and IL-6 in the serum of rats in each group.

Immunofluorescence staining to detect CD31 expression in traumatized tissues: issues were removed from liquid nitrogen, OCT-embedded, and frozen sectioned at a thickness of 6 μm and patched. -20℃ pre-cooled acetone fixed at room temperature for 10 min; TBS solution rinsed for 5 min × 3 times; 0.025% TritonX-100 TBS solution rinsed for 5 min × 2 times; 10% goat serum + 1% BSA 0.2 mL, closed at room temperature for 2 hours; add the primary antibody (1% BSA, 1:200 dilution) 0.2 mL, placed in the kit, incubated at 4℃ overnight ; 0.025% TritonX-100TBS rinsed for 5 min×2 times; add secondary antibody (1% BSA, 1:1000 dilution) 0.2 mL, incubate at room temperature, avoiding light for 2 h; TBS solution rinsed for 5 min×3 times; after sealing with sealing agent with DAPI and coverslips, the sections were used for image acquisition by fluorescence microscope camera system.

Western blot for protein expression of LC3-II, PINK1, Parkin in traumatized tissue: Western blot for protein expression of LC3-II, PINK1, Parkin. The weighed tissues were removed, ground in liquid nitrogen and added to the ready-made protein lysate to EP tubes, and stored at -80℃. After removal from the refrigerator, incubate at 4°C for 5 min with melting, shake vigorously for 30 s, and repeat 4 times. Protein concentration was determined after centrifugation at high speed pre-cooling using the BCA protein analysis kit. Proteins were cooked according to each time point in an amount of 20 μl/tube, shaken, mixed, centrifuged and sealed, then cooked at 100°C for 10 min, cooled on ice, shaken and mixed, centrifuged and stored at -20°C. Separated by 5% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was closed at room temperature for 2h and washed 3 times with TBST for 10min/time. The primary antibody was prepared with 5% BSA dilution, i.e. LC3-II (1:500), PINK1 (1:500), Parkin (1:500), shaking bed for 1h, overnight at 4℃. Rewarm, wash the membrane, add secondary antibody (1:1 000), 5% BSA prepared dilution, incubate at room temperature for 2h. β-actin was used as internal reference. Prepare ECL reagent kit, filter paper and turn on the gel imaging instrument in advance. Prepare liquid A and B in 1:1 ratio according to the instructions of ECL chemiluminescence reagent, and avoid light. Make the AB liquid in full contact with the PVDF membrane, make exposure and save the picture.

Statistical Analysis

SPSS26.0 statistical software was applied to analyze the experimental data, and GraphPad Prism 8.0 software was used to draw statistical graphs. Measurement data were expressed as mean±standard deviation, one-way ANOVA was used for comparison between multiple groups, independent samples t-test was used for comparison between two groups, and repeated-measures ANOVA was used for comparison between model groups measured at different time points. Differences were considered statistically significant at $P<0.05$.

Results

Screening of Active Ingredients and Targets of Action of Danhuangsan and Targets Related to Diabetic Ulcers and Iron Mortality

After screening and de-emphasizing in TCMSP database, BATMAN-TCM database and ETCM database, 93 potential targets of Danhuangsan were obtained. Among them, 65 active ingredients of rhubarb, with 3501 potential targets; 137 active ingredients of Salvia miltiorrhiza, with 5101 potential targets; 12 active ingredients of angelica, with 508 potential targets; 23 active ingredients of incense, with 1606 potential targets; 18 active ingredients of rosin, with 671 potential targets; and 12 active ingredients of myrrh, with 456 potential targets. The active ingredients of 6 drugs were combined and de-emphasized with the targets, and 1,179 potential targets were finally obtained. We screened the targets of diabetic ulcer in the database, merged the targets of three databases, deleted the duplicate targets, and obtained 1886 potential targets of diabetic ulcer; we screened 1669 targets related to iron death in the database, and all of the above targets were standardized in the UniProt database. The targets of Danhuangsan, the potential targets of diabetic ulcer and the targets related to iron death were imported into Microbiotics to draw the Wayne diagram online, and 93 targets of the intersection of the three were obtained, see Figure 1.

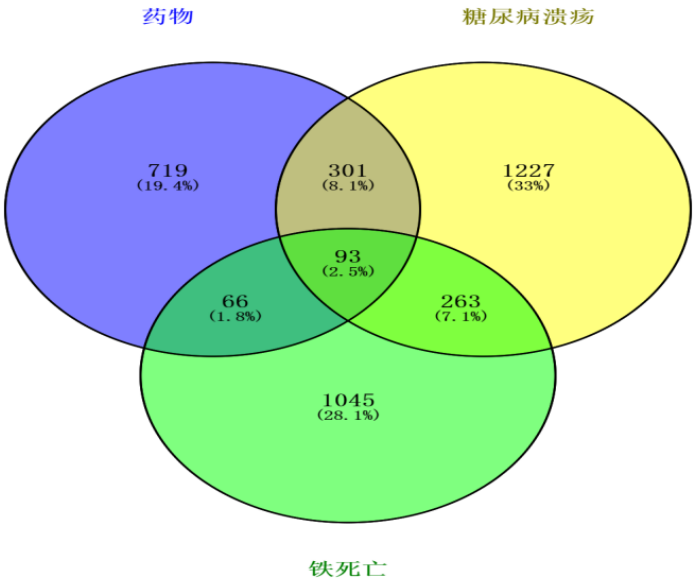


Figure 1: Dan Huang San - diabetic ulcer - iron death Wayne diagram

Building a "Drug-Active Ingredient-Target-Disease" Network: Cytoscape 3.9.1 software was used to construct the relationship network of "Danhuangsan-targets-diabetic ulcers", and the topological structure of the network was analyzed by using the cyto-NCA plug-in, and the core components and targets were screened according to the topological parameters, as shown in Figure 2.

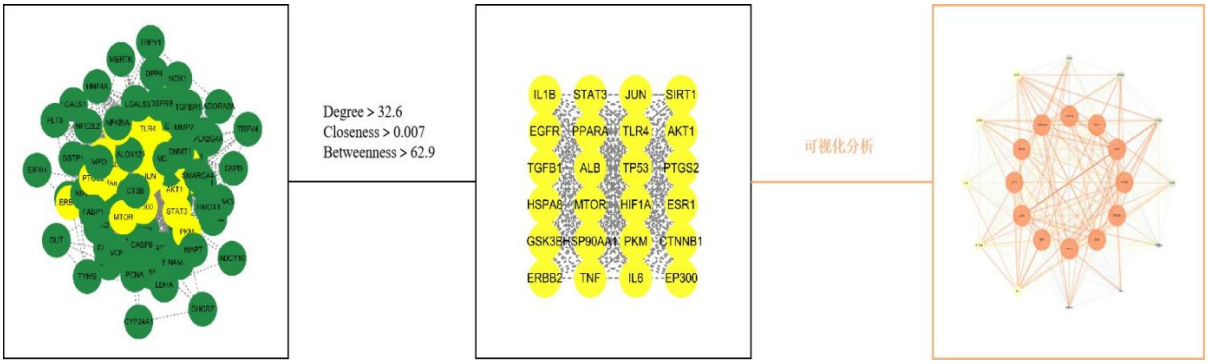


Figure 2: Network diagram of "Danhuang San - active ingredients - targets of action".

GO Functional Analysis and KEGG Pathway Enrichment Analysis Map

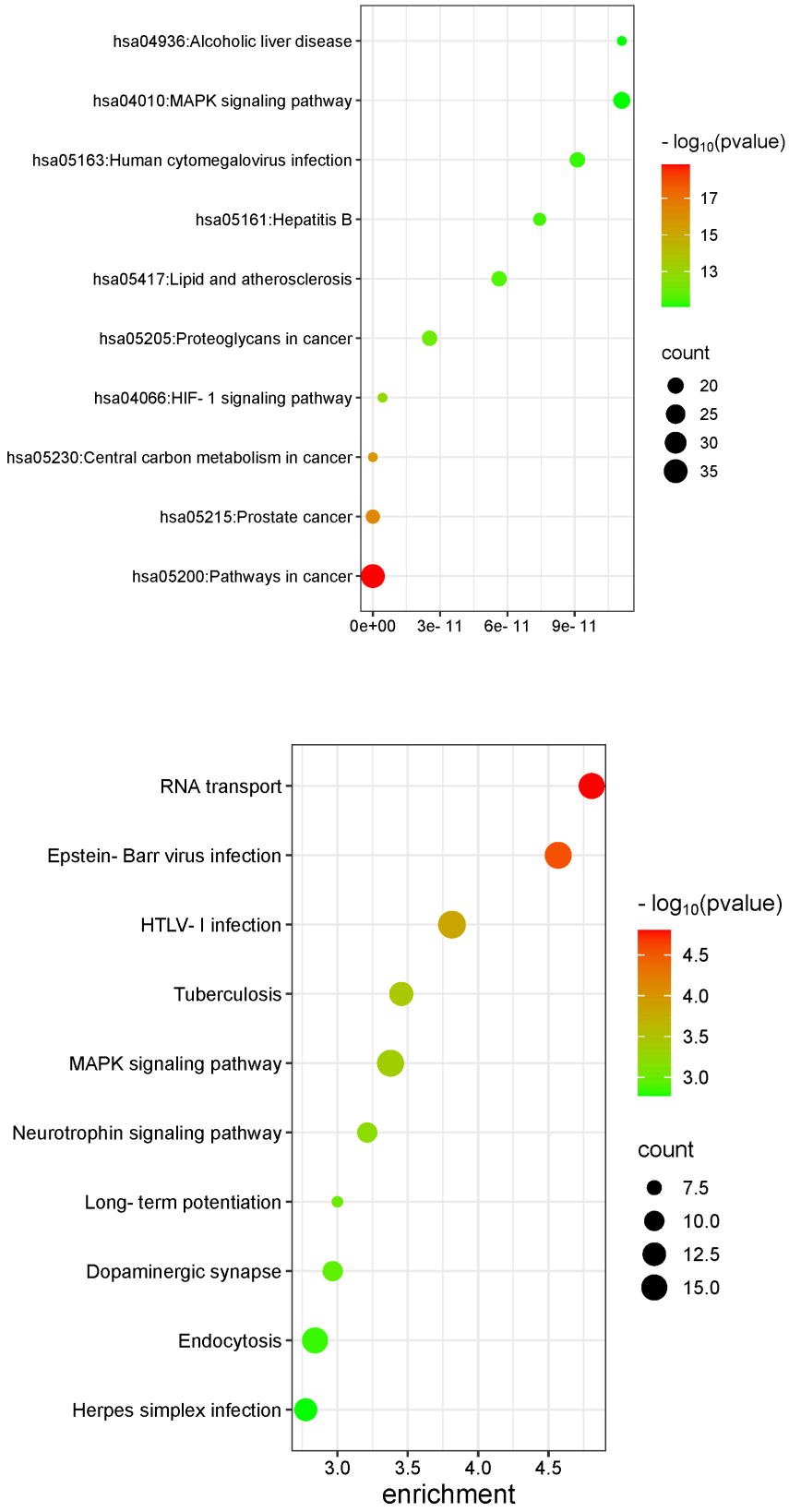
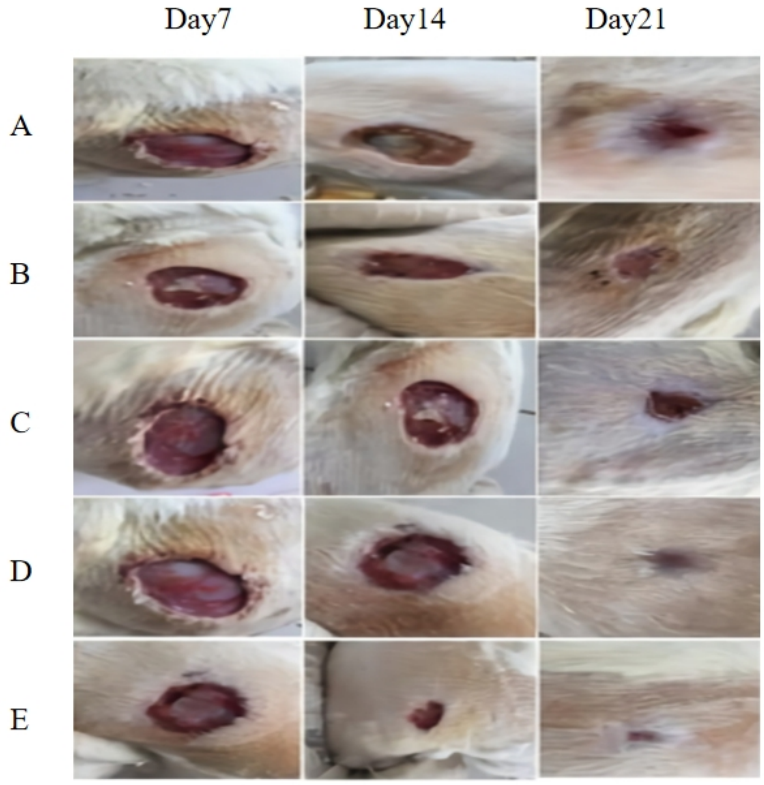


Figure 4: Bubble map for KEGG pathway enrichment analysis

Observation of Wound Healing Rate in Rats

Compared with the control group, the wound healing rate of the model group decreased; compared with the model group, the

wound healing rate of the antibiotic group, the growth factor group and the Danhuangsan group increased in the 7th, 14th and 21st d of administration; and the wound healing rate of the Danhuangsan group in the 14th and 21st d of administration was significantly higher than that of the antibiotic group and the growth factor group. See Figure 5 and Table 1.



A: control group; B: model group; C: antibiotic group; D: growth factor group; E: danhuangsan group

Table 1: Comparison of wound healing rate among groups (☒±s, %)

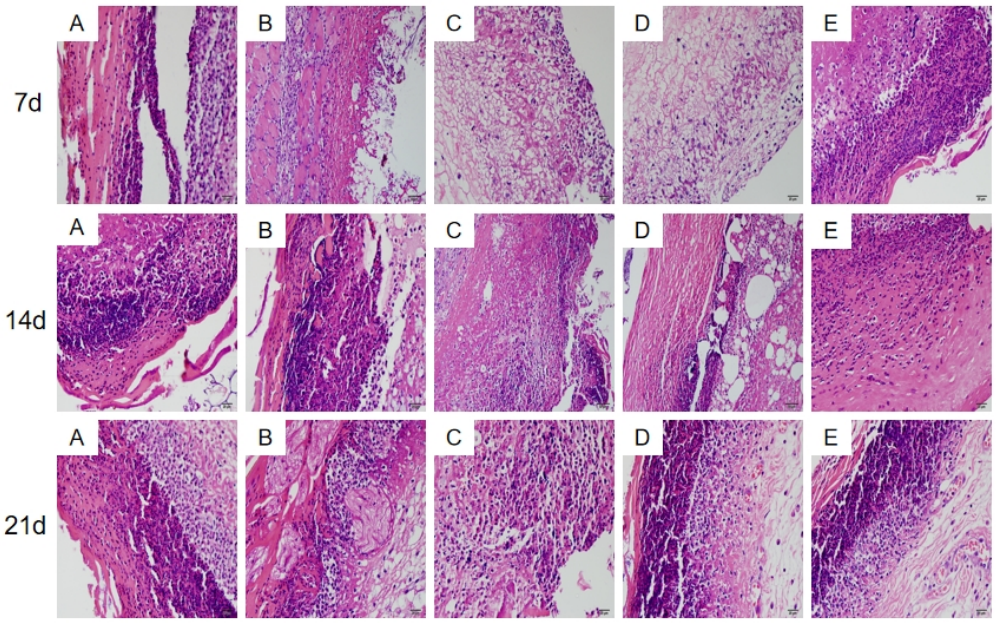
group	healing rate		
	7d (n=12)	14d (n=9)	21d (n=6)
Control	17.83±1.30	29.95±1.50	63.08±1.35
Model	15.34±1.33[a]	20.20±1.01[a]	49.49±3.22[a]
Antibiotic group	20.68±0.87[b]	45.87±2.21[b]	76.75±2.04[b]
Growth factors	22.19±1.04[b]	48.16±1.30[b]	80.41±4.28[b]
DHS	25.00±1.36[b]	54.71±1.99[bcd]	84.72±2.45[bcd]

Note: Compared with the control group,[a] P<0. 05; compared with the model group,[b] P<0.05; compared with the antibiotic group,[c] P<0.05; and compared with the growth factor group,[d] P<0.05. Twelve rats were tested in each group at day 7, nine rats were tested in each group at day 14, and six rats were tested in each group at day 21.

Histopathological Morphology of Trauma In Each Group

HE staining showed that in the control group, granulation tissue was formed on the wounds of rats, a small number of discrete capillaries could be seen distributed therein, and collagen fibers were proliferated; in the model group, the wounds of rats could be seen to be thickened and edematous in the epidermis layer, with interstitial laxity, inflammatory cell infiltration, and disorganized arrangement of proliferation of collagen fibers; in all the intervention groups, the number of inflammatory cells could

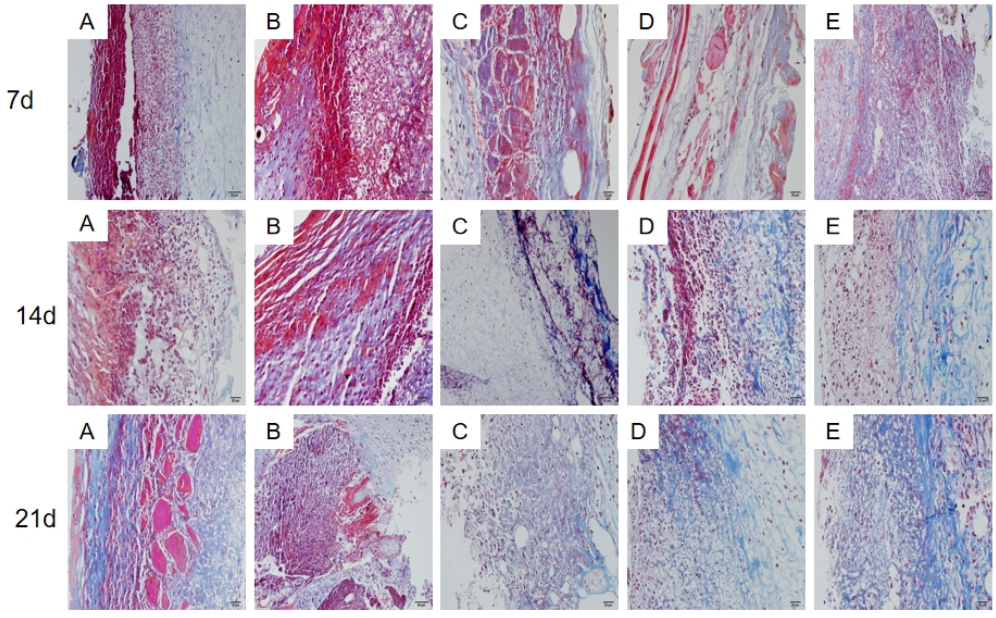
be seen to be significantly reduced, the number of fibroblasts was increased in large quantities, with tight arrangement of the inter-cells, and the formation of nascent granulation tissue was Significantly increased, the epidermis covers a larger area of trauma. Figure 6.



A: control group; B: model group; C: antibiotic group; D: growth factor group; E: danhuangsan group

Wound Collagen Formation in Each Group

Masson staining showed that the model group had lighter blue staining, less collagen fiber formation, and sparsely arranged tissue matrix; the control group had darker blue staining, collagen fiber formation, and more tightly arranged tissue matrix; and collagen fibroblasts were seen to be significantly neoplastic in each intervention group. Figure 7.



A: control group; B: model group; C: antibiotic group; D: growth factor group; E: danhuangsan group

Comparison of the Number of Traumatic Colonies of Rats in Each Group

Compared with the control group, the number of traumatic colonies in the model group was elevated; compared with the mod-

el group, the number of traumatic colonies in the growth factor group, Danhuangsan group, and Danhuangsan group were all reduced in the 7th, 14th, and 21st d of the administration of the drug, and in the 14th and 21st d of the administration of the drug, the number of colonies in the Danhuangsan group was significantly lower than that in the growth factor group and the Danhuangsan group. See Table 2.

Table 2: Comparison of the number of traumatic colonization in each group (⊠±*s*, n=3, logCFU/g)

group	7d	14d	21d
Control	2.78±0.62	2.22±0.30	1.24±0.24
Model	12.41±1.02[a]	11.14±0.81[a]	9.69±0.65[a]
Antibiotic group	9.26±0.75[b]	8.32±0.51[b]	7.54±0.41[b]
Growth factors	9.38±0.79[b]	8.40±0.30[b]	7.43±0.55[b]
DHS	8.65±3.29[b]	7.69±0.62[bcd]	6.71±0.38[bcd]

Note: Compared with the control group,[a] P<0. 05; compared with the model group,[b] P<0.05; compared with the growth factor group,[c] P<0.05; compared with the Dan Huang San group,[d] P<0.05.

Comparison of Inflammatory Factors in Serum of Rats in Each Group

Compared with the control group, serum hs-CRP, IL-1 and IL-6 levels were significantly increased in the model group ($P < 0.05$). Compared with the model group, the serum hs-CRP, IL-1, IL-6 levels of the growth factor group, Dan Huang San group and Dan Huang San group were significantly reduced, and the reduction was more significant in the Dan Huang San group ($P < 0.05$). See Table 3.

Table 3: Comparison of inflammatory factors in serum of rats in each group (⊠±*s*, n=3)

group	hs-RCP (μg/L)	IL-1 (ng/L)	IL-6 (ng/L)
Control	2.23±0.32	17.93±1.56	34.39±1.75
Model	8.69±0.63[a]	51.51±2.12[a]	93.78±3.43[a]
Antibiotic group	7.70±0.47[b]	41.15±1.64[b]	74.89±2.74[b]
Growth factors	7.15±0.55[b]	39.19±1.60[b]	73.93±2.27[b]
DHS	6.63±0.24[bcd]	35.67±2.02[bcd]	70.68±1.39[bcd]

Note: Compared with the control group,[a] P<0. 05; compared with the model group,[b] P<0.05; compared with the growth factor group,[c] P<0.05; compared with the Dan Huang San group,[d] P<0.05.

Neovascularization of Ulcerated Wounds in Rats of All Groups

CD31 fluorescence staining showed that: no obvious positive fluorescence signal was detected in the model group; multiple positive fluorescence signals (green) were visible in each intervention group compared with the model group, revealing that Danhuangsan could effectively promote traumatic neovascularization.Figure 8.

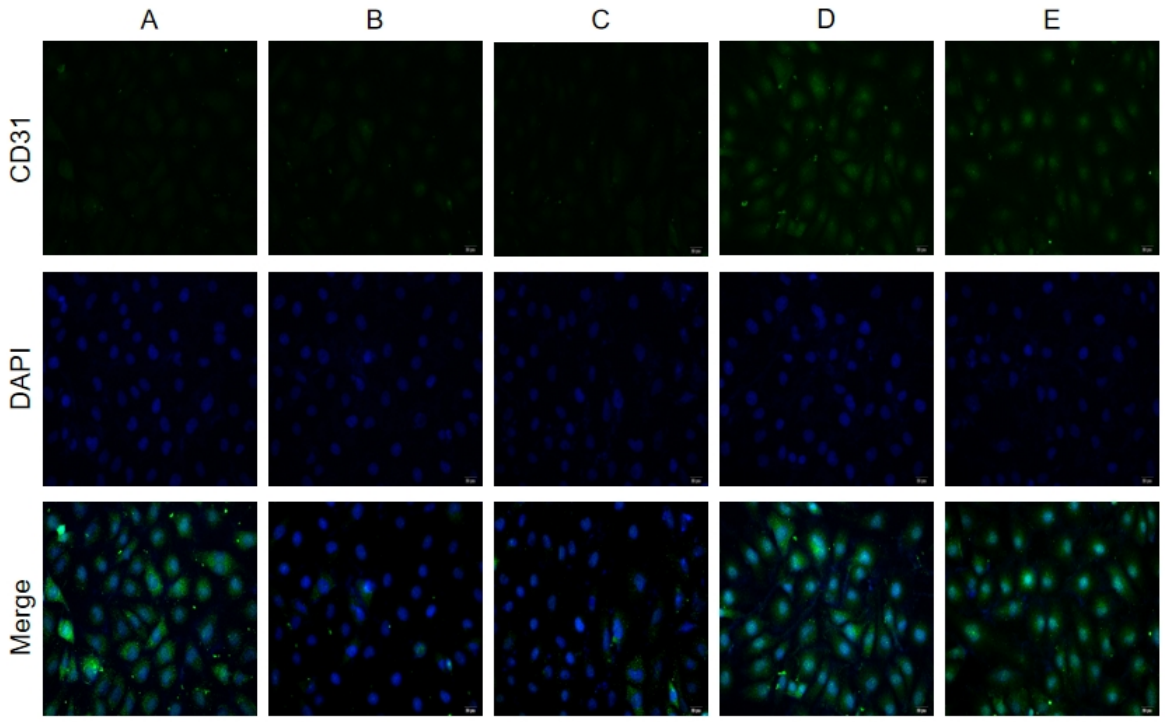
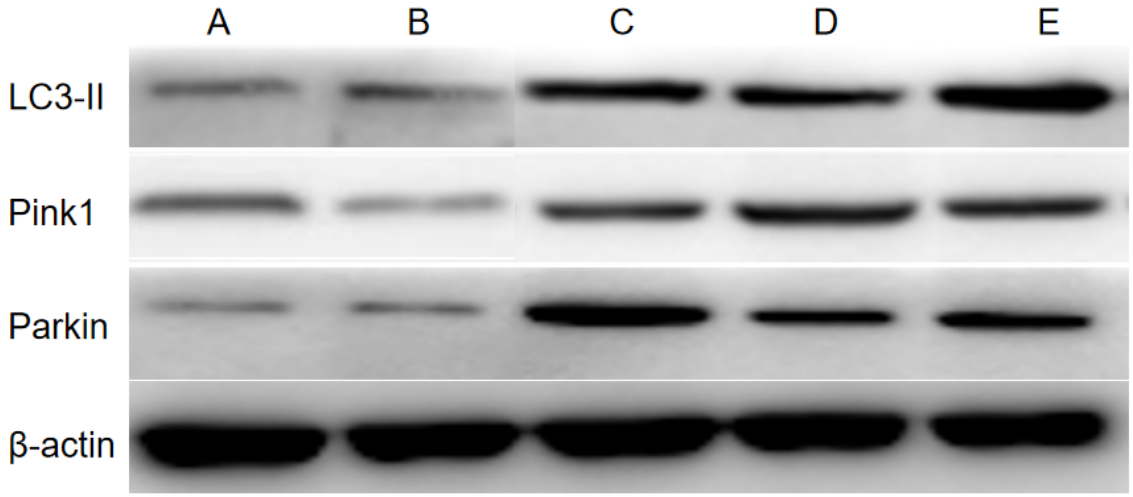
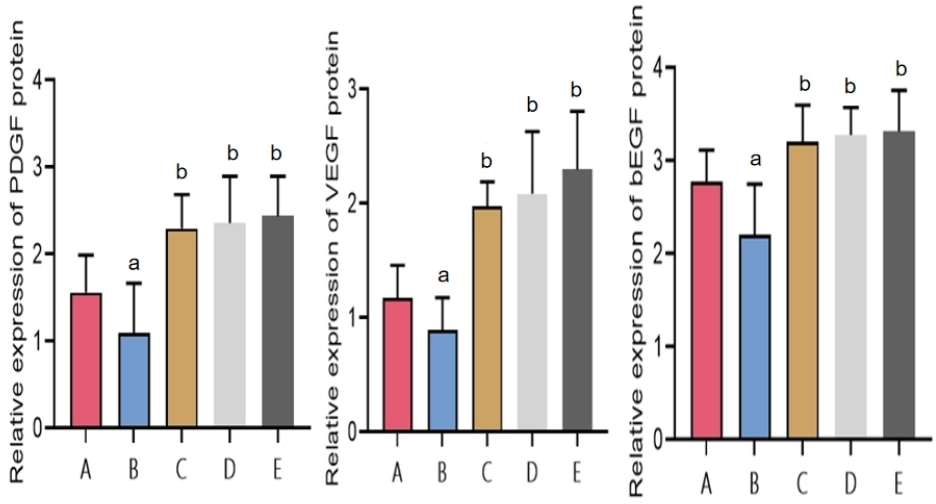


Figure 8: Immunofluorescence staining of CD31 on skin ulcer wounds of rats in each group (×20 μm). Note: A: control group; B: model group; C: antibiotic group; D: growth factor group; E: danhuangsan group

Comparison of LC3-II, PINK1 and Parkin Protein Expression In Trauma Tissues of Rats from Various Groups

After 21 days of intervention, compared with the control group, the protein expression of LC3-II, PINK1 and Parkin in the model group was decreased ($P < 0.05$); compared with the model group, the protein expression of LC3-II, PINK1 and Parkin in the growth factor group, the danhuangsan group and the danhuangsan group were increased ($P < 0.05$), and the differences were all statistically significant. Figure 9.





Note: A: control group; B: model group; C: antibiotic group; D: growth factor group; E: Dan Huang San group. Compared with the control group,[a] P<0. 05; compared with the model group,[b] P<0.05; compared with the growth factor group,[c] P<0.05; compared with the Dan Huang San group,[d] P<0.05.

Discussion

The aim of this study was to investigate the mechanism of action of Dan Huang San in the treatment of diabetic foot ulcers using a rat model. The results showed that Dan Huang San could effectively promote the healing of infected wounds in diabetic rats, and its pathway of action may be related to the inhibition of inflammation and activation of PINK1/Parkin expression.

Diabetic foot ulcers (DFUs) are foot infections, ulcers and/or deep tissue destruction in diabetic patients due to abnormalities of the distal nerves of the lower limbs and various degrees of vascular pathology. In line with the current treatment concepts, early intervention in the treatment of diabetic foot ulcers has been recognized as an effective way to avoid the deterioration of the condition of DFUs, and to reduce the rate of disability and mortality of DFUs in the past and present. DFU belongs to the category of "gangrene" in Chinese medicine research, and its related description can be found in the "Huang Di Nei Jing" (The Yellow Emperor's Classic of Internal Medicine), "Ling Shu - Gangrene and Carbuncle Chapter" - "It occurs in the toes of the foot, and is called a detached carbuncle. In Chinese medicine theory, diabetes mellitus is categorized as the category of "thirst", and studies have shown that prolonged thirst will deplete the body's positive qi and yin, leading to qi and yin deficiency, and when qi is deficient, blood and fluids are unable to be transported to the ends of the limbs, leading to insufficient qi, blood, essence and fluids in the feet, and the tendons and veins are not nourished, which leads to symptoms such as numbness, pain, and even necrosis.The result is numbness, pain, and even necrosis. Prof. Xu Yunsheng[20]Professor Xu Yunsheng believes that diabetic foot originates from congenital or acquired weakness, resulting in deficiency of vital energy, stagnation of blood and internal dampness, which interact with each other and ultimately lead to damage of foot tissues and formation of ulcers. To summarize, the formation of DFU mainly originates from long-term poor control of diabetes mellitus, which leads to the pathological state of "prolonged thirst, deficiency of qi and yin". The patient's body has weakened positive qi, evil qi (dampness, heat, poison) breeding, stagnation of qi, blood and veins, resulting in poor blood supply to the ulcerated surface of the foot. This pathological environment is not conducive to the removal of putrefied flesh and the growth of new granulation tissue, resulting in the formation of chronic hard-to-heal ulcers. Therefore, early prevention and treatment of DFU is crucial. Through timely intervention and treatment, it can effectively control the development of the disease, reduce the occurrence of complications and improve the quality of life of patients.

Dan Huang San, as one of the empirical formulas in our hospital, is effective in the treatment of diabetic foot ulcers[21]. Dan

Huang San, as one of the empirical formulas in our hospital, is effective in treating diabetic foot ulcer. Among them, active ingredients such as danshen and rhubarb have multiple pharmacological effects in Danhuangsan, especially in the following aspects showing significant potential: ① Antioxidant effect Danshen and rhubarb in Danhuang San are natural antioxidants with the ability to neutralize free radicals, which is expected to reduce the level of oxygen free radicals in patients with diabetic foot ulcers. This is critical in mitigating cellular and tissue damage caused by oxidative stress, especially in the development of diabetic foot ulcers, where a hyperglycemic state triggers the overproduction of oxygen free radicals, making tissues more susceptible to damage. This is important to reduce the level of inflammation in the foot tissue and improve the healing environment. ② Promoting Wound Healing[22] : The active ingredients within Dan Huang San promote wound healing. Salvia miltiorrhiza and Angelica sinensis are believed to stimulate cell proliferation, collagen synthesis and neovascularization, which are key processes in wound healing. For patients with diabetic foot ulcers, accelerated wound healing not only helps prevent infection, but also mitigates the risk of further tissue necrosis. ③Anti-inflammatory and anti-bacterial effects[23] : Inflammation plays a key role in the development of diabetic foot ulcers. The sedum, rosin and myrrh components within Dan Huang San are believed to have anti-inflammatory and anti-bacterial effects, inhibiting bacterial reproduction, reducing inflammatory responses, and decreasing inflammatory cell infiltration and the release of inflammatory mediators.

Wound healing in diabetic foot ulcers is a complex and extremely challenging biological and molecular process involving the coordinated efforts of multiple cell types[24, 25] . Research has found[26, 27] that chronic inflammation is one of the key factors preventing normal wound healing. A hyperglycemic state weakens the immune system and reduces the body's defenses against pathogens . At the same time, neutrophils in diabetics release more neutrophil extracellular traps (NETs), which not only exacerbate the inflammatory response, but may also cause unwanted damage to surrounding tissues . In wounds, excessive accumulation of inflammatory immune cells and inflammatory cytokines leads to an imbalance in the expression of matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP), and this imbalance can damage the extracellular matrix (ECM), thus impeding wound healing[28, 29] . In addition, diabetic wounds provide a favorable environment for biofilm formation, and multidrug resistance and biofilm formation are important factors in the development of diabetic foot ulcer (DFU) infections[30] . In this study, we combined the traditional Chinese medicine Dan Huang San preparation with silver nano dressing with antibacterial effect. It was found that nanosilver exhibited its unique antibacterial and anti-inflammatory properties after direct contact with bacteria, and these properties played a key role in accelerating the wound healing process[31,32] . The present study showed that there was a significant decrease in the levels of IL-1, IL-6, and hs-CRP in the Danhuangsan group, suggesting that Danhuangsan has a significant effect in promoting wound healing, and its mechanism may be related to the reduction of infections, lowering the level of inflammatory factors, and improving the environment of the wounds.

CD31, a prominent marker of neovascular endothelial cells, is valuable in assessing neovascularization in ulcerated wound tissues[33] . The results of the study showed that multiple positive fluorescent signals (green) were observed in each intervention group compared with the model group, suggesting that Dan Huang San could promote CD31 expression in ulcerated wound tissue, promote neovascularization of the wound tissue, and improve the local blood supply of the wound.The PINK1/Parkin pathway is one of the classical pathways regulating mitochondrial autophagy, and plays a key role in the initiation of mitochondrial autophagy, signaling, and selective degradation, as well as in the maintenance of normal cellular metabolism. normal cellular metabolism[34]. PINK1 is a highly conserved mitochondrial protein involved in the regulation of mitochondrial function, and Parkin is an E3 ubiquitin ligase that increases mitochondrial activity. Activated Parkin can connect with LC3, etc. to form mitochondrial autophagosomes to initiate the mitochondrial degradation program.Xi, J et al[35] It was found that baicalein upregulates mitochondrial autophagy via the PINK1/Parkin signaling pathway, thereby protecting vascular endothelial cells from hyperglycemia-induced injury.Hong, K et al[36] showed that cinnamaldehyde could promote wound healing in diabetic rats by regulating the expression of PINK1/Parkin signaling pathway, activating mitochondrial autophagy, inhibiting inflammatory responses, and increasing vascular neogenesis and collagen synthesis. The results of the present study showed that the expression levels of PINK1/Parkin were significantly up-regulated in the Danhuangsan group compared with the model group. This find-

ing suggests that Danhuangsan may play an anti-inflammatory role by activating the PINK1/Parkin pathway, which in turn accelerates the healing process of localized ulcers.

In conclusion, Danhuangsan accelerated the healing process of wound ulcers in diabetic rats, effectively eliminated wound bacteria, and promoted the expression of wound growth factors, the mechanism of which may be related to the activation of the PINK1/Parkin pathway. The mechanism may be related to the activation of PINK1/Parkin pathway. In the future, we can further investigate the mechanism of its action and lay the foundation for the drug development of Danhuangsan.

Constraints on Interests

There is no conflict of interest in the submission of this manuscript.

Cecilia Zhao: writing-origin drafts, participated in data analysis, participated in the design of animal experiments, completed most of the experiments. Chunling Zhang and Lu Chen: project management, conceptualization, methods, and revision of the manuscript. Xinghui Wang: writing-original draft, investigation, data organization and editing. Long Yi: supervision. Tietao Di and Qing Shi: formal analysis and investigation. Lei Zhu, Zhiqin Luo and Xuan Yang: methods, validation.

Data Availability

The data used to support the results of this study are available through the corresponding author.

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