Qingre Baidu mixture-induced effect of AI-2 on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms in chronic and refractory wounds

WEI SHAN, YUNFEI WANG, ZHEN ZHANG, JIE XING, JIENAN XU, WEN XIAO, YITING SHEN, SHUYU GUO and HUAFA QUE

Department of Surgery of Traditional Chinese Medicine, Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200032, P.R. China

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Abstract. The present study aimed to investigate the effect of the traditional Chinese medicine Qingre Baidu mixture (QBM) on the regulation of various parameters, including the morphology of bacterial biofilms, the bacterial density sensing system, the self-induction of the molecule autoinducer (AI)-2 and the hypoxia-inducible factor (HIF)-vascular endothelial growth factor (VEGF) signaling pathway. For that purpose, Sprague Dawley rats were administered the QBM, the Wu Wei Xiao Du Wan drink (WXD) and cefoperazone (Cef) prior to drug isolation from serum. *Vibrio harveyi* BB170 was employed as a reporter strain to detect the AI-2 signaling pathway in *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The expression of HIF-1α and VEGF expression was examined by immunohistochemistry. ELISAs were used to measure the expression of HIF-1α, HIF-2α, HIF-3α and VEGF. The level of inflammation was evaluated by hematoxylin and eosin staining. Biofilm formation and the number of macrophages were detected by immunofluorescence. The results revealed that the QBM could reduce the concentration of AI-2 derived from *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and markedly increase the levels of HIF-1α, HIF-2α and VEGF in chronic and refractory wounds. The QBM strongly inhibited the formation of bacterial biofilms and the number of macrophages, therefore promoting wound healing. In conclusion, the QBM could inhibit the biofilm formation of *Staphylococcus aureus* and *Pseudomonas aeruginosa* through decreasing the levels of AI-2 while upregulating the expression of HIF-1α, HIF-2α and HIF-3α, which increased the levels of VEGF, thereby promoting angiogenesis and wound healing in chronic and refractory wounds.

Correspondence to: Dr Huafa Que, Department of Surgery of Traditional Chinese Medicine, Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, 725 South Wanping Road, Xuhui, Shanghai 200032, P.R. China
E-mail: huafaque16@sina.com

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Introduction

Chronic and refractory wounds do not heal normally, and the integrity of the skin of the affected patients is not achieved in 1 month, leading to an increased inflammatory response and tissue damage (1). Chronic refractory wounds pose a great psychological burden to patients and seriously affect their quality of life and are therefore are considered one of the most urgent problems to be resolved in the field of surgery (2).

Chronic and refractory wounds are usually caused by microbial colonization and are treated as serious infections. One of the main factors that influences the wound healing process is bacterial infection (3). The formation of bacterial biofilms is an important factor in the development of bacterial resistance and avoidance of the body's immune defense mechanisms. The bacterial density sensing system serves an important role in the formation of biofilms, which directly affects the formation and dissociation of biofilms (2).

The self-induction of the molecule autoinducer (AI)-2, which is the only molecule currently known to be capable of conducting signal transduction during intra- and interspecific interactions of bacteria, serves a key role in the formation and stability of the bacterial density induction system (2,4). AI-2 regulates the expression pattern of genes that promote biofilm development. An increased concentration of AI-2 leads to a high level of bioluminescence in *Vibrio harveyi*. AI-2 has been reported to be involved in numerous important processes in a number of Gram-positive and Gram-negative bacteria, including biofilm formation, excretion of poison, production of antimicrobial agents, migration and genetic competence (5,6). It is well known that hypoxia-inducible factors (HIFs) serve a transcriptional role under conditions of hypoxic stress and activate a number of target genes, including phosphoglycerate kinase and vascular endothelial growth factor (VEGF) in cells (7-9). These processes contribute to endothelial proliferation, glycolysis and angiogenesis, which facilitates the generation of new cells (8). Therefore, there is close association between HIFs, VEGF and wounds healing.

Clinical observations of patients with chronic refractory wounds have revealed that these patients can significantly control and eliminate bacterial infection of the wound upon oral treatment with the QBM. The authors' previous results revealed that the traditional Chinese medicine QBM served an antibacterial role by regulating the immune function in mice with *Staphylococcus aureus* infection (10). Based on the clinical efficacy of the QBM, the present study focused on the effect of the self-inducer molecule AI-2 on chronic and refractory wounds in rats with *Staphylococcus aureus* and *Pseudomonas aeruginosa* infection.

Materials and methods

Animals. Male Sprague-Dawley rats (n=30; age, 6-8 weeks) weighing 200-250 g were obtained from Shanghai SLAC Laboratory Animal Co., Ltd., (Shanghai, China). Rats were housed under conventional conditions at an appropriate temperature (22.0±0.5˚C) and humidity (50-60%), and under a 12-h light/dark cycle (lights were switched on from 8:00 a.m. to 8:00 p.m.). the rats were allowed free access to food and water. All experiments and procedures were carried out according to the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China [animal production license number: SCXK (Shanghai) 2012-0002].

Drugs and reagents. The QBM (cat. no. 160120) was purchased from the LongHua Hospital Shanghai University of Traditional Chinese Medicine (Shanghai, China). Its main ingredients are Chinese medicines, including *Angelica sinensis*, *Viola mandshurica*, honeysuckle, Red Peony root, *Salvia miltiorrhiza*, *Forsythia suspensa*, Astragali radix, *Gleditsia sinensis* and liquorice. The WXD (cat. no. 160413) was purchased from the LongHua Hospital Shanghai University of Traditional Chinese Medicine. The main ingredients are Chinese medicines including honeysuckle, wild chrysanthemum, *Viola mandshurica*, *Gynura bicolor* and dandelion. *Pseudomonas aeruginosa* was purchased from the American Type Culture Collection (cat. no. 27853; Manassas, VA, USA).

The bacteria culture medium contained 1.5% tryptone (g/100 ml), 0.5% soy peptone (g/100 ml) and 0.5% NaCl dissolved in distilled water (pH 7.2±0.2).

Establishment of a refractory wound model. Rats were anesthetized with an intraperitoneal (IP) injection of sodium pentobarbital (50 mg/kg) and their lumbar spine was then sheared. The wound area was marked with a plastic bottle cap of 2 cm in diameter. A 2 cm-deep skin defect was produced by a surgical method under aseptic conditions for intramuscular injection of cortisone sodium succinate (8 mg/100 g). A *Pseudomonas aeruginosa* suspension wrapped in alginate, which was used as a type of biofilm, was prepared with a microball maker (quantitative quality control strain; Hangzhou Microball Science & Technology Co., Ltd., Hangzhou, China). The bacterial concentration was 10^9 colony-forming units/ml. Next, 0.5 ml artificial *Pseudomonas aeruginosa* was injected into the wound surface of the animals to create a refractory wound model with bacterial biofilm infection.

The rats were randomly divided into five groups, namely a control group, model group, Cef control group, WXD control group and the QBM group, and treated for 20 days (n=6). The control group received water every day. The model and other groups were injected with 0.5 ml artificial *Pseudomonas aeruginosa* via the wound surface. Then, drug administration commenced at day 1 subsequent to the establishment of the model. The stomach and the wound surface were treated with saline every day. The wound surface was cleaned with 1:5,000 nitrofurazone solution prior to switching drugs. The rats in all groups had saline gauze topically applied according to the size of the wound. Then, two layers of aseptic and disinfected dry gauze were put on the wound, which was fixed with medical tape once daily. Concomitantly, each group was orally administrated with the corresponding drug (40 mg/kg) or physiological saline (10 ml/kg, once per day). The rats were anesthetized with an IP injection of sodium pentobarbital (50 mg/kg) and euthanized by decapitation following collection of the plasma at days 1, 3, 8, 15 and 20. One rat from each group was euthanized at each indicated time point. An area of 3x3 cm^2 containing wound tissue and surrounding skin was cut. Part of the tissue was fixed with 4% formaldehyde at room temperature for 24 h and part was frozen at -80˚C.

Measurement of AI-2 production in bacteria by Vibrio harveyi BB170 bioluminescence. Luminescence-based broth assays for AI-2 production were performed using *Vibrio v* BB170 as a biosensor strain. Briefly, *Vibrio harveyi* was cultured for 36 h on marine agar plates (cat. no. BD2216) at 26˚C. Single colonies were cultured overnight on a rotary shaker (150 rpm) in 5 ml Marine Broth medium (Shanghai Canspec Scientific Instruments Co., Ltd., Shanghai, China) until the reaching stationary phase at 26˚C. Then, the colonies were diluted 1:500 and cultured overnight. *Pseudomonas aeruginosa*, DH5α and *Vibrio harveyi* BB170 were cultured overnight. The indicated bacteria, while growing at stationary phase, were centrifuged at 12,000 x g for 10 min at 26˚C and then the supernatant was purified with 0.22-μm filtration membranes. A total of 180 μl diluted BB170 bacterial solution was mixed with the target bacterial supernatant at 26˚C for 2, 4 or 6 h.

Immunohistochemistry (IHC). IHC was performed on 5 μm sections of paraffin-embedded tissue with the indicated enzyme-labeled antibody at room temperature. Briefly, IHC using anti-HIFα monoclonal (1:200; cat. no. 36169; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-VEGF polyclonal (1:400; cat. no. AF-293-NA; R&D Systems, Inc., Minneapolis, MN, USA) antibodies was performed according to the manufacturer's protocol. For detection, the paraffin embedded sections (5 μm) were heated at 60˚C for 2 h and then deparaffinized with toluene and rehydrated in a graded alcohol series. To retrieve antigens, the sections were boiled for 15 min at 120˚C in 10 mM citrate buffer (pH 6.0). The sections were then washed 3 times with PBS at room temperature and pre-incubated with goat immunoglobulin G (IgG; cat. no. ab150077; 1:5,000, Abcam, Cambridge, UK) dissolved in PBS containing 1% bovine serum albumin (BSA; Thermo Fisher Scientific, Inc., Waltham, MA, USA; pH 7.4) for 1 h. Upon incubation with the primary antibodies overnight, the sections were washed

with PBS and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (cat. no. ab6721; 1:5,000; Abcam) in PBS containing 1% BSA for 50 min. Upon washing with PBS 3 times, the reaction was visualized with 3,3'-diaminobenzidine and H_2O_2 in the presence of nickel and cobalt ions. Nonspecific rabbit IgG (cat. no. ab188776; 1:5,000; Abcam) was used as the negative control and the sections were imaged by a fluorescent microscope (Olympus CX23; Olympus Corporaion, Tokyo, Japan).

ELISA. ELISA kits were used to determine the concentration of HIF-1α (cat. no. DYC1935-2; R&D Systems, Inc.), HIF-2α (cat. no. DYC1997-2; R&D Systems, Inc.), HIF-3α (Spbio, Wuhan, China) and VEGF (cat. no. MMV00; R&D Systems, Inc.) or interleukin-27 (cat. no. DY2274; R&D Systems, Inc.) in the bacterial supernatants. The indicated sample solutions were added to ELISA well plates and the specific-kit cytokine was bound via the immobilized antibody on the plate. Upon washing off the unbound substances, an enzyme-linked polyclonal antibody that was specific to each cytokine was added to the wells. Upon washing the unbound antibody-enzyme molecules, a substrate solution was added to the wells and the intensity of the color in the reaction developed proportionally to the quantity of specific cytokine bound in the initial step, as indicated in the manufacturer's protocol. The reaction was terminated and the color intensity was determined using a microplate reader at 450 nm.

Hematoxylin and eosin staining. The sections were heated at 60˚C for 2 h, deparaffinized with toluene and rehydrated in graded alcohol prior to being immersed in hematoxylin for 5 min at room temperature. The sections were then washed with acidic alcohol (0.5% HCl in 70% ethanol), immersed in PBS for 5 min and stained by several immersions in eosin for 3 min at room temperature. Upon removing the excess eosin, the slides were dehydrated with ethanol (75-100%), cleared with xylene and mounted with a xylene-based mounting medium. The stained sections were observed under a light microscope and images were captured.

Immunofluorescence detection of biofilm formation. For detection of biofilm formation, the sections were heated at 60˚C for 2 h, deparaffinized with toluene and rehydrated in graded alcohol. To retrieve antigens, the sections were boiled for 15 min at 120˚C in 10 mM citrate buffer (pH 6.0). The sections were washed 3 times with PBS at room temperature and then pre-incubated with goat IgG dissolved in PBS containing 1% BSA (pH 7.4) for 1 h. Subsequently, the sections were incubated with concanavalin A-fluorescein isothiocyanate (0.3 mg/ml) at 4˚C for 30 min. Upon washing with PBS, the sections were incubated for 15 min with propidium iodide (50 μg/ml). The sections were washed with PBS and mounted on slides (75x25x1 mm) with a drop of mounting medium. After sealing the edges with nail polish, the slides were stored in the dark at 4˚C. Antibody localization and cell structures were visualized with a confocal microscope.

Immunofluorescence detection of macrophages. For detection of macrophages, the sections were heated at 60˚C for 2 h, deparaffinized with toluene and rehydrated in graded alcohol. To retrieve antigens, the sections were boiled for 15 min at 120˚C in 10 mM citrate buffer (pH 6.0). The sections were washed 3 times with PBS at room temperature and then pre-incubated with goat IgG at room temperature dissolved in PBS containing 1% BSA (pH 7.4) for 1 h. Upon overnight incubation with primary antibodies against cluster of differentiation (CD) 68 (cat. no. ab125212; 1:1,000, Abcam), the sections were washed with PBS and incubated with HRP-conjugated goat anti-rabbit IgG in PBS containing 1% BSA for 50 min at room temperature. Upon washing with PBS, the sections were incubated for 7 min with DAPI at room temperature. Then, the sections were washed with PBS and mounted on slides (75x25x1 mm) with a drop of mounting medium. Upon sealing the edges with nail polish, the slides were stored in the dark at 4˚C. Antibody localization and cell structures were visualized with a confocal microscope.

Statistical analysis. Each experiment was performed at least three times, independently. A one-way analysis of variance followed by Dunnett's test analysis was used to compare the values in multiple-groups. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were conducted with the SPSS 19.0 software (IBM Corp., Armonk, NY, USA).

Table I. Healing rate of refractory wounds regulated by Cef, WXD or QBM.

	Day (%)				
Group	1	3	8	15	20
Model	3.10	18.10	38.00	45.00	55.00
QBM	4.50	23.50	42.00	54.10	65.50
WXD	4.40	21.40	38.60	53.20	68.60
Cef	7.10	28.40	45.00	68.20	90.20

QBM, Qingre Baidu mixture; WXD, Wu Wei Xiao Du drink; Cef, cefoperazone.

Results

QBM increases the healing rate of refractory wounds. The effect of the QBM on the healing rate of refractory wounds in rats was first evaluated. The results revealed that the QBM-treated group exhibited an increased healing rate of refractory wounds. However, there was no obvious difference between the Cef group and the WXD group at day 8 (Fig. 1A and Table I). No abnormalities were observed in the morphology of the skin of the sham-operated group during the whole experimental period, while the animals in the other groups displayed varying degrees of damage (Fig. 1B). Specifically, on day 1, the inflammatory cells were markedly concentrated in the wound tissue of these groups, while on day 3, the animals in these groups exhibited diffuse distribution of inflammatory cells. There was an increased concentration of fibroblasts in the Cef, WXD and QBM groups. Telangiectasia and granulated tissue were observed in the QBM group. On day 8, the granulated tissues and newborn epithelia were increased in the other 3 groups. On

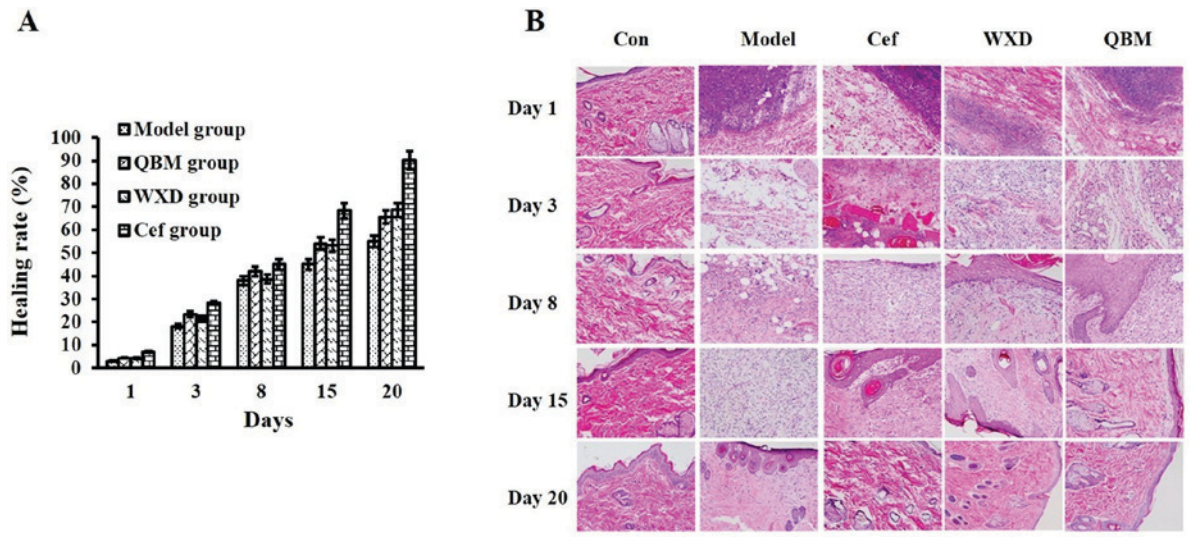

Figure 1. QBM promotes chronic and refractory wound healing. Rats were randomly divided into 5 groups and treated for 20 days. Next, the healing speed of the indicated groups was examined. (A) Different effects of the indicated treatments on the healing rate of refractory wounds. (B) Morphological changes of skin tissue in chronic and refractory wounds were examined by hematoxylin and eosin staining (magnification, x400). QBM, Qingre Baidu mixture; WXD, Wu Wei Xiao Du drink; Cef, cefoperazone; Con, control.

day 15, there was an increased number of capillary vessels in the granulated tissue of the model group. The epithelium increased in the Cef and WXD, while the fibers in the heat-clearing group increased. The number of collagen cells increased prior to returning to normal levels. The tissue expressed more fibroblasts and collagen, and it almost returned to a normal state, in the QBM group. On day 20, the epithelium expressed in the model group, while the tissue expressed more fibers and collagen prior to almost returning to a normal state in the other groups. In summary, the healing speed of each group was faster than the healing speed of the model group. The QBM group exhibited the best curative effect, since it promoted granulation growth and improved healing speed (Fig. 1B).

QBM downregulates AI-2 and inhibits the formation of bacterial biofilms in chronic and refractory wounds. Group differences in terms of the expression of AI-2 are presented in Fig. 2. Following wound infection with *Pseudomonas aeruginosa*, the expression of AI-2 peaked at day 8 in the control group. Compared with the model treatment, the QBM markedly downregulated the expression of AI-2. The level of AI-2 in the Cef and WXD groups was lower compared with in the control group, but it remained higher than that in the QBM group (Fig. 2A). To detect bacterial biofilm formation, immunofluorescence was performed. The results indicated that the biofilm formation rate was downregulated at day 20 in the QBM-treated group compared with the model group. This result indicated that QBM has a strong inhibitory effect on the formation of bacterial biofilms (Fig. 2B).

HIF-1α, HIF-2α and VEGF expression levels are upregulated by the QBM in chronic and refractory wounds. The present results revealed that the expression of HIF-1α, HIF-2α and VEGF in the serum of animals in the refractory wound model with bacterial biofilm infection was markedly upregulated by the QBM. The WXD also increased the level of HIF-1α, HIF-2α and VEGF, although not as markedly as the QBM. The effect of Cef on the expression of HIF-1α, HIF-2α and VEGF was decreased compared with the model group (Fig. 3A). Unexpectedly, the expression of HIF-3α in the serum of animals in the refractory wound model with bacterial biofilm infection was markedly upregulated by the WXD. The QBM also increased the level of HIF-1α, HIF-2α and VEGF, although not as markedly as the WXD. The effect of Cef on the expression of HIF-1α, HIF-2α and VEGF in the Cef group was decreased compared with in the model group (Fig. 3A). The same results were obtained by IHC for the HIF-1α and VEGF groups (Fig. 3B).

QBM recruits macrophages to chronic and refractory wounds. CD68 is a marker of macrophages. Macrophages rapidly engulf bacteria to restrict autoimmune disease or inflammation. The present study examined the content of macrophages in wounds. The results indicated that the QBM and WXD significantly recruited numerous macrophages to the wound compared with the model group ($P<0.01$). However, in QBM, WXD and Cef groups, the content of macrophages in the Cef-treated group was the lowest compared with the QBM and WXD groups (Fig. 4).

Discussion

Chronically refractory wounds require considerable medical resources every year. The key factor responsible for the failure of healing of such wounds is the presence of multiple species of bacterium, including *Staphylococcus aureus* and *Pseudomonas aeruginosa* with highly organized biofilms (11). Biofilm protection prevents the body's immune response from reaching the bacteria, which is an important factor in the failure of therapeutic treatment (12). The present study has demonstrated for the first time that the expression of AI-2 induced by the QBM on the biofilm of *Staphylococcus aureus* and *Pseudomonas aeruginosa* promoted the healing of chronic and refractory wounds (13).

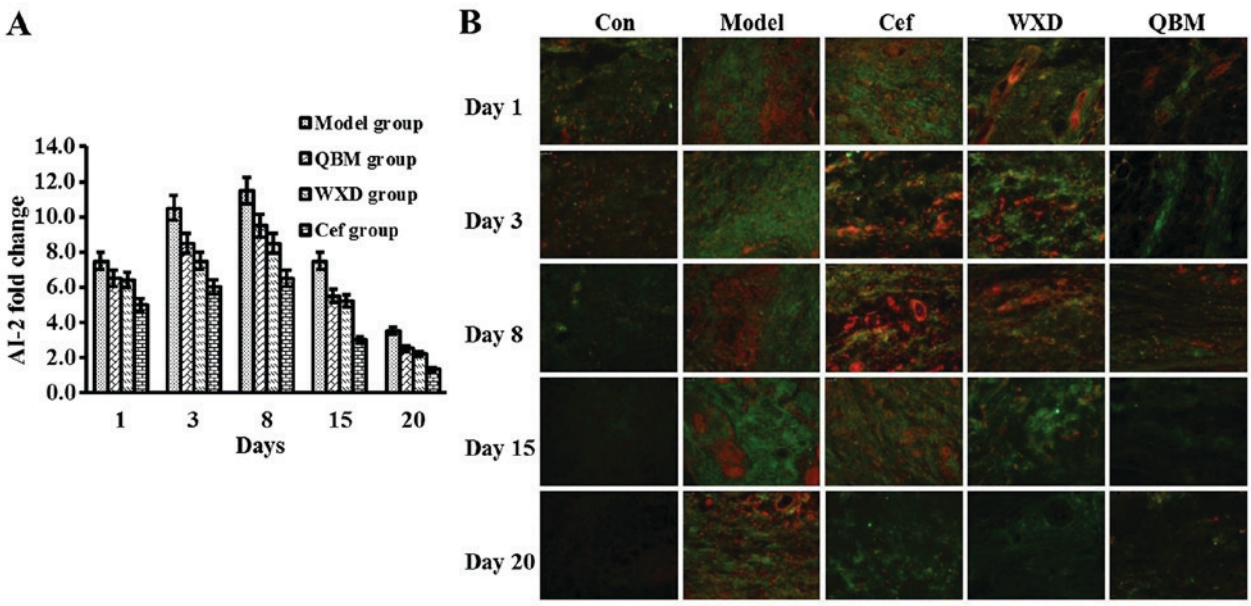

Figure 2. Detection of AI-2 activity during the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The expression of AI-2 on the biofilm of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was downregulated by the QBM. (A) AI-2 production in bacteria was measured by a *Vibrio harveyi* BB170 bioluminescent method. Data are presented as the mean ± standard deviation; n=3. (B) Biofilm formation was examined by immunofluorescence (magnification, x400). AI-2, autoinducer-2; QBM, Qingre Baidu mixture; WXD, Wu Wei Xiao Du drink; Cef, cefoperazone; Con, control.

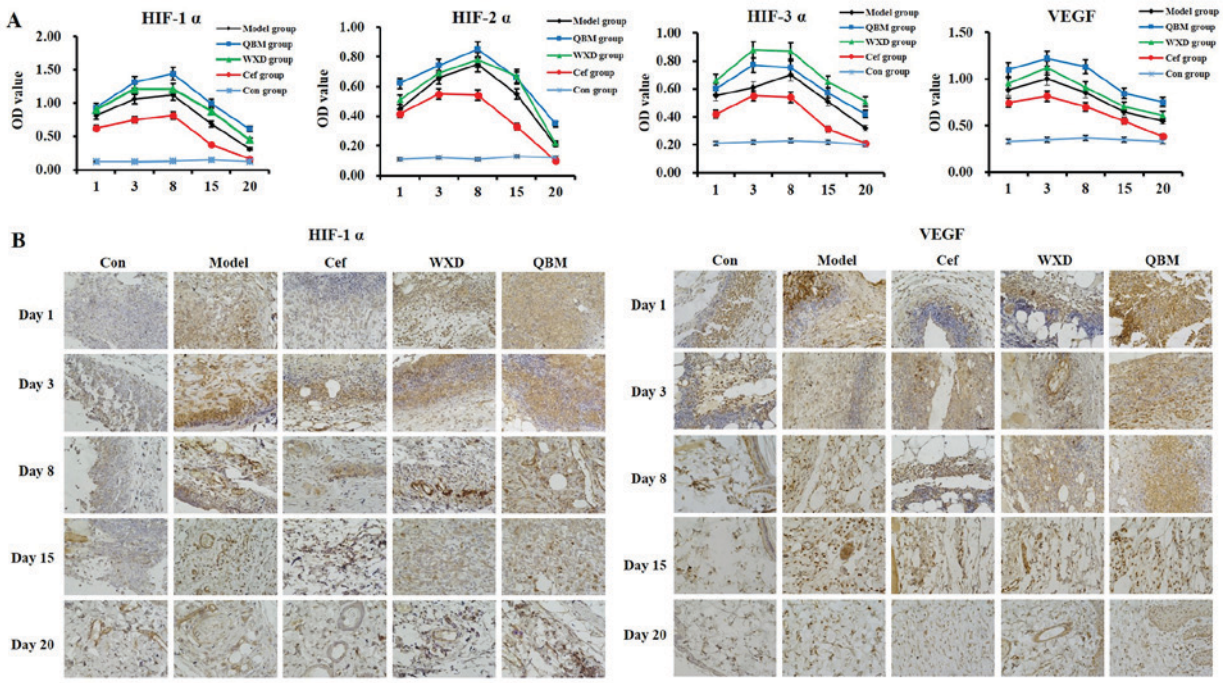

Figure 3. The expression level of HIF-1α, HIF-2α and VEGF in the serum of animals in the refractory wound model group with bacterial biofilm infection is increased by the QBM. (A) The protein content of HIF-1α, HIF-2α, HIF-3α and VEGF in the serum of animals in the refractory wound model was measured by ELISA. (B) Immunohistochemical staining of HIF-1α and VEGF was performed in the refractory wound animal model (magnification, x400). Data are presented as the mean ± standard deviation; n=3; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; QBM, Qingre Baidu mixture; WXD, Wu Wei Xiao Du drink; Cef, cefoperazone; Con, control.

The QBM is mainly composed of 10 herbs, including Radix Astragali, the root of red-rooted salvia and honeysuckle, which are traditionally used in the treatment of furunculosis and erysipelas. In the chronic refractory wound, the main types of bacterium detected are *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Therefore, the present study compared the effect of the QBM with another traditional Chinese medicine or Cef on the bacterial biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The QBM-treated group displayed a significantly increased healing rate in refractory wounds. However, there was no obvious difference between the Cef group and the WXD

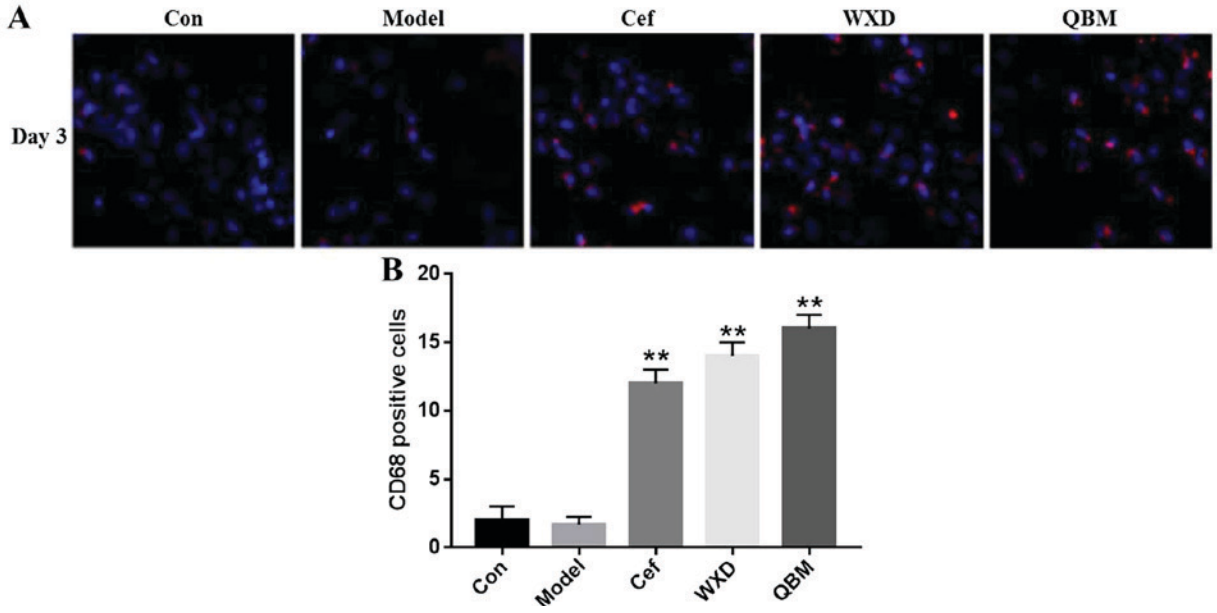

Figure 4. Macrophages are recruited to chronic and refractory wounds by the QBM. Antibody localization and cell structures were visualized with a confocal microscope. (A) Immunofluorescence staining of CD68 (red) and nuclear staining with DAPI (blue) were observed with a confocal microscope. (B) Quantification of CD68-positive cells in each group. Data are presented as the mean ± standard deviation; n=3; **P<0.01 vs. control group. CD, cluster of differentiation; QBM, Qingre Baidu mixture; WXD, Wu Wei Xiao Du drink; Cef, cefoperazone; Con, control.

group. Furthermore, the tissue expressed increased levels of fiber and collagen, and almost returned to a normal state, in the QBM group.

Quorum sensing (QS) is the cell-to-cell signaling system that reflects the ability of bacteria to respond to small signaling molecules secreted by various microbial species (14,15). The QS system is activated by extracellular receptors and is important in the formation of biofilms (16). Therefore, the QS system may be a key target for antimicrobial treatment. Numerous anti-infectious approaches against *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms have been studied during the past decade, including antibiotic combinations. However, several problems remain to be resolved (17), including the extended use of antibiotics, which results in a high prevalence of bacterial resistance. Currently, traditional Chinese medical compounds that inhibit QS systems are being investigated. AI-2 is one of the QS molecules that mediates intra- and interspecies communication. AI-2 is formed from spontaneous rearrangement of 4,5-dihydroxy-2,3-pentanedione, which is produced by the enzyme LuxS and is the primary QS molecule produced by Gram-positive and Gram-negative bacteria (18). AI-2 has been demonstrated to serve a pivotal role in biofilm formation, including in the initial bacterial aggregation and the production of virulence factors. AI-2 inhibits biofilm formation in *Bacillus cereus* (19), *Candida albicans* (20) and *Eikenella corrodens* (21), and promotes biofilm formation in *Escherichia coli* (22) and *Streptococcus mutans* (23). The present study demonstrated that the QBM down-regulates AI-2 in biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and inhibits the formation of bacterial biofilms in chronic and refractory wounds (24).

HIF-α and VEGF serve important roles in cancer progression in various cancer cell lines (25). Previously, HIF-α and VEGF have been demonstrated to exhibit a high level of expression when a bacterial infection occurs (26). The results of the present study revealed that the levels of HIF-1α, HIF-2α and VEGF were upregulated by the QBM. The WXD also increased the levels of HIF-1α, HIF-2α and VEGF, although not to the same extent as the QBM. However, the effect of Cef on the expression of HIF-1α, HIF-2α and VEGF was decreased compared with the model group.

Macrophages have been reported to participate in the induction of inflammation (27). Infections with human pathogens that require macrophages in the infected wounds for control and recognition include infections with *Staphylococcus aureus* and *Pseudomonas aeruginosa* (28). In addition to recognition of bacteria by the host immune system, macrophages are recruited to the wound site and phagocytose the invading organisms. In particular, deficiencies in macrophage phagocytosis, as well as bactericidal potential, have been associated with reduced bacterial clearance, as well as chronic and refractory wounds (29). The present study used CD68, a marker of macrophages, to detect the content of macrophages, and observed that the QBM recruited macrophages to chronic and refractory wounds. This result demonstrated that the QBM serves a prominent effect in anti-bacterial defense.

A previous study reported that bacterial biofilms served a key role in the emergence of resistance to antibacterial agents (30). However, it is widely known that a mature biofilm matrix may provide the conditions for bacteria to elude the host immune response while forming a barrier against the majority of conventional antimicrobial treatments (31). Furthermore, numerous biofilm-associated mechanisms of drug resistance should also be considered. Increased mutation frequencies have been described in biofilm cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, suggesting that the biofilm matrix provides an important environment to promote mutational resistance to antibiotics (32). In the present study, the effect of Cef on inflammation and wound healing in chronic

and refractory wounds was decreased compared with the QBM.

The present study examined the effect of the QBM-regulated biofilm formation of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in chronic and refractory wounds. The QBM upregulated the expression of AI-2, HIF-1α, HIF-2α and HIF-3α and increased the levels of VEGF. These results provide novel insight into the complex interrelationships between chronic and refractory wounds and bacterial biofilms, and open the possibility of treating chronic and refractory wounds with traditional Chinese medicines.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WS, YW, ZZ, JXi and JXu participated in experiment design, tissue collection and experiment execution. WX, YS and SG analyzed and interpreted the patient data, and were major contributors to the development of the first draft of the present manuscript. HQ conceived and designed the current study. WS and HQ reviewed and approved the final draft of the manuscript prior to submission.

Ethics approval and consent to participate

Ethics approval for the present study was provided by the Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine Animal Experimental Ethics Committee. The National Institutes of Health guide for the care and use of laboratory animals was strictly followed by the authors.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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