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RESEARCH ARTICLE

Promotive effects of four herbal medicine ARCC on wound healing in mice and human

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Abstract

Background: Traditional Chinese medicine (TCM) had been extensively used in China for wound management and had shown great potential in wound treatment while its mechanism is still needed to be addressed.

Objective: The present study sought to investigate the therapuetic effect of the TCM ARCC on acute and chronic wounds.

Methods: Here, using the ultra-low temperature preparation method, the mixed ultramicro powder prepared with Angelica (A), Angelica (R), Calcined Gypsum (C) and Caleramide (C) named as ARCC. The effects of ARCC on wound healing in adult and aged mice were comparatively evaluated through a full-thickness skin defect model. In addition, we randomly selected 10 patients aged 55 to 70 years from a cohort of 500 patients with diabetic feet to assess their prognosis.

Results: As the results showed that the healing rate had delayed in aged mice compared to adult mice, while ARCC prominently augmented the healing process in aged mice. Moreover, ARCC treatment wounds in aged mice showed accelerated re-epithelization, enhanced granulation tissue formation, and increased vascularization, which was similar to that of adult mice. Furthermore, ARCC also achieved therapeutic effects in diabetic foot patients, accelerating wound healing. The results found that foot ulcers improved significantly 7 days after the ARCC administration, and 80% of patients were healed within 1 month.

Discussion: In the present study, ARCC was found to have therapeutic effects on both acute and chronic wounds in animal models. ARCC also demonstrated

therapeutic effects in diabetic feet, which promoted wound healing, prevented wound infection, and avoided the risk of further surgery or amputation. All these evidences suggested ARCC was a promising approach for wound treatment.

Conclusions: ARCC might be recommended as a promising therapeutic medication in diabetic and chronic refractory wounds.

KEYWORDS

angiogenesis, diabetic foot, epithelization, traditional Chinese medicine, wound healing

1 | INTRODUCTION

Skin is the largest organ of our body and protects us against injury which is crucial to maintaining homeostasis. Restoration of skin integrity and function after the damage is of great importance for the underlying organs. The wound repair process is a natural biological response that involves three sequential and highly orchestrated phases, including inflammatory, proliferation, and tissue remodeling to heal the wound as fast as possible.[1,2] However, Disruption of this fine regulatory process can interrupt the precisely programmed cells and signaling pathways that are necessary for wound repair. It has been proved that a variety of pathophysiological conditions such as age, diabetes, and vascular disease can negatively regulate the wound healing process.[3] Along with the increasing global aging and the incremental diabetic patients, the incidence of chronic wounds increases each year.[4] Diabetes inhibits cellular infiltration, angiogenesis, and attenuation of wound contraction and closure.[5] Diabetic foot ulcers alone account for 15% of diabetics and are the most common cause of non-traumatic major amputations.[6]

Although chronic wounds are not life-threatening in the short term, they can seriously affect the patient's quality of life and carry a risk of amputation or extensive surgery because of infection.[7,8] Therefore, the primary goal of wound treatment is to heal the wound as soon as possible. Presently, topical negative pressure (TNP), skin substitute grafts, silver ion dressing, and synthetic, biological dressing were common current treatments for chronic wounds. TNP applies negative pressure on the wound, promotes a moist wound environment, removes necrotic tissue, keeps the wound surface clean, reduces edema, and increases blood flow.[9] Studies have shown that the MMP9/TIMP-1 ratio and phosphorylated MMP-9 level of wounds treated by negative pressure suction decreased significantly.[10] Grafts, such as autografts (derived from the patient's skin), allografts (derived from another person's skin), xenografts (derived from animals) may be a lifesaving intervention in burn patients and patients with the good basic situation.[11] Covering the wound, biological dressing can not only protect the new granulation tissue of the wound but also promote the granulation growth of the wound, thus promoting the wound healing.[12] The role of biological dressings in promoting wound healing is mostly limited to local areas, while patients with chronic wounds are mostly caused by systemic problems that lead to local wound nonhealing, so systemic comprehensive treatment is more critical. Comprehensively, patients with chronic wounds often respond poorly to the most advanced therapeutic treatments which suggest us to look for more alternative therapies. Currently, the use of traditional herbal medicine has increased for clinical management. According to the World Health Organization, it is estimated that more than 80% of the world's patients rely on traditional medicines for the treatment of various skin diseases.[13] In particular, multiple medicinal plants have been proven to promote wound healing in different experimental studies, such as excision, incision, and burn wound models.[14-16] In addition, herbal medicine has been used to treat patients with chronic wounds in China.[17-19] The effectiveness of herbal medicine treatment mainly depends on the traditional Chinese compound and the diagnosis and distinguish from disease status. According to Traditional Chinese medicine diagnostics, chronic wounds are characterized by blood stasis induced by the deficiency in Qi and Yin and then lead to blocking the channels. Compared with biological dressings, Traditional Chinese medicine plays the role of promoting blood circulation and removing stasis, not only locally, but also systemically.[20] To ameliorate and treat these pathological features, we selected four herbal medicines, Angelica Sinensis (A), Radix Rehmanniae (R), Calcined gypsum (C), and Calamine (C). Angelica, the medicinal site is the root, has the effects of removing blood stasis and clearing blood congestion.[21] Radix Rehmanniae, the dry root tuber of Rehmannia glutinosa, a family of black ginseng, owns the function of clearing away bacteria and anti-infection.[22] Calcined gypsum was heated quickly from gypsum block, calcined until red, and then was removed, cooled, and crushed. It was composed of anhydrous calcium sulfate, which shows the effect of stopping bleeding, restraining sores, and relieving itching.[23] Calamine, which is a kind of carbonate mineral calcite magnesite, possesses the effect of antipruritic with a debonaire feeling.[24,25] These four herbs appeared separately in a variety of ancient prescriptions and reported in a few kinds of literature, as the main pharmaceutical ingredients for the treatment of skin-related diseases, including various wounds and other skin diseases including secondary skin desquamation, neurodermatitis, and psoriasis.[23,26-30] Nevertheless, the combination of the four herbs (ARCC) has not yet been evaluated for their efficacy in wounds, and the possible mechanism remains unclear. In addition, the herbal medicine of ARCC has the advantages of wide sources, low price, minor toxic, and side effects. Therefore, here, we apply ARCC on the wound to find answers to these questions from both animal and human levels and further explore the promotion of wound healing.

2 | MATERIALS AND METHODS

2.1 | Materials and ARCC powder preparation

The decoction pieces of ARCC were purchased from Beijing Qiancao Medicine (Beijing, China), and the preparation of ARCC

superfine powder refers to previous technology with patent right protection (201210467065.8).[31] The superfine powder was sieved with a 200-mesh sieve and then was mixed by the ratio of 1:1:1:1:1 (ARCC). Rabbit anti-mouse CD31 (ab28364) and TGF-β1 (ab92486) polyclonal antibodies were purchased from Abcam (USA). Goat horseradish peroxidase (HRP)-labeled anti-rabbit immunoglobulin G (IgG) was purchased from Zhongshan Jinqiao Biotechnology (ZB-2301, Beijing, China).

2.2 | Animal model

All experiments were conducted with the institutional animal care and use committee (IACUC) of the Chinese PLA general hospital. Male C57BL/c mice (Vital River, Beijing, China, 4-6 weeks and 16-18 weeks) were kept in a semi-shielded environment and used to evaluate the effect of ARCC. All mice were raised and anesthetized based on international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The mice were anesthetized by intraperitoneal injection of 90 mg/kg 1% sodium pentobarbital. After shaving the dorsal portion of the body, a circular defect of full-thickness was made with a skin biopsy punch (10 mm diameter). Animals were allocated into four groups: Group 1: adult mice without treatment; Group 2: adult mice with ARCC treatment; Group 3: aged mice without treatment; Group4: aged mice without ARCC treatment. ARCC mixed with petroleum jelly was used to just fill the wound immediately after surgery and was administered every other day until healing. After surgery, animals were raised in separate and cages, and the rearing environment was the same as pre-operation. There were three mice in each group, and each experiment was repeated three times.

2.3 | Histological and immunohistochemistry examination

Three mice of each group were sacrificed to 7th/14th days (adult mice) and 7th/14th/21st days (aged mice), the wound area was

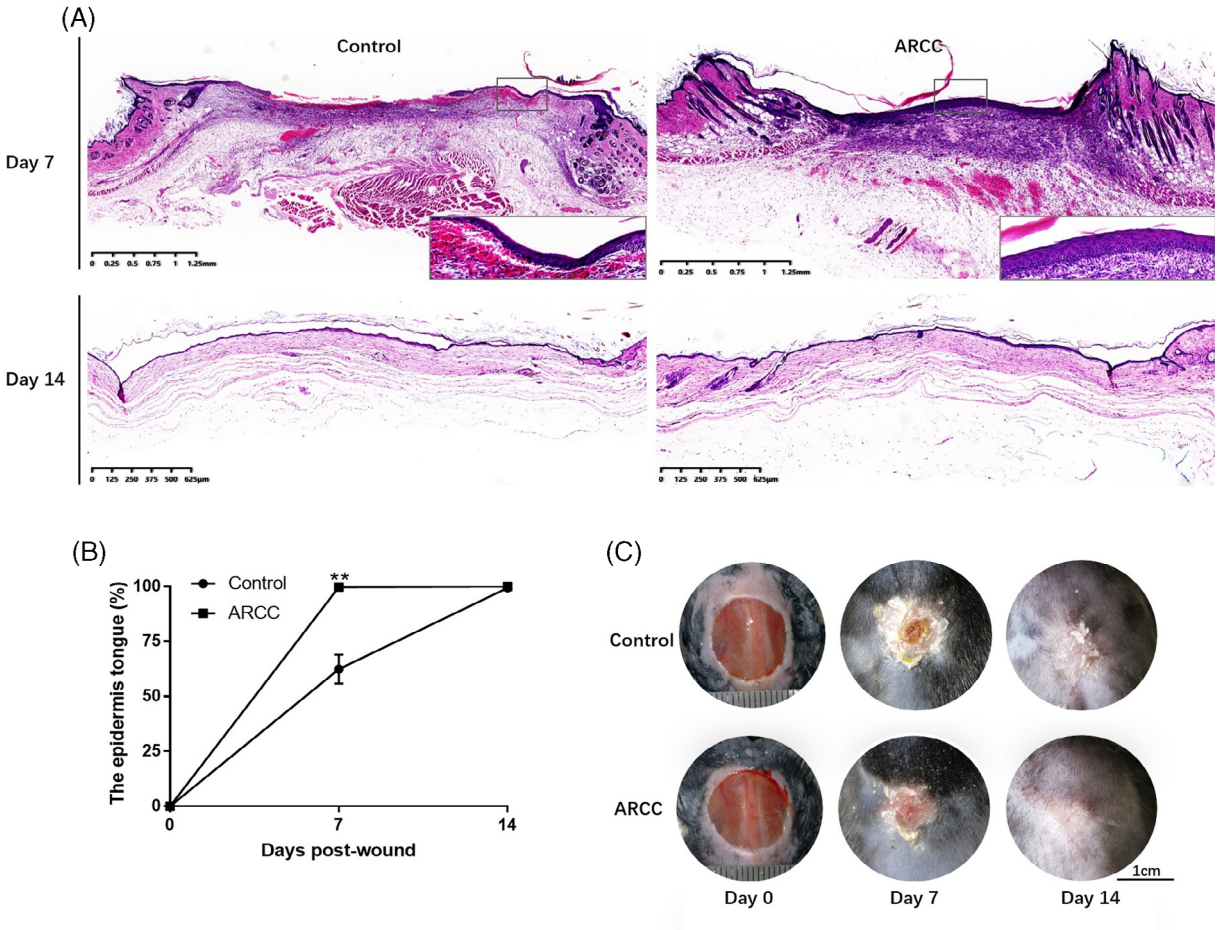

FIGURE 1 Histological and General observation of wound healing on mice (4-6w) on day 7 and 14 after wounding with or without ARCC treatment. (A) Histological photographs of full-thickness skin wounds at various time points. The enlarged part of the rectangle shows the new epidermis. (B) Proportion of new epidermal tongue at various time points. The data are expressed as the mean ± SD, N = 3, $^{**}P < .01$. (C) General observation of wounds at various time points

measured and the wound healing rate was calculated.[32] The collected wound tissues were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Each tissue block was cut to several 5-μm sections and was stained with hematoxylin and eosin (H&E) and Masson's trichrome, and was observed under a light microscope.[33] Sections also were immune-stained for anti-CD31/TGF-β1 to assess the formation of new blood vessels and the deposition of collagen.

2.4 | Wound healing assessment in humans

Between December 1, 2019 and 30, 50 diabetic patients aged 55 to 70 years with lower limb ulcers due to local persistent wounds were enrolled. Enrolled patients admitted to the surgery department of the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, and the herb medicine to treat wounds is a routine treatment method there. The baseline data of patients including sex, age, and ulcer size were collected. The ointment made mainly from ARCC and Vaseline was applied to the wound and observed and photographed every day.

2.5 | Statistical analysis

All calculations and statistical analyses were performed by using the GraphPad Prism 7.0 software (San Diego, California). The Student *t*-test was used to analyze the significance of any differences between the two groups. $P < .05$ was considered statistically significant.

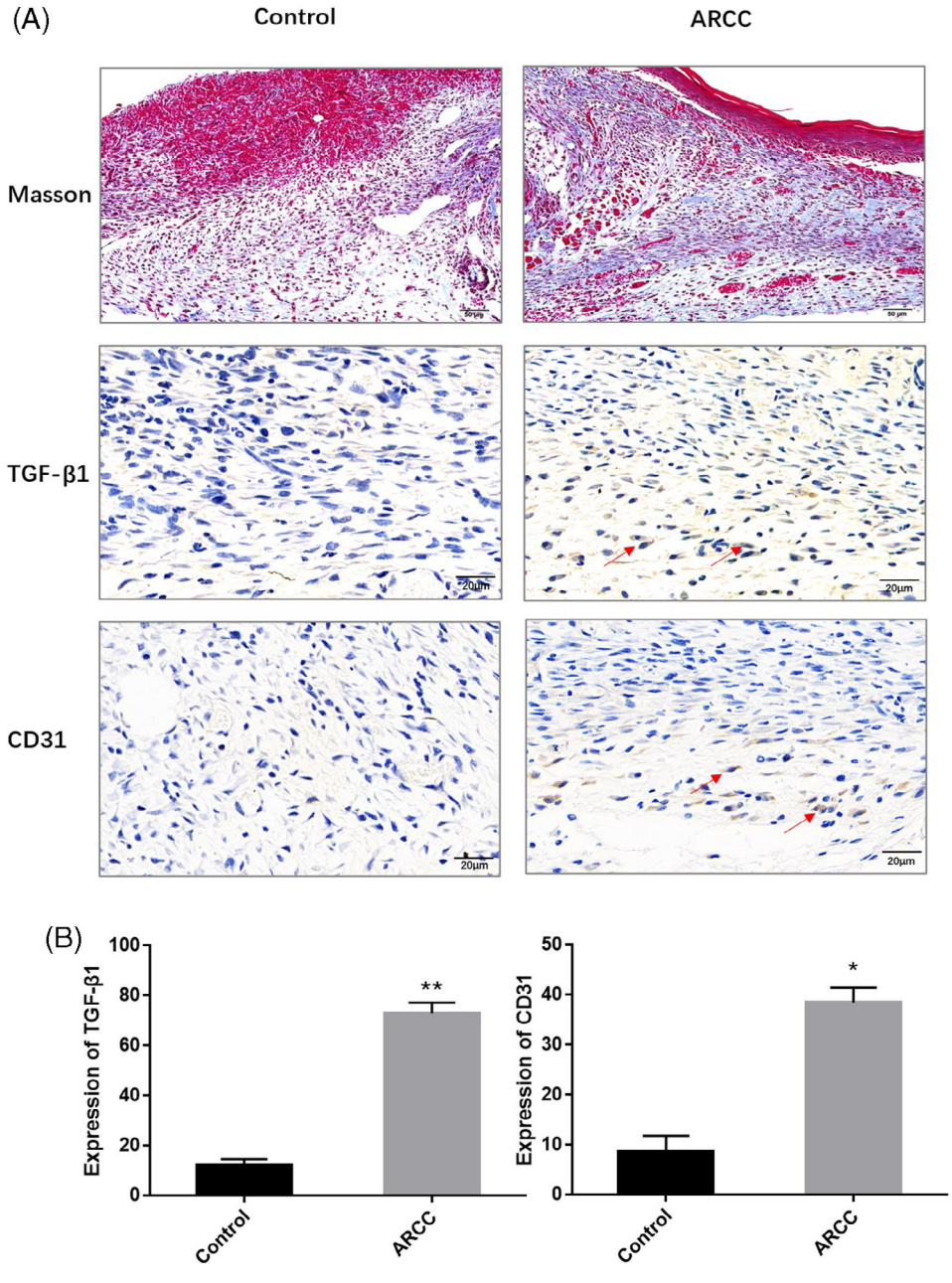

FIGURE 2 (A) Photomicrographs using Masson's trichrome and Immunohistochemical staining. The red arrows show cells that are positive for TGFβ-1/CD31 expression. (B) Quantitative analysis of TGFβ-1/CD31 expression at 7 days post wounding with or without ARCC powders. The data are expressed as the mean ± SD, N = 3, $^{*}P < .05$ and $^{**}P < .01$

3 | RESULTS

3.1 | ARCC accelerated wound closure and epithelization in adult and old mice

Mice in both groups were given the same modeling treatment with a circular skin defect (10 mm) of full-thickness on day 0. The wound-healing effect of ARCC was evaluated by tracing the wound on day 7. As shown in Figure 1B, wounds treated with ARCC (group 2) had completely healed on the seventh day, while there was a remaining unhealed wound area in group 1. On the 14th and 21st days, the wounds of both groups had healed. There was no significant difference in HE staining. In addition, crawling distance (the length of the epidermal tongue) covering the wound was used to evaluate the rate of re-epithelialization. As shown in Figure 1A, compared with group 1, the rate of re-epithelialization in group 2 was much faster than group 1 at different detection points. Also, the thickness of the epidermal tongue (59.34 μm) was significantly thicker than that of group 1 (22.74 μm), as shown in the enlarged area of Figure 1 A. Similarly, skin appearance in the ARCC treated group was also better than control in Figure 1C. Thus, ARCC exhibited a promotion role in wound healing of adult mice.

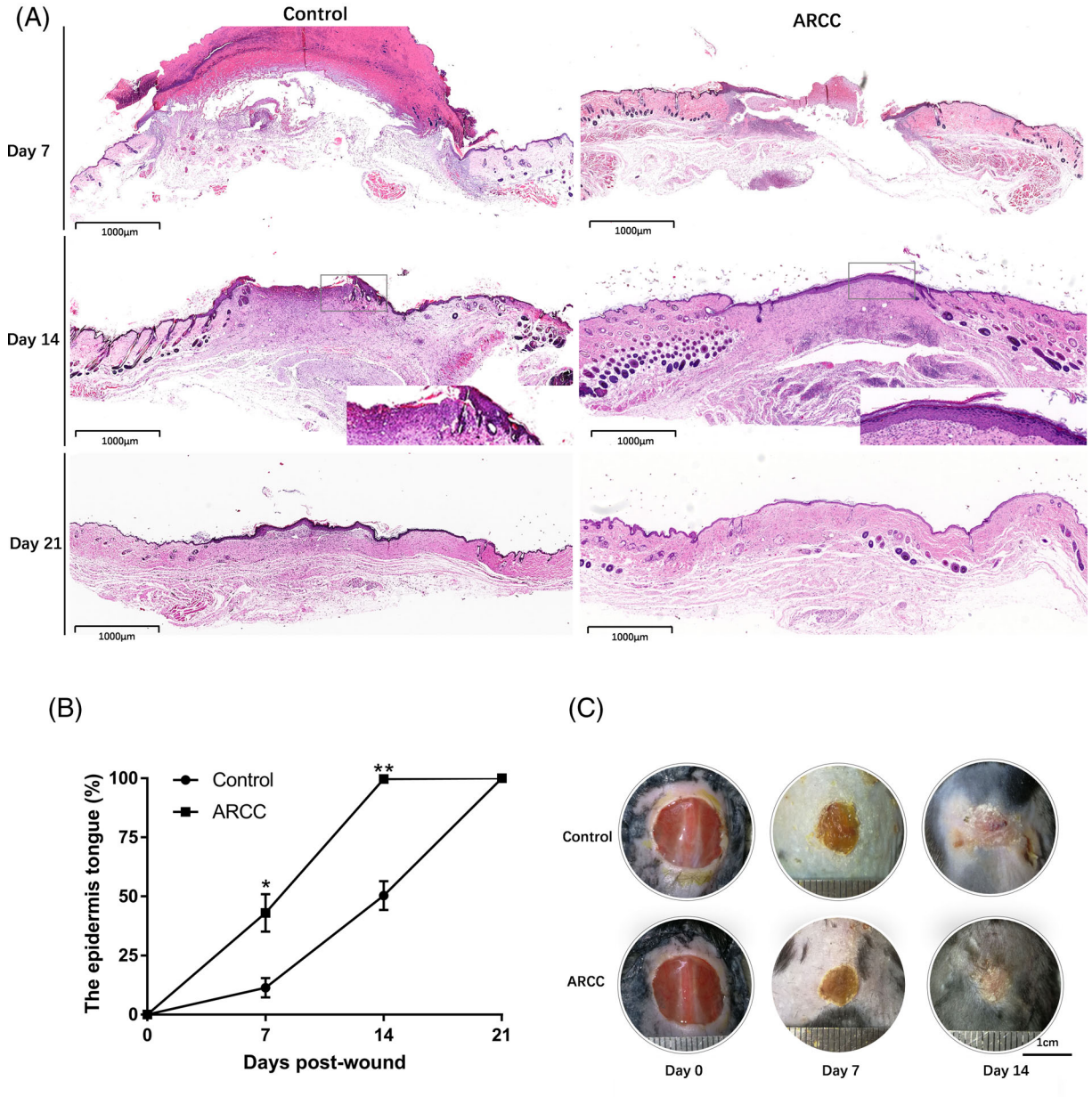

FIGURE 3 Histological observation of wound healing on mice (16-18w) on day 7, 14 and 21 after wounding with or without ARCC treatment. (A) Histological photographs of full-thickness skin wounds at various time points. The enlarged part of the rectangle shows the new epidermis. (B) Proportion of new epidermal tongue at various time points. The data are expressed as the mean ± SD, N = 3, $^{*}P < .05$ and $^{**}P < .01$. (C) General observation of wounds at various time points

3.2 | ARCC induced fibrosis and vascularization in wound healing in adult and old mice

Fibrosis can provide scaffolds for the repairing cells to grow, and rapid vascularization of the wound is conducive to the supply of nutrients. Masson's trichrome staining is one of the indicators for evaluating wound fibrosis. In acute wounds, day fifth-seventh is the remodeling period, which is the main period of collagen fiber formation. Similarly, mice in both groups were given the same modeling treatment on day 0. On day seventh after injury, numerous collagen fibers were gathered in wounds of Group 2, compared with sparse collagen deposition in Group 1 (Figure 2). Transforming growth factor 1 (TGF-β1) is a pleiotropic cytokine involved in multiple important events in wound repair, such as inflammation, angiogenesis, and collagen formation. In this study, the immunohistochemical results showed that the TGF-β1 positive cells in Group 2 were markedly more than those in Group 1 (Figure 2). It was previously found that the most angiogenesis occurred on the seventh day after wound formation. The new blood vessels in the granulation area were evaluated based on CD31 immunohistochemical staining in different groups on day seventh. And the CD31 positive cells in the ARCC treated group was significantly higher than that of the control. These results demonstrated that ARCC might promote wound repairing through fibrosis and revascularization.

3.3 | ARCC promoted wound healing in aged mice

Delayed or pathological healing conditions are often accompanied by pathophysiological features such as ischemia, chronic inflammation,

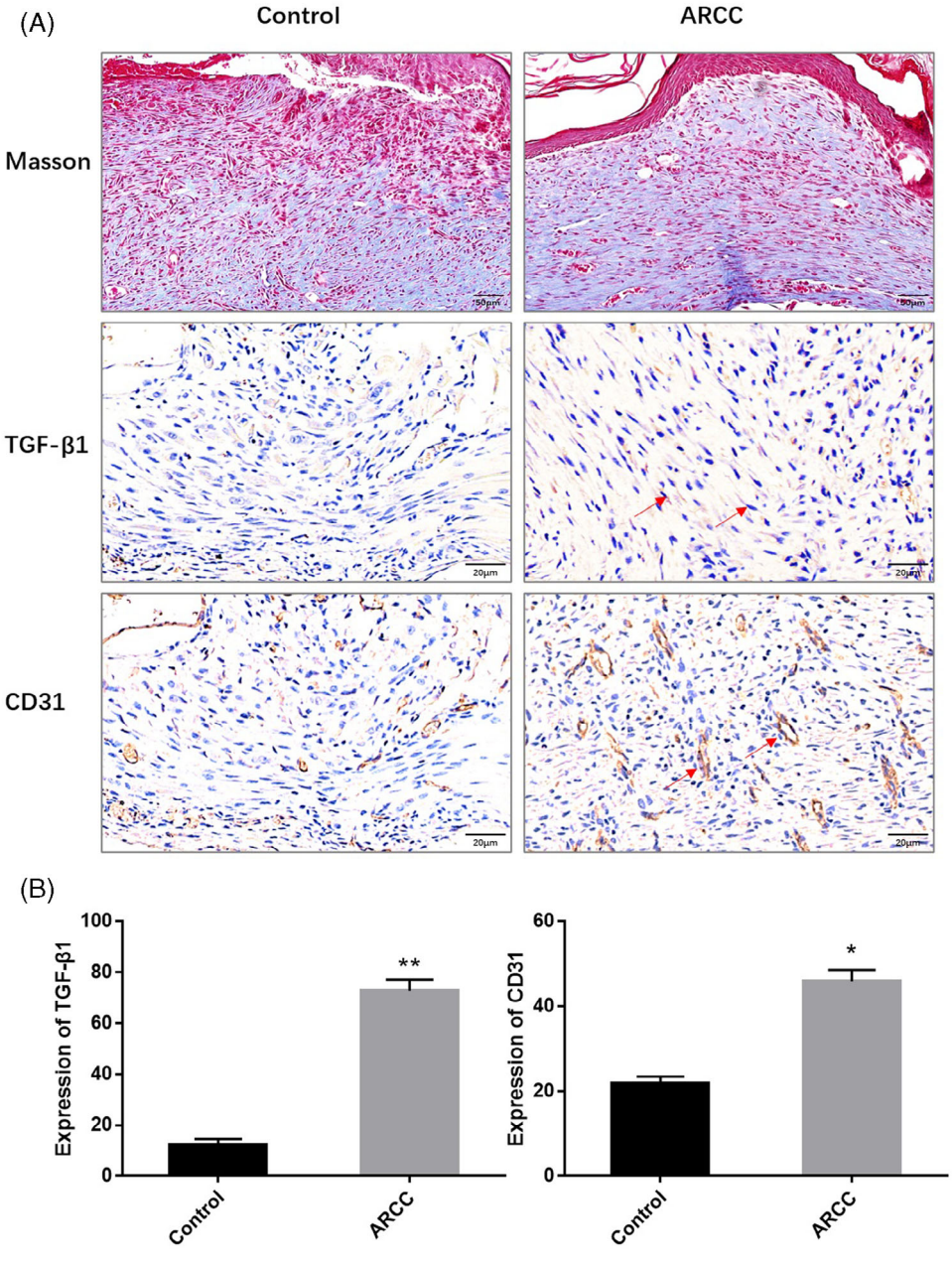

FIGURE 4 (A) Photomicrographs using Masson's trichrome and Immunohistochemical staining. The red arrows show cells that are positive for TGFβ-1/CD31 expression. (B) Quantitative analysis of TGFβ-1/CD31 expression at 14 days post wounding with or without ARCC powders. The data are expressed as the mean ± SD, N = 3, $^{*}P < .05$ and $^{**}P < .01$

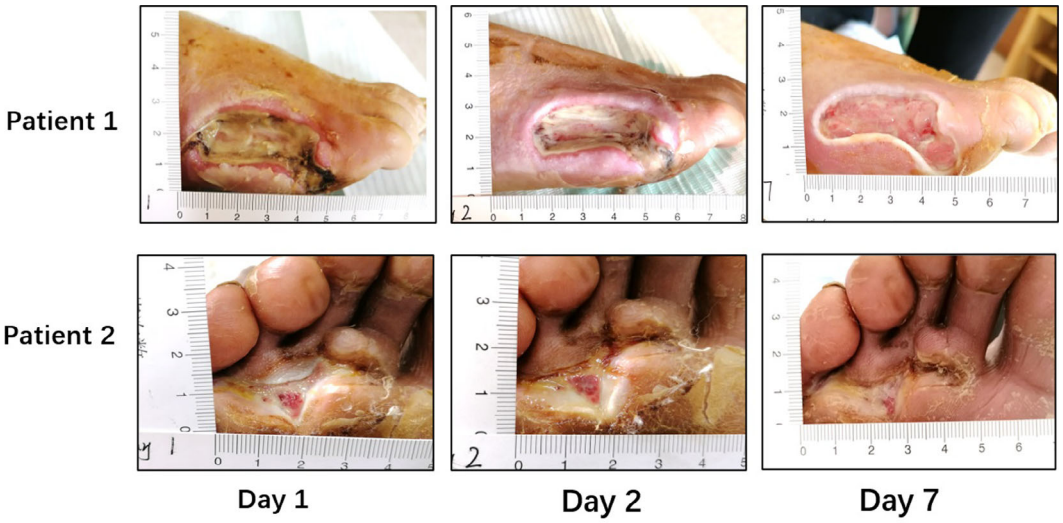

FIGURE 5 Representative skin photographs of diabetic foot at various time points after treating with ARCC containing ointment

TABLE 1 The baseline data of patients

Patient	1	2	3	4	5	6	7	8	9	10
Age	63	57	69	59	70	68	64	68	66	58
Sex	Female	Male	Male	Female	Female	Male	Female	Male	Male	Female
Duration (diabetic foot)	2 Weeks	1 year	1 Month	1 Week	1 Month	1 Month	1 Year	1 Week	3 Months	6 Months
Necessity of surgery	Yes	No	Yes	No	No	No	Yes	No	No	Yes
First attack (diabetic foot)	Yes	No	Yes	Yes	Yes	Yes	No	Yes	No	No
Wagner classification	Three	Two	Three	Two	Three	Three	Four	Two	Three	Four
Size of the wound (cm)	5 × 3	2 × 2	3 × 3	5 × 3	6 × 2	1 × 2	2 × 3	2 × 2	2 × 2	2 × 2
Wound position	Acrotarsium	Sole of Foot	Toe	Acrotarsium	Lower Leg	Sole of Foot	Toe	Sole of Foot	Heel	Toe
Healing rate (/week)	35%	50%	35%	35%	30%	35%	10%	35%	25%	10%
Healing time	1 month	14 days	21 days	14 days	1 month	21 days	No healing	1 month	1 month	No healing

and cell dysfunction.[34-36] Rapid vascularization can not only supplement the wound with more immune cells to remove necrotic tissue, but also provide nutritional support for repairing cells.[37,38] Therefore, we choose blood-activating and muscle-promoting natural medicines to improve the local and peripheral blood circulation of the wound. The wounds of aged mice showed similar pathophysiological characteristics of chronic wounds. In this study, full-thickness skin defects were made in aged C57BL/c mice of 16 to 18 weeks on day 0. And ARCC immediately applied to wounds after modeling. As expected, the healing rate of the aged mice was significantly reduced. As shown in Figures 1A and 3A, Wounds in adult mice were almost healed on day 14th, while the wounds in aged mice were still left with scabs. This suggested that wound healing in older mice was delayed. Nevertheless, after being applied to ARCC (group 4), the speed of the epidermis crawling to the center of the wound was significantly faster than that of the non-ARCC group (group 3) on the seventh day. The healing rate of wounds in Group 4 on day seventh (47%) was much higher than that of Group 3 (11%) (Figure 3B). Similar to adult mice, the wound was completely healed and re-epithelialization was also completed on day 14th in Group 4, which was faster than the 52% healing rate in Group 3 (Figure 3B). On day 21st, both groups completed healing. Furthermore, the thickness of the newly formed epidermis was significantly thicker in Group 4 than in Group 3, which was shown in Figure 3A. We further evaluated the vascularization and fibrosis effects of ARCC on wounds in aged mice. The immunohistochemical staining density of the pro-fibrotic cytokine TGF-β1 in Group 4 was significantly stronger than that of Group 3 on

the 14th day after injury (Figure 4). Moreover, the ARCC group showed greater collagen deposition with a more regular arrangement of bundles than those in the control group (Figure 4). More CD31 positive cells and a higher density of capillaries were also observed in the ARCC treated aged mice (Figure 4). These results demonstrated that ARCC could also promote the repair of chronic wounds through enhancing vascularization and fibrosis.

3.4 | Ointment with ARCC promote the healing of chronic foot gangrene

The patients diagnosed with diabetic feet were applied the myogenic ointment mainly containing ARCC and Vaseline on local wounds and got a daily dressing change. We treated more than 500 of these similar patients over 3 months. We randomly selected 10 patients during 55 to 70 years old and photographed them for recording every day. Considering the patients may require additional treatment, we set the endpoints of clinical studies to be 2 months. During the observation period of 1 month, except 2 patients showed insensitive to treatment, the wounds in other patients exhibited a significantly reduced exudation, a rapidly appeared red granulation, and a quickly shallow wound depth after ARCC treatment for 7 days, which was shown in Figure 5. The two patients with unhealed wounds still had not been cured by the end of the study. The results showed that the ARCC containing ointment could improve wound healing and help patients to avoid further operations or amputations. The basic information of these patients was shown in Table 1.

4 | DISCUSSION

The treatment of chronic wounds has been one of the most challenging issues in the field of regenerative medicine. Poor local blood supplies and insufficient epithelialization are the main obstacles for nonhealing wounds.[39] As mentioned above, there are various ways to treat chronic wounds at present,[9,11] but these treatments are mostly limited to the local area, which is difficult to solve the nonhealing of chronic wounds caused by systemic diseases, while the use of traditional Chinese medicine just could play the systematic and systemic role.[40] Moreover, compared with expensive advanced dressings, natural herbal medicine has the advantages of wide sources, low price, minor toxic and side effects.[41] In addition, the Micrograft technique in reconstructive surgery which minces tissue into micro-fragments 50 μm at least is an effective method.[42] In recent years, as a relatively efficient way in the field of surgery, the micrograft technique is useful for treating an intractable wound in clinical situations, which could accelerate the process of wound healing by promoting granulation tissue formation, regeneration of neovascularization, and wound contraction.[43,44] One of the few defects is that this method results frequently in unexpected scar formation.

As a conservative treatment, herbal medicine has attracted more and more attention and becomes an available resource for treating a variety of diseases.[45,46] Traditional Chinese medicine and extracts have been used for wound treatment and could significantly promote skin healing after external application, such as rosemary,[47] essential oil of peppermint.[48] Peppermint essential oil has effective antibacterial activity and accelerative effects on infected wound healing.[49,50] In addition, it was reported cinnamon essential oil has antioxidant and antibacterial properties and could accelerate wound healing through shortening the inflammatory phase, increasing collagen deposition, and promoting re-epithelialization.[5,51] The combination of herbs can achieve complementary effects and increase curative effects. Angelica Sinensis and Radix Rehmanniae as the natural botanical medicine, have a wide range of biological functions, including preventing infection,[52] regulation of immunity[53,54] and inhibition of tumor growth,[55-57] as well as treatment of skin diseases.[58] As natural minerals, calcined gypsum and calamine are widely used as wound dressings and biological materials.[59-62] However, the above four herbal medicine is rarely combined and applied to skin disease. The purpose of this study was to investigate the effect of a combination of traditional medicines (Angelica Sinensis, Radix Rehmanniae, Calcined gypsum, and Calamine, ARCC) on wound healing in an animal model and patients.

Here, whether it was an acute wound or a chronic wound, the application of ARCC could accelerate the speed of wound closure and re-epithelialization compared with the untreated group. Of course, the ointments only control group or another commercial dressing was not included, the ointments themselves may simply provide and maintain a moist environment. Just like the possible mechanism of the above-mentioned herbal medicines reported in the literature,[63,64] we have also observed corresponding evidence for the acute and chronic wounds of animals. It was found that the topical application of ARCC increased the density of neovascularization and enhanced the deposition of collagen as suggested by increased CD31 and TGF-β1 positive cells. These results confirmed that ARCC could accelerate the healing of both acute and chronic wounds. In addition, further application of the compound to diabetic foot wounds showed that ARCC could significantly improve the wound environment, promote wound healing, alleviate pain, which would further prevent wound infection, and avoid the risk of further surgery or amputation of patients. However, there have been cases of poor response to ARCC, which may be related to poor blood sugar control, significant blood vessel obstruction, and the location of the wound. All this evidence suggested ARCC was a promising approach for wound treatment, especially for a chronic wound. And these natural medicines have the advantages of fewer side effects, a wide range of sources, and economical cost, and are more easily accepted by elderly patients. Of course, one major limitation of this natural product is the lack of strict quality control due to the complexity of the active ingredients and variations in different production batches, which requires us to purchase from the formal Chinese herbal factory. In addition, the extraction of the main active components of Chinese herbal compounds is needed in the future.

In addition, in future research, we will mainly focus on two issues for ARCC: the first is the molecular mechanism of ARCC to promote wound healing. We should expand the clinical sample size to further clarify the therapeutic effect of ARCC in the clinic. ARCC-related key

signal molecules should be found through a large number of molecular and cell biology experiments. The second is the effective ingredients in ARCC that play therapeutic roles. A large number of researches show that traditional Chinese medicine achieves therapeutic effects mainly through its secondary products after metabolisms such as flavonoids, glycosides, alkaloids, and tannins. These metabolites show the property of anti-inflammatory, antioxidant, and promotion of collagen synthesis and re-epithelialization. Given that, it's worth identifying the main active components in ARCC.[65]

5 | CONCLUSION

We investigated the topical application of ARCC on wound healing in animals and humans. The results showed positive outcomes as the promoted indicators of healing. ARCC could accelerate the healing of acute and chronic wounds in adult and aged mice through accelerating wound closure and epithelization, inducing fibrosis, and vascularization. Meanwhile, it could promote the healing of chronic wounds in human diabetic foot ulcers. Therefore, it may be recommended as a routine treatment of wounds.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: Rungong Yang, Meirong Li.
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Formal Analysis: Lingzhi Zhong.
Funding Acquisition: Lingzhi Zhong.
Methodology: Lingzhi Zhong, Cuijuan Shi, Meirong Li, Qian Hou.
Project Administration: Meirong Li, Xiaobing Fu.
Resources: Cuijuan Shi.
Supervision: Meirong Li.
Validation: Cuijuan Shi.
Visualization: Cuijuan Shi, Rungong Yang.
Writing - Original Draft Preparation: Lingzhi Zhong.

All authors have read and approved the final version of the manuscript.

Corresponding author, Prof Meirong Li had full access to all the data in this study and takes complete responsibility for the integrity of the data and the accuracy of the data analysis.

DATA AVAILABILITY STATEMENT

The manuscript is an honest, accurate, and transparent account of the study being reported. The data that support the findings of this study are available from the corresponding author upon reasonable request.

TRANSPARENCY STATEMENT

The corresponding author of the article affirms that this manuscript is honest, accurate, and transparent.

ETHICS STATEMENT

Research involving animal and human rights: All animal protocols were approved by the ethics committee of PLA Hospital (The number of Welfare & Ethical Review in Animal Experimentation, 2019-x15-80). All surgeries and measurements were performed with sodium pentobarbital anesthesia, and maximum efforts were made to minimize suffering. All human subjects were by the ethical standards of the Helsinki Declaration of the World Medical Association and the skin images were collected from patients after obtaining signed informed consent forms by the ethics committee of First Teaching Hospital of Tianjin University of Traditional Chinese Medicine.

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