Biochemistry & Pharmacology: Open Access

Xichao et al., Biochem Pharmacol 2014, 3:4
DOI: 10.4172/2167-0501.1000140

Research Article Open Access

A Novel Application of Wutou (*Aconitum carmichaeli*) and Banxia (*Pinellia ternat*) Aqueous Extract on Wound Healing of Rats

Xia Xichao[1], Liu Hongyang[2], Wang Weina[1], Liu Fei[3], Hu Qingfu[1], Liang Guina[1], Zhang Dong[1] and Liu Rongzhi[1]*

[1]*Department of Basic Medicine, Nanyang Medical College, Nanyang, 473041, Henan Province, China*
[2]*Department of Plant Pathology, Nanyang Agriculture School, Nanyang, 473002, Henan Province, China*
[3]*Department of Clinical Medicine, Nanyang Medical College, Nanyang, 473041, Henan Province, China*

Abstract

Objective: The purpose of current study was to investigate effects of application of Wutou and Banxia aqueous extract in the wound rats.

Methods: Rats were fulfilled a surgical lesion with a 2.0 cm resecting tissue in the dorsal fascia. Following, animals were divided into 3 groups, including model group, control group treated with 1 mg/mL of Yunnan Baiyao, and Wutoubanxia group administrated 1 mg/mL of Wutou and Banxia extract. Wound contractions in day 0, 3, 7, 11 were calculated by an image analyzer. The histological analysis was detected using hematoxilin and eosin. The levels of tumor necrosis factor α (TNF-α), interleukin-2 (IL-2), transforming growth factor-β1 (TGF-β1), and basic Fibroblast Growth Factor (bFGF) transcripts in the wound tissue were determined by real-time quantitative PCR.

Results: Compared with the control group, rats in the model group showed poor re-modeling and re-epithelization characterized by a significant decrease of neovascularization, epithelialization and fibroblast. Furthermore, the expression levels of TNF-α, IL-2 were significantly increased, and TGF-β1 and bFGF significantly decreased in the model group in contrasted with that in the control group. By contrast, the treatment of Wutoubanxia extract reversed the above-mentioned conditions caused by wound.

Conclusion: The results suggest that administration of Wutou and Banxia extract has a promoting role in wound healing of rats possibly through enhancing anti-inflammatory ability and inducing fibroblast formation.

Keywords: Wutou and Banxia aqueous extract; Wound healing; Rat; Anti-inflammatory ability; Fibroblast formation

Introduction

In the resent years, wounds showed a gradually elevating-trend, and perhaps last for this status in the future with respect of unpredictable of natural disasters, increasing traffic accidents, abruptly physical injuries and so on [1,2]. Therefore, great efforts have been required to explore the candidates for wound healing and elucidate their mechanism in wound healing [3-5]. Process of wound healing is divided into three overlapping phases: inflammation (0-3 days), cellular proliferation (3-12 days), and remodeling (3-6 months) [6-8]. During the inflammatory stage, the pro-inflammatory cytokines are considered as key mediators to promote cutaneous inflammatory events [6]. In the second stage, the fibroblasts facilitate the formation of granulation tissue, proliferation moves into the wound, causing extracellular matrix production, connective tissue fibers and neovessels [8]. Accordingly, a new therapeutic candidate with more efficiency applied in the two stages should have a direct impact on the wound healing [9-11]. Meanwhile, it is exciting that great progresses have achieved along application of herbs in the wound healing [12-15].

Wutou, the axial root of *Aconitum carmichaeli*, has been extensively used to treat colds, polyarthralgia, diarrhea, heart failure, beriberi, and edema for thousands of years [16,17]. Banxia, the rootstock of *Pinellia ternate*, has a therapeutic effect on treatment of cough, infection and inflammation [18]. In Traditional Chinese Medicine (TCM), if several herbs are prescribed for treat a well-defined disease they should comply with formulas in which some herbs can't be used together *in vivo*, such as 18-against and 19-fear recorded in a classical TCM book [19]. Wutou against Banxia is an example of 18-against, but their use together in vitro remains large unknown [16,17,20,21]. Considering here, the current study was performed to investigate effects of Wutou and Banxia aqueous extract on the wound rats by analysis of the expressions of pro-inflammatory cytokines and fibroblast growth factors.

Methods

Preparation of aqueous extract

Wutou and Banxia were kindly provided by Hospital of Traditional Chinese Medicine of Nanyang Medical University. 350 g Wutou and 50 g Banxia were weighted, chopped and dried in shade, and powered mechanically. Power was immersed in water at room temperature for 4 h with constant stirring, boiled distilled water for 2 h, filtered through a filter paper, concentrated by rotary evaporator R52002K (Changyu Biochemical Instrument Factory, Shanghai, China). Residual was collected and dissolved in sterilized water to form Wutou and Banxia aqueous extract with a concentrate at 1 mg/mL.

Animals and treatment

Adult male Sprague-Dawley rats (150-200 g) were obtained at Henan Animals Center for Medical Science and Research, housed under standard conditions of temperature (22 ± 2°C), relative humidity (55 ± 5%) and light (12 h light/dark cycles). The animals care and use in

***Corresponding author:** Rongzhi Liu, Department of Basic Medicine, Nanyang Medical College, Nanyang, China, Tel: +86-0377-635-261-21; E-mail: liurongzhi@sina.com

Received May 01, 2014; **Accepted** May 22, 2014; **Published** June 02, 2014

Citation: Xichao X, Hongyang L, Weina W, Fei L, Qingfu H, et al. (2014) A Novel Application of Wutou (*Aconitum carmichaeli*) and Banxia (*Pinellia ternat*) Aqueous Extract on Wound Healing of Rats. Biochem Pharmacol 3: 140. doi:10.4172/2167-0501.1000140

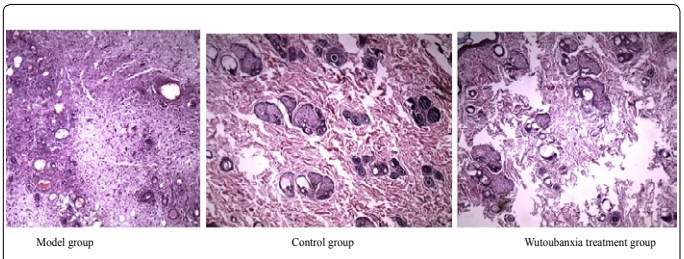

Figure 1: Histopathological view of epidermal/dermal in wound healing rats on day 11. Haematoxylin–eosin stain × 400. Control animals showing poor collagenization, fibroblasts and poor neovascularization. Control animals and Wutoubanxia treatment animal showing better healing and higher collagen deposition.

the experiment were approved by Nanyang Medical University Animal Care and Use Committee.

Wound healing activity was evaluated using excisional wound model. On the day 0, animals were anesthetized with 80 mg/kg ketamine and 10 mg/kg xylazine intramuscularly, and placed on operation table in its natural position. An impression was performed on area of 5 cm long × 4 cm wide, located in the thoracolumbar region. In the center trichotomized area was treated antisepsis with alcoholic clorexedine and fulfilled a surgical lesion with metallic punch with 2.0 cm in diameter resecting all tissue in the dorsal fascia. The rats were divided into 3 groups 30 animals per group, including model group, control group treated with 1 mg/mL of Yunnan Baiyao, and Wutoubanxia group administrated 1 mg/mL of Wutou and Banxia extract. All drugs were sprayed on wound by injector for twice daily in a volume of 100 ul.

Wound assessment and sample collection

Wound assessment starting from day 0, digital photograph was taken at day 0, day 3, day 7, and day 11. Wound size was measured by an image analyzer (image measurement standard v4.01, Bersoft, Puerto Plata, Dominican Republic) to assess the size changes during healing process. At corresponding days, 6 animals per group were deeply anaesthetized with chloralhydrate at a concentration of 350 mg/kg, the full-thickness biopsy specimens of dorsal skin including periwound margin were dissected either for histological analysis or immediately frozen in liquid nitrogen, and then stored at -80°C for real-time PCR analysis.

Wound contraction % = (original wound area – specific day wound area)/ original wound area × 100.

Histological analysis

The specimen sample were fixed in fixative (60% of absolute alcohol, 30% of formaldehyde, 10% of glacial acetic acid) and embedded with paraffin. The specimens were cut in 4 um thick sections, and then stained by routine Hematoxilin and Eosin (H&E) for histological analysis. Briefly, the slides were deparaffinized in xylene, and rehydrated in descending order of ethanol. Following, the slides were dipped in hematoxylin, washed in tap water and dehydrated in ascending order of ethanol. Finally, the slides were stained with eosin and washed with absolute ethanol and xylene. The sections were analyzed under a light microscope (Olympus BX50, Barcelona, Spain) at 400 × magnifications.

Quantitative real-time PCR

Total RNA was isolated from the wound tissue using TRIzol (Invitrogen Life Technologies, Shanghai, China) according to the protocol set by manufacturer. The quality of RNA was confirmed by 1.2% agarose gel electrophoresis. The first-strand cDNA was synthesized using RTase M-MLV reverse transcriptase (Takara, Dalian, China). The cDNA was used as the template for real-time PCR reaction.

To determine the gene transcription levels of tumor necrosis factor α (TNF-α), interleukin-2 (IL-2), transforming growth factor-β1 (TGF-β1) and basic Fibroblast Growth Factor (bFGF) transcripts in the wound tissue, real-time quantitative PCR was performed using SYBR Premix Ex Taq™ (Takara, Dalian, China) according to the manufacture's instruction with the primers that were synthesized by Sangon (Shanhai, China). PCR was performed using an ABI 7500 Real-Time Detection System (*ABI*, Foster City, CA, USA). The expression levels of TNF-α, IL-2, TGF-β1 and bFGF were calculated using $2^{-\Delta C_T}$.

Statistical analysis

The data were expressed as the mean ± S.E.M. The results of the study were analyzed by one-way analysis of variance (ANOVA) with the aid of Statistical Package for Social Sciences (SPSS) software. P Values with $P<0.05$ were considered as statistically significant.

Results

Effects of Wutou and Banxia extract on wound healing in excisional rat

Animals contractions in the Wutoubanxia treated groups were significantly increase from day 3 to 7 at a level 98.61%, day 7 to day 11 at 47.73%, and the complete wound healing occurred at day 11 compared with that of the model group. Similar results were also observed in the control group animal those s were received Yunnan Baiyao. Poor re-modeling and re-epithelization were detected in the model group. Faster keratinization characterized with minor intraepithelial cornification was occurred in the control group and the Wutoubanxia treatment group. Histopathological sections from Wutoubanxia treated group showed significant increase of neovascularization, epithelialization and fibroblast in which showed matured epidermis with keratinization and mature hair follicles, fibroblasts in dermis that are the proof of completion of healing in contrasted with the model group (Figure 1).

Effects of Wutou and Banxia extract on mRNA levels of TNF-α and IL-2 in excisional rats

TNF-α and IL-2 expression levels in rats of the model group, the control group and the Wutoubanxia treatment group were within the significant increase throughout the experiment compared with that of day 0. The mRNA levels of TNF-α and IL-2 was significantly increase in the model group (Figures 2 and 3) compared with that of the control group. After administration of Wutou and Banxia extract, the TNF-α expression decrease by 34.9% ($P<0.05$) during whole experiment (Figure 2). Moreover, the IL-2 expression reduced 28.9% ($P<0.05$) and 41.46% ($P<0.01$) at day 3 and day 7, respectively (Figure 3).

Effects of Wutou and Banxia extract on mRNA level of TGF-β1 in the excisional rats

TGF-β1mRNA levels in rats of the model group, the control group and the Wutoubanxia treatment group showed a fluctuation throughout the experiment. Compared with the model group, TGF-β1 expressions in rats of the Wutoubanxia treatment group were increased 114.3% ($P<0.01$) in the day 3, 72.3% ($P<0.01$) in the day 7, but decreased 55.4% ($P<0.01$) in the day 11 (Figure 4). The change tread in animals of the Wutoubanxia treatment group was also detected in those of the control group at corresponding time.

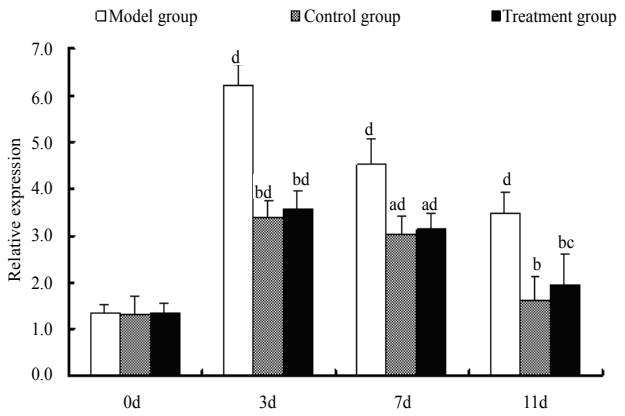

Figure 2: Effects of Wutou and Banxiag aqueous extract on the TNF-α mRNA levels in wound healing rats
N=6, [a]*P*<0.05, [b]*P*<0.01 vs model group, [c]*P*<0.05, [d]*P*<0.01 vs 0 day.

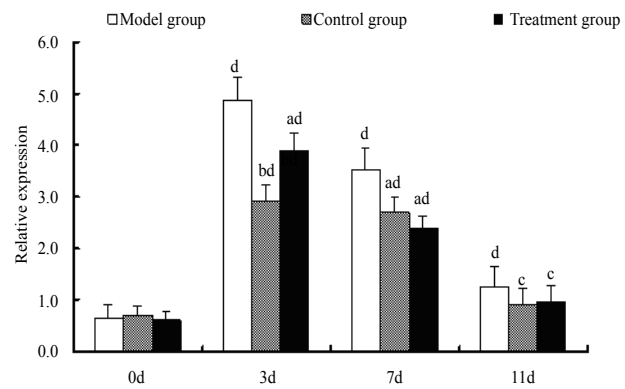

Figure 3: Effects of Wutou and Banxiag aqueous extract on the IL-2 mRNA levels in wound healing rats
N=6, [a]*P*<0.05, [b]*P*<0.01 vs model group, [c]*P*<0.05, [d]*P*<0.01 vs 0 day.

Effects of Wutou and Banxia extract on mRNA level bFGF in the excisional rats

Like the TGF-β1 mRNA levels change trend, the bFGF mRNA levels of rats in the three groups were also showed a fluctuation. Compared with that of the model group, the bFGF expression level in rats of the Wutoubanxia treatment group increased 74.7% (P<0.01) on day 3. On day 7, the bFGF mRNA level has not significant difference in contrasted with that of the model group, but was still significantly increase than that of day 0. Finally, it decreased 36.7% (P<0.05) in contrasted with that of the model group (Figure 5). Similar change tread was also detected in the control group.

Discussion

Tissue repair in the wound healing is a complex cascade of biological events regulated by numerous cytokines and growth factors that provide local signals to mediate cellular migration, angiogenesis, matrix synthesis, collagen deposition, formation of granulation and tissue remodeling [22,23]. In the CTM, many herbs play a positive role in the wound healing through acting on cytokines and growth factors [24,25].

Our results show that the administration of Wutou and Banxia aqueous extract contribute to wound healing in the incisions rats: an elevation of contraction, an acceleration of neovascularization, epithelialization and fibroblast, a down-regulation of TNF-α and IL-2 expression were observed. These results suggest that the application of Wutou and Banxia extract in wound rats has a promoting-role on wound healing. Inflammation cascade, occurring in the original stage of wound healing, is initiated by the innate immune system that pro-inflammatory cytokines, such as TNF-α, IL-2, IL-6 and IL-8, have a key role in the inflammatory response [26,27]. Inflammatory response is taken as a direct pathogenic-signal in the traumatized tissues [28]. Previous studies demonstrated that over-expression of these pro-inflammatory mediators may lead to wound condition deterioration [29]. Meanwhile, inflammatory response of wound tissue is coordinated by the interplay of anti-inflammatory cytokine and pro-inflammation cytokines, where a low level of pro-inflammation cytokines and/or a high level of anti-inflammatory cytokines should be helpful to tissue repair [30]. So, one key factor of wound healing acceleration from Wutou and Banxiag treatment is attribute to suppress of TNF-α and IL-2 expression.

In addition, remarkable gene up-regulation of TGF-β1 and bFGF in response to Wutou and Banxia extract treatment that reveals administration of Wutou and Banxia results in an acceleration of fibroblast formation in the wound healing through regulating certain

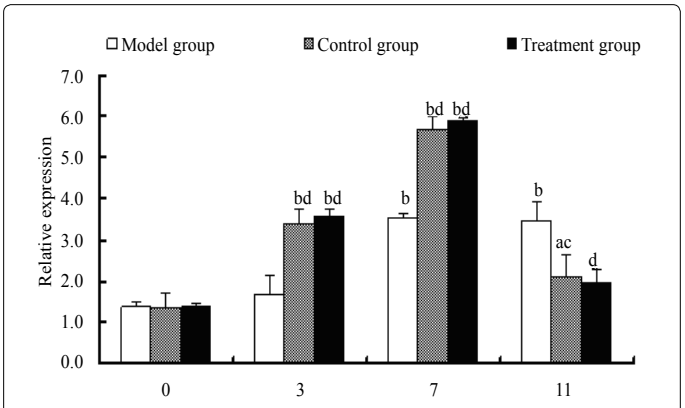

Figure 4: Effects of Wutou and Banxiag aqueous extract on the TGF-β1 levels in wound healing rats
N=6, [a]*P*<0.05, [b]*P*<0.01 vs model group, [c]*P*<0.05, [d]*P*<0.01 vs 0 day.

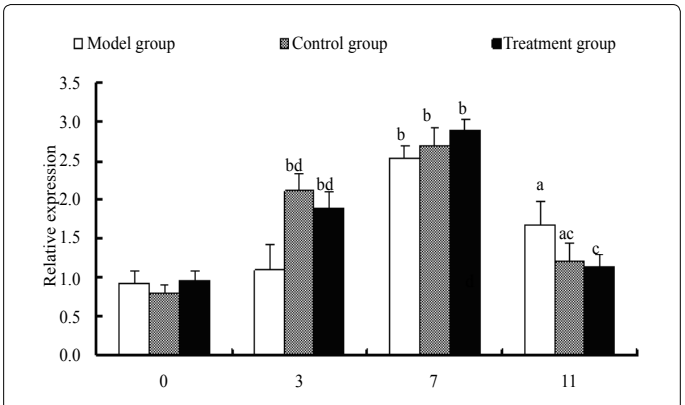

Figure 5: Effects of Wutou and Banxiag aqueous extract on the bFGF mRNA levels in wound healing rats
N=6, [a]*P*<0.05, [b]*P*<0.01 vs model group, [c]*P*<0.05, [d]*P*<0.01 vs 0 day.

genes expression. Literature has indicated that induction of TGF-β1 expression contributes to wound healing because TGF-β1 may initiate fibroblast, collagen synthesis and extracellular matrix formation [31,32]. In addition, TGF-β1 also has an ability to stimulate the expressions of other cytokines, such as matrix metalloproteinase 9 vascular endothelial growth factor-A and monocyte/macrophage chemotactic protein-1 [33]. bFGF, an important Fibroblast Growth Factor, can stimulate re-epithelialization and mediates in mesenchymal-epithelial interactions to promote epithelial proliferation and migration within the wounded area, which facilitates differentiation of new epidermis once combination with its receptor through [34-36]. Based on mentions, administration of Wutou and Banxia extract causes the induction of TGF-β1 and bFGF that may lead to fibrosis in the cellular proliferation stage of wound rat [23,37,38].

Wutou and Banxia are considered as an instance of 18-against in the classical TCM book [39,40]. In the present study, Wutou co-administration with Banxia plays a positive role in wound healing by application in vitro that is paradoxical with the traditional view. In the earliest pharmacopeia of China, Wutou possesses an evil reputation in duce it is consider as an extremely toxic plant in which toxic aconite alkaloids are contained which serve as highly toxic to myocardium, nerves, stomach and intestine through hyperpolarization and activation effect on the voltage-dependent sodium channels of the [41,42] Banxia also has few side effects and can leads to tongue numbing and swelling, salivation, slurred speech, and hoarsenes [42]. For a long time, the exact reasons of that Wutou and Banxia can't be used together in vivo remain large unknown [39,40,43]. But, the medical affect of Wutou combination with other herbs in vitro are constantly explored in resent years. The combinational use of *A. carmichaeli* and *Paeonia lactiflora* was showed to produce better efficacy in preventing and curing secondary adjuvant arthritis in rats [44,45]. Modern clinical studies have evidenced that the application of Fuzi, the lateral root of *A. carmichaeli*, in combination with other herbs have anti- arthritis property in the therapy of patients, indicating its promising therapeutic potential against arthritis [46,47]. These studies reveal the application of Wutou co-administration with Banxia in vitro should has potential scenario and is deserved to shed light of mechanism. So, there is still a long and exciting road ahead to fully understand and explain the mechanism of Wutou and Banxiag aqueous extract in the wound healing.

Nevertheless, the features of TNF-α, IL-2, TGF-β1, and bFGF acted by Wutou and Banxiag extract is not similar, for example the disparity of these genes expression in different time of sampling, imply that is likely related with order of these elements involved in cascade reaction in wound healing. On another hand, it is likely that many components existed in the Wutou and Banxiag have different regulatory pathways on these growth factors.

The current study demonstrated that treatment of Wutou and Banxia aqueous extract promote wound healing activity in rat incision. Further study shows the down-regulation of TNF-α and IL-2 expressions, and up-regulation TGF-β1 and bFGF expressions from Wutou and Banxia extract treatment is one value pathway to promote the wound healing.

Acknowledgement

We gratefully acknowledge the contribution of Pro Liu Rongzhi to this research program. This research was funded by the National Natural Science Foundation of Henan (No. 12B35002).

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