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Flavonoids accelerate wound healing of pressure sore by promoting angiogenesis: Potential mechanism

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Abstract: Pressure ulcers can progress in four stages based on the level of tissue damage from discoloration of the upper layer of skin to the ulcers of the deep skin. These sores extend below the subcutaneous fat into your deep tissues like muscle, tendons and ligaments. Pressure ulcers bed sores, are the most common complications after surgery and are difficult to treat. Flavonoids Liquid is a typical traditional Chinese medicine, which composed of *Rheum officinale and Abelmoschus manihot.* Recent study showed that the aqueous extract of *Rheum officinale (da huang)* possesses cytoprotective, antioxidant and wound healing effects. In this study, we investigated the role of flavonoids on pressure ulcer wound healing and its potential mechanism. A rat model mimicking pressure ulcer was developed and used to test the efficacy of flavonoids in ulcer healing. It was found that flavonoids significantly increase wound healing of pressure ulcer. Moreover, flavonoids promoted the expression of biomarkers related to angiogenesis in the rat model. In conclusion, flavonoids could increase the vasculature in wounds and could be used for treatment of pressure ulcer or bed sores.

Keywords: Ulcer, wound healing, *Rheum officinale and Abelmoschus manihot.*

INTRODUCTION

Pressure ulcer or bed sores are very common localised skin damage results in long term pressure and friction. Due to which the injured skin losses the barrier function to provide defence against infections, it requires rapid medical care and treatment. Usually, synthetic drugs and artificial skin are used to cover the wound site for wound healing (Mariam *et al*., 2018). However, these materials are expensive and may cause allergic reaction and drug resistance. Therefore, there are needs for alternative drugs and new treatment methods (WU *et al.*, 2012). Treatment of pressure ulcers requires a multidisciplinary strategy based on the underlying diseases, infections and wound conditions. Traditional nursing practice, including topical antibiotics, antibiotic-containing dressings, preservatives and even debridement, are used routinely to control infections and promote wound healing. However, these treatment methods are not efficient for every wound. Some wounds do not respond to these therapies and eventually worsens the wound healing process ((Robert *et al*., 2015) Trivedi *et al*., 2014). Currently, the emergence of multidrug-resistant bacteria has become a serious and common problem in pressure wounds, resulting in longer hospital stays, increased financial burden, morbidity and, in some cases, higher mortality rates (Trivedi *et al*., 2014). Therefore, there is an urgent need to develop novel treatment for pressure sores.

Traditional Chinese Medicine (TCM) is the main source and asset for alternative medicine. Flavonoids Liquid is a typical TCM, which composed of *Rheum officinale and Abelmoschus manihot. Rheum officinale (da huang)* has been used for hundreds of years for a variety of purposes ((Hong *et al*., 2020). Due to its flavonoids, tannins and triterpenes, the extract of *Rheum officinale* has antioxidant, anti-stress, immunoregulatory and anti-inflammatory properties (TRUJILLO *et al*., 2017). In fact, the aqueous extract of *Rheum officinale* shows cytoprotective, antioxidant and wound healing effects. Recently, there is a report that the extract of *Rheum officinale* has a positive effect on the different stages of wound healing in rats and has a significant healing potential in wounds (Lopez-Lopez *et al*., 2014). However, the mechanism of wound healing after flavonoids treatment remains unclear. Moreover, flavonoid has not been fully investigated in the treatment of pressure ulcer. Vascular lesions or peripheral arterial dysfunction are major risk factors for ulcer healing (WU *et al*., 2007). Impaired skin perfusion leads to impaired wound healing and the ability to produce sufficient immune response, as well as the inadequate delivery of antibiotics to the site of infection. Individuals with impaired perfusion express angiogenic molecules to promote angiogenesis (Trujillo *et al*., 2017). Angiogenesis refers to the formation of new blood vessels that respond to tissue growth, hypoxia, and repair. Normal angiogenesis relies on the complex balance between angiogenic and antiangiogenic i.e tyrosine kinase 1 (sFlt-1) and activin A and is associated with endothelial cells (ECs) and keratinocytes, which are characterized by extracellular matrix rearrangements, EC proliferation, migration, vessel wall morphology change and match (Carmeliet, 2005). Extensive microangiopathy delays cell infiltration, granulation tissue formation, angiogenesis, collagen synthesis and recruitment of vascular progenitor cells that interfere with normal

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healing processes (Lopez-Lopez *et al.,* 2014; Gonzalez-Curiel *et al*., 2014). Although several angiogenic molecules have been reported in the pathology of pressure ulcer, there are few reports on the expression of vascular endothelial growth factor (VEGF) and angiogenin (ANG) after flavonoids treatment.

In this study, we investigated the effect of flavonoids on wound healing in a pressure ulcer rat model and explored the potential mechanism of wound healing process.

MATERIALS AND METHODS

Animal model

The pressure ulcer rat model was established by cyclical magnetic compression as previously reported. (Reid, R. JSR, 2004). Briefly, 230g-250g SPF SD rats were anesthetized by isoflurane inhalation, followed by fur shaving to form a 4cm × 3cm rectangular isolated area on the back. The isolated skin area was sterilized by 0.5% iodine solution. Thereafter, 0.5cm inferior to the left shoulder, a 3cm skin transverse incision was generated and blunt dissected. A sterilized magnet was tunnelled under the skin and fixed by the fascia. Finally, the incision was sutured by 6-0 polyglycolic acid thread. The ulcer was achieved by cyclical magnetic compression where a permanent magnet was applied extra corporeally to the implantation area of the rats for 2hr to cause ischemia. After that, the magnet was removed for blood flow reperfusion of the skin for 0.5 hr. The procedure was repeated 5 times per day for 3 days. Phase means stages 3 pressure sore and above pressure sore were judged 3 days after operation by wound area erosion and needling without bleeding. r\

Group were settled as follows: Negative control treated with physiological saline (K); 50mg/ml of Total Flavones of A. manihot (ZD); 100mg/ml of Total Flavones of A. manihot (ZG); positive control treated with 50mg of rhVEGF165 (P).

After the establishment of the pressure ulcer rat model, rats were randomly divided into 4 groups treated with various conditions of drugs. There are 4 treatment periods in each group (3, 7, 14 and 21 days) and 4 rats in each period.

Granulation tissue proliferation or fibrosis was assessed according to criteria listed in table 1.

Regeneration of epithelial tissue around the ulcer was assessed based on criteria listed in table 2.

The wound closure rate

The ulcers of rats were imaged at day 0 means 1st day and the end of treatment. The area of sore was measured by Computer Aided Design (CAD) software. The original sore area at day0 and sore area at the end of treatment were recorded as S0 and Sn respectively. The wound closure ratio (%) = (S0-Sn)/S0 x 100%

Detecting the expression of angiogenesis related biomarkers by ELISA

The expression of VEGF, AngI, AngII, NO and Endothelin in wound tissues were quantified using corresponding ELISA kits (Elabscience), according to manufacturer's instructions. Briefly, 50 µL of standard or samples were added into each well and subsequently mixed with 50µL Biotinylated Detection Antibody. After incubation for 45 min, wells were washed 3 times by wash buffer. Thereafter, 100µL of HRP Conjugate were added into each well followed by 30 min incubation at 37^{o}C. After 4 washes, 90µL of Substrate Reagent were applied and incubated for 15 min at room temperature. Following addition of 50µL of Stop Solution, expression levels of the indicated molecules were determined immediately by measuring the OD_{450} using Multiskan MK3 plate reader (Thermo Fisher Scientific).

Immuno Histochemistry

Standard immune histochemical staining was used for detection of VEGF and eNOS. Fresh tissues were made into paraffin sections followed by dehydration. Heat-mediated antigen retrieval was achieved in citrate buffer solution (pH=6). Tissue sections were incubated with 3% H_2O_2 for 25min to block endogenous peroxidase. Following 30min blocking in 3% BSA, tissue sections were incubated overnight at 4^{o}C with primary antibodies probing VEGF and eNOS. The next day, sections were washed with PBS and subsequently incubated with corresponding HRP labelled secondary antibodies for 50 min at room temperature. The target antigen was detected by 3,3'-Diaminobenzidine (DAP) and nucleus was stained by Harris hematoxylin for 3 min. The statistical analysis of IHC was performed by measuring integral optical density (IOD) in each image.

Real time PCR

Total cell RNAs were extracted using Trizol reagent (TAKARA) based on manufacturer's recommendation. 6µl extracted RNA was reversely transcribed using the Prime Script™ RT reagent Kit with gDNA Eraser (TAKARA) according to the provider's protocol. Quantitative PCR was performed using SYBR® Green Realtime PCR Master Mix (TAKARA) in the Step One Plus Real-Time PCR System (Applied Biosystems). GAPDH housekeeping gene was measured as an internal control for all samples. The forward and reverse primers of indicated genes are in table 3.

Ethical approval

This study was conducted as per NIH guideline for Lab animal and was approved by the ERB of Ezhou Central Hospital, Ezhou City, Hubei Province, PR China with reference number 152/ECH-CH/2017.

Table 1: Assessment for Proliferation or Fibrosis in Granulation Tissue **(**Caroline *et al*., 2016)

Score	Criteria
1-3	No minimal cell aggregation. No granulation tissue or epithelial migration
4-6	Thin, immature granulation tissue, which mainly are inflammatory cells. There are small amount of fibroblasts, capillaries or collagen deposition. Minimal epithelial cell migration.
7-9	Medium thickness of granulation tissue. Fibroblasts and collagen deposition become dominant instead of inflammatory cells. There are massive neovascularization and moderate epithelialization migration.
10-12	Thick vascular granulation tissue. Fibroblasts and extensive collagen deposition are the main components. The wound is partially or completely covered by epithelium.

Table 2: Assessment of Regeneration of Epithelial Tissue around the Ulcer (Eitaro *et al*., 2016).

Score	Criteria
0	No ulcer surface epithelial tissue coverage
1	Epithelial regeneration on one side of the ulcer
2	Epithelial regeneration on both sides of the ulcer
3	Full ulcer surface epithelial tissue coverage but no skin appendages
4	Full ulcer surface epithelial tissue coverage and skin appendages formation

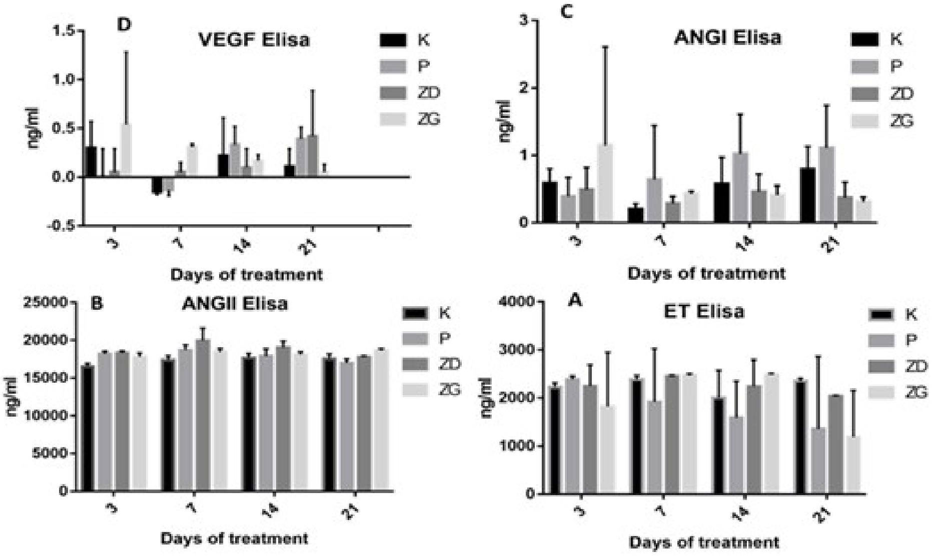

Fig. 1: The expression of angiogenesis related biomarkers in wound tissues. Rats were applied with different treatments for 3, 7, 14 and 21days. At indicated points, the wound tissues on the rats were collected and analysed for indicated protein expression by Elisa. A. VEGF; B. ANGI; C. ANGII; D. ET. Data were analysed by student t-test. n=4.

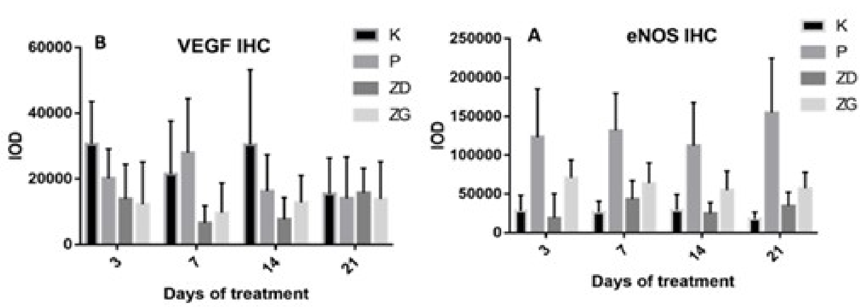

Fig. 2: The quantification of VEGF and eNOS expression of rats in IHC. Rats were applied different treatments for 3, 7, 14 and 21days. At indicated points, the wound tissues on the rats were collected and analysed for indicated protein expression by IHC. A. VEGF; B. eNOS. The data were quantified by measuring IOD value and analysed by student t-test. n=4.

**There was significant difference in the results when compared to physiological saline treatment group

STATISTICAL ANALYSIS

Spss 22.0 was used for analysis. Among all the data, (%) class count data were tested by X^2 test, ($\bar{x} \pm s$) class measurement data were tested by t test. $p<0.05$, indicating significant difference.

RESULTS

Flavonoids treatment altered the expression of angiogenesis related biomarkers

The level of VEGF in wound tissues before treatment was extremely low by ELISA measurement. After 3 and 7 days of treatments, the ZG groups showed the highest expression of VEGF compared with other treatments. After the wounds were treated for 14 days, the expression of VEGF in P group increased. The ZD group presented the highest expression of VEGF than that in any others groups (fig. 1A). The expression of AngI was different from that of VEGF. ZG treatment induced the highest AngI expression in 3 days, while P group had the most AngI in other treatment periods (fig. 1B). In the first 3 days' treatment, ZD groups presented the highest expression of AngII while AngII expression became more dominant in ZG group compared with other groups (fig. 1C). For the expression of ET ZG group has the most dominant expression with 14 days' treatment while K group possessed the highest reading in different treatment time period (fig. 1D).

Wound tissues were collected and analyzed by IHC staining using antibodies against VEGF and eNOS. The expression of VEGF was low in CG group with 3-day treatment, but VEGF expression increased to the peak with 7-day treatment. The expression of VEGF was low at day7 and then fluctuated with 14 and 21 days' treatment in K group (fig. 2A). The IHC result of eNOS is different from that of VEGF. In The expression of eNOS is much higher in P group than other groups. The expression of eNOS remained at low level and did not change during all treatment periods in ZG group (fig. 2B).

Control group always showed much higher expression of AngI mRNA than other treatment groups. P, ZG and ZG treatment did not significantly affect AngI mRNA expression, which decreased continuously with prolonged treatment period. Control group also demonstrated the highest level of AngII among all other treatment groups. ZG and ZD treatment did not present obvious impact on the expression of AngII mRNA. There was an increased expression of VEGFR mRNA from day3 but then the expression decreased from day7 in all the treatment groups except control group. The majority of treatment groups demonstrated higher mRNA level than that in control group except ZG treatment group between day3 and day14. ZG group showed rapidly decreased while other groups reduced slowly. Expression of VEGFR mRNA in control dropped at day7 but then experienced fluctuation until day21. The eNOS expression shared similar pattern with VEGFR. ZD and ZG showed a different pattern where bFGF mRNA expression rise from day3 to day7 but then reduced at day21. P treatment induced more mRNA expression of bFGF from day3 to day14. It Increased from day3 and reached the plateau at day7 but it dropped quickly at day21. As for the expression of ET-1 mRNA, K, P and ZG shared similar pattern where they diminished from day3 to day7 but then amplified at day14 and dropped at day21. ZD had a different pattern where ET-1 mRNA expression remained no change during all treatment periods (fig. 3).

Flavonoids treatment improved wound healing of pressure ulcer

The photos of wound area with different treatments on the rats were taken and measured at indicated time points. ZG showed faster wound healing effect than other groups at all time d P groups. The wound on the rats in K group remained open at day 21 (fig. 4).

Sore bearing rats were treated with different drugs for 3, 7, 14 and 21 days. Interestingly, with less than 14 days' treatment, wounds in groups ZG and ZD were closed. However, with 21 days' treatment, there were still around 16% opened sore area in K group but sores in other groups were all nearly recovered (fig. 5).

Three days after modelling, lamellar granulation tissue was found, which consisted of nascent capillaries and fibroblasts with infiltration of inflammatory cells and small amount of collagen fibres. Less blue cords were revealed with Masson staining with no epithelial tissue regenerated at the edge of ulcers. After 3 days of establishment of ulcer model in rats, ulcers were seen in all treatment groups where the skin surface was slightly bleeding and covered with inflammatory necrotic tissues. Under the ulcers, three rats had thin granulation tissue with inflammatory cells. Hyperaemia and edema in interstitium were observed in K group. Fibroblasts, capillaries and a small amount of collagen deposition were seen. Masson stained blue revealed short filaments. There was no epithelial regeneration at the edge of ulcer. The granulation tissue and fibrosis got 4.25 points by average. In P group, one rat had thin granulation tissue under the ulcer with interstitial edema. A few fibroblasts, capillaries and collagen deposition were observed. Masson staining revealed a small amount of blue, short, filamentous deposited collagen. Other rats showed slight granulation tissue and collagen deposition. There were inflammatory cells, capillaries and collagen deposition in granulation tissue. Masson staining presented blue, short filamentous. The granulation tissue and fibrosis got 3.75 points by average. In ZG group, one rat did not show granulation tissue formation while others presented

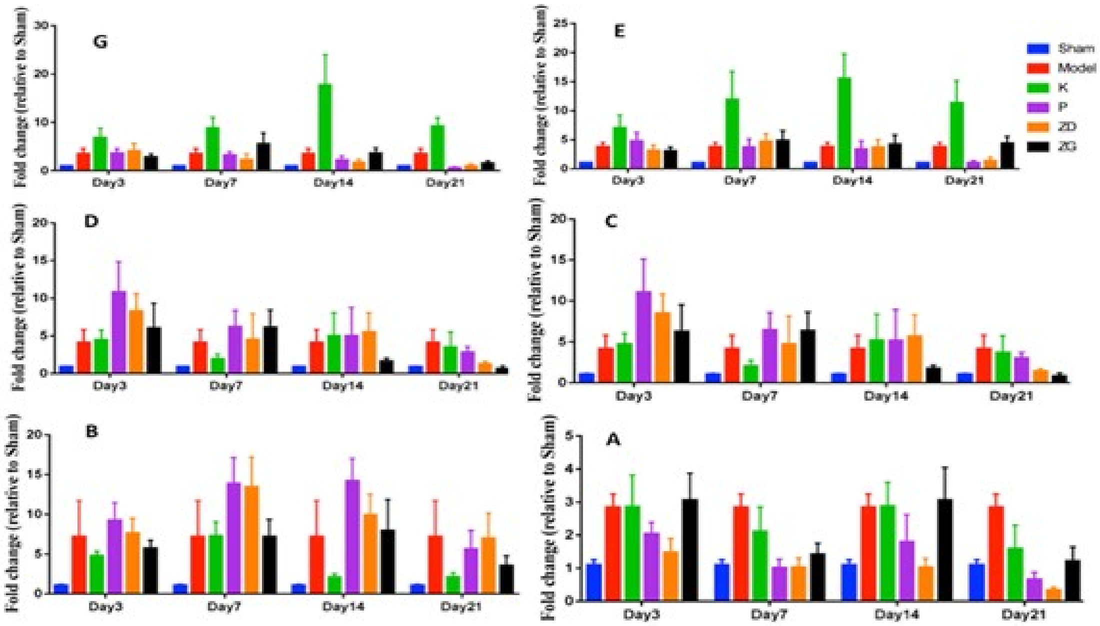

Fig. 3: The expression of mRNAs in flavonoids treated rats. Rats were applied with different treatments for 3, 7, 14 and 21days. At indicated time points, the wound tissues of the rats were collected and analysed for indicated mRNA expression by RT-PCR. A. ANGI; B. ANGII; C. VEGFR; D. eNOS; E. bFGF; F. ET-1. The data were quantified by measuring IOD value and analysed by student t-test. N=4

immature granulation tissue around the ulcers. Increasing number of inflammatory cells and capillaries were observed. These three rats all showed fibroblasts and collagen deposition. A small amount of blue collagen was found by Masson staining. The granulation tissue and fibrosis got 4.5 points by average. In ZD group, rats in this group demonstrated similar granulation tissue regeneration as ZG group. The granulation tissue and fibrosis got 3.75 points by average. No regeneration of epithelial tissue was observed in all groups.

Granuloma tissue was observed 7 days after modelling and the number of inflammatory cells in this tissue reduced. Fibroblasts had produced and deposited collagen fibers. Masson stained blue cords. Some rats showed of epithelial regeneration at ulcer edge. All rats showed defective skins and ulcer formation. But the surface was covered with decreased necrosis compare with day3. Ulcers on all rats shared similar situation of granulation tissue proliferation or fibrosis at day 7. Medium granulation tissue was observed under ulcers, and the number of inflammatory cells was significantly reduced compared with the day3. Fibroblasts had produced and deposited collagen fibers. Masson staining showed blue short cords mixed with fibroblasts. The granulation tissue and fibrosis got 8.25, 6.5, 7.0 and 7.0 points in K, P, ZG and ZD respectively by average. Two rats presented regeneration of epithelial tissue on one side of the ulcer but no skin appendages formation in K and ZD group. No regeneration of epithelial tissue was observed in P group.

Fourteen days after modelling, some rat ulcers were covered with intact or incomplete regenerated epithelium, with moderate thickness of granulation tissue underneath the ulcer. The number of inflammatory cells in the ulcer decreased but the deposition of collagen fibers increased. Masson staining presented large amount of blue short cords. The majority of rats showed epithelial regeneration at ulcer edge or surface coverage by intact epithelium. Additionally, regenerated epithelial skin appendages were seen is some rats. ZG and ZD can facilitate ulcer healing, granulation tissue proliferation and fibrosis especially after 14 days' treatments. Three rats showed local skin defection and ulcer formation in K group. The surface covered with decreased number of inflammatory necrosis tissue compared with day7. One rat had obvious hemorrhage at ulcer site and one rat fully healed. In P group, three rats showed local skin defection and ulcer formation. The surface covered with decreased number of inflammatory necrosis tissue compared with day 7. One rat had obvious hemorrhage and necrosis tissue at ulcer site. In ZG and ZD groups, three rats showed local skin defection and ulcer formation. The surface covered with decreased number of inflammatory necrosis tissue compared with day7. Ulcer on the other one rat had been covered with intact regenerative epithelium. In K group, thick layer of granulation tissue was observed under 3 ulcers and the number of inflammatory cells decreased compared with day7. Fibroblasts had produced and deposited large amounts of collagen fibers. Masson stained revealed more and thicker blue staining than day7.

Wound on the remaining one rat had been completely covered by regenerated epithelium, with mature granulation tissue and dominant fibroblasts and extensive collagen deposition. The granulation tissue and fibrosis got 12 points by average. In P group, ulcers in this group showed similar granulation tissue proliferation or fibrosis as K group. The granulation tissue and fibrosis got 12.5 points by average. In ZG and ZD groups, thick layer of granulation tissue was observed under 3 ulcers. Fibroblasts had produced and deposited large amounts of collagen fibers. Masson staining revealed more and thicker collagen deposition than day7. Wounds on remaining one rat had been completely covered by epithelial and connective tissue with abundant collagen deposition. Masson staining showed blue thick cords. The granulation tissue and fibrosis of ZG and ZD groups got 10.5 and 11.25 points by average, respectively. Three rats presented regeneration of epithelial tissue on one side of the ulcer but no skin appendages formation in K group. The remaining one rat presented healed ulcer where surface was fully covered with intact regenerated epithelium and differentiated epithelial cells. The formation of hair follicles was also observed in this rat. Two rats presented regeneration of epithelial tissue on one or both sides of the ulcer but no skin appendages formed in P group. One rat presented healed ulcer where surface was fully covered with intact regenerated epithelium and differentiated epithelial cells. The formation of hair follicles was also observed in this rat. No regeneration of epithelial tissue was observed in the last rat. In ZG group, all rats presented regeneration of epithelial tissue on one side of the ulcer but no skin appendages formation. Wound on one rat was fully covered with intact regenerated epithelium and differentiated epithelial cells. In ZD group, all rats presented regeneration of epithelial tissue on one side of the ulcer. One rat showed skin appendages formation but others not.

Twenty-one days after modelling, ulcer covered by intact regenerated epithelium with thick granulation under was seen in most of the rats (3/4). Masson staining revealed abundant blue collagen cords. One rat in K group presented defective skin and ulcer formation. Ulcers on other rats were fully healed. In K group, vascular granulation tissue and small amount of inflammatory cells can be observed in one rat. Fibroblasts produced and deposited large amounts of collagen fibers in this rat as shown by the blue Masson staining. The ulcers on other

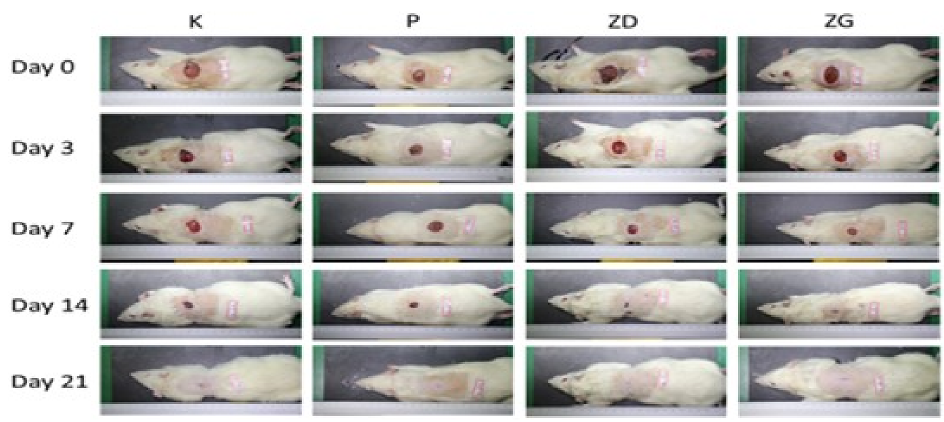

Fig. 4: The wound healing of rat model after flavonoids treatments. The typical images of wound in rats was shown at the given time points. K, non-treatment control; P, positive treatment; ZD, low concentration of flavonoids; ZG, high concentration of flavonoids

**There were significant differences in the results when compared to physiological saline treatment group

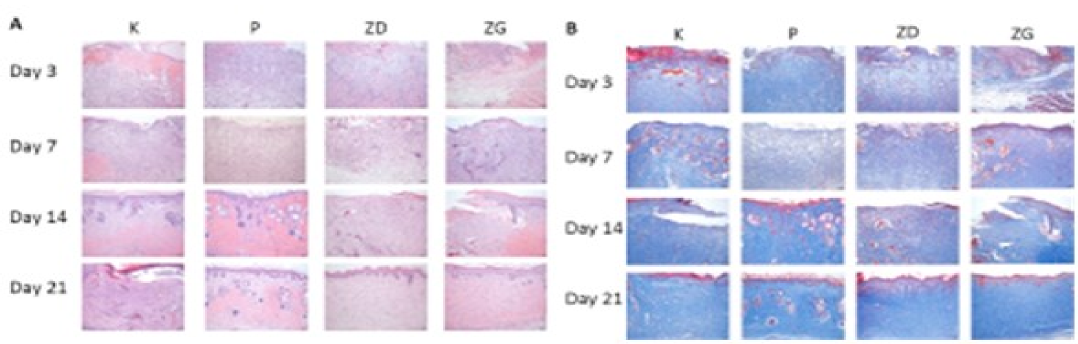

Fig. 5: Typical pathological images of wound across 21 days treatment. A, HE staining. B, Masson staining

**There were significant differences in the results when compared to physiological saline treatment group three rats had been completely healed but small amount of blood vessels and inflammatory cells are still visible. The granulation tissue and fibrosis got 13.75 points by average. In P, ZG and ZD groups, granulation tissue in 4 rats had been transformed into connective tissue with fewer inflammatory cells and fibrous cells and large amount of collagen fibers. Masson stained showed blue bar. The granulation tissue and fibrosis of P, ZG and ZD groups got 15.5, 15.25 and 15 points by average, respectively. In K group, ulcers on three rats were healed. Epithelial cells well differentiated. There was no skin appendages formation. In the last rat, new epithelium was observed but no skin appendages formed while in P group ulcers on three rats were fully healed. Epithelial cells well differentiated and skin appendages formed. New epithelium was observed but no skin appendages formed in the last rat. In ZG group, ulcers on all rats were fully healed. Epithelial cells were well differentiated and two of them showed skin appendages formation. In ZD group, ulcers on all rats were fully healed. Epithelial cells were well differentiated. One of them showed skin appendages formation but others not.

DISCUSSION

Pressure ulcer is a serious complication in patients with limited mobility and insufficient blood flow. Several studies have reported that the use of *rhubarb* and *hollyhock* can reduce ulcer swelling and stimulate angiogenesis (Mariam *et al*., 2018, Hong *et al.,* 2020, Lidong *et al*., 2018). Flavonoids, the active ingredient in Astragalus, have attracted attention for improving wound healing of pressure ulcer. In this study, we evaluated the mRNA expression of angiogenic molecules VEGF, ANGI, ANGII and NO in pressure ulcer after treatment with flavonoids. Our ELISA results showed that flavonoids treatment increased the expression of these angiogenesis molecules. Compared with the control group, the expressions of VEGF, ANGI and ANGII were not significantly increased, but flavonoids significantly increased NO expression at 7 and 14 days after treatment, indicating that flavonoids may activate the expression of NO. Similarly, PCR results showed that VEGFR and eNOS did not increase in the group of flavonoid treatment, but AngI and AngII expression were significantly up-regulated after flavonoid treatment, indicating that flavonoid treatment can promote the expression of Angl and Angll.

Wound healing is a normal, complex process that can be divided into four stages, including haemostasis, inflammation, proliferation and tissue remodelling (Grace *et al*., 2018). This process requires a coordinated interaction among different cell types, biochemical mediators, and extracellular matrix molecules at a particular time (Maquart, 2015). Unlike acute wounds, pressure ulcers, which are typically chronic wounds, show many molecular abnormalities in phagocytic fibroblasts and keratinocytes (Surajit & Mishra, 2015; Gefen 2016), angiogenesis disorders (Lidong *et al*., 2018), and phagocytic activity (Rizov *et al*., 2017). This study revealed that pressure ulcer damage re-epithelization, cell migration and proliferation, and granulation tissue formation, leading to worsening of the wound healing process. During the proliferative phase, fibroblasts are stimulated to migrate and proliferate into the wound to produce matrix proteins, hyaluronic acid, fibronectin, proteoglycans and collagen fibres. Previous studies have shown that fibroblasts isolated from pressure wounds have lower migration activity and mitogenic responses. Abnormalities in these cells impede the formation of granulation tissue and extracellular matrix molecules, resulting in non-healing wounds. After flavonoid treatment, an improvement in wound healing in pressure sores was observed with abundant fibroblasts, accumulation of granulation tissue around wounds, and extracellular matrix formation. One of the key stages of wound repair is epithelial remodelling, which involves the movement of keratinocytes through the wound bed to restore epidermal activity. Due to the high glucose levels, the migration and proliferation of keratinocytes are impaired, leading to a lack of reepithelialisation (Surajit & Mishra, 2015). Fibroblast formation is another key factor in wound healing and is involved in multiple steps, such as creating new extracellular matrix (ECM) and collagen structures to support other cells associated with effective wound healing as well as contractions of wound (Bainbridge, 2013). Angiogenesis is critical to the wound healing process allows new capillaries to deliver nutrients to the wound and promote fibroblast proliferation. The wound was initially hypoxic due to the loss of vascular perfusion, but vascular perfusion was restored as new capillary networks developed. Regulating traumatic angiogenesis may itself be a means of improving healing in some cases, especially in the case of delaying or defective angiogenesis involving a cure disorder (Mehdi *et al*., 2017; Darby *et al*., 2014; Raluca *et al*., 2020). Due to the expression of α-SM actin in microfilament bundles or stress fibers, these cells exhibit contractile properties that play a major role in the contraction and maturation of granulation tissue. Prokaryotic fibroblasts generally evolve into differentiated myofibroblasts, the most common variant of this cell, containing α-SM actin stress fibers (Darby *et al*., 2014). Depending on experimental or clinical conditions, myofibroblasts may express other SM-associated contractile proteins, such as SM myosin heavy chain or desmin; however, the presence of α-SM actin represents the most fibroblastic phenotype reliable marking (Darby *et al*., 2014; Raluca *et al*., 2020). Mitochondria are a major source of reactive oxygen species (ROS) in fibroblasts and are therefore considered as key regulators of wound healing that may affect the expression of nuclear genes involved in this process. Mitochondrial ROS in the cytoplasm is regulated by antioxidants that quench ROS to check its ability to close the wound. Studies have shown that the level of mitochondrial ROS affects the centre of some nuclear-encoded genes on the expression of wound healing and oxidative stress, and the antioxidant regulation of mitochondrial ROS has a positive effect on wound closure in-vitro (Janda *et al*., 2016). Therefore, fibroblasts can be considered an effective natural redox-based strategy to help treat unhealed wounds.

CONCLUSION

Taken together, our results indicated that flavonoids accelerated the healing of pressure ulcer in a dose-dependent manner in a rat model. Flavonoids may improve wound angiogenesis by activating endothelial cell proliferation and mitochondrial ROS. This study provides a rationale for using flavonoids to enhance wound healing or as a potential therapeutic agent for pressure ulcers.

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