Department of Burn and Plastic Surgery[1], General Hospital of Ningxia Medical University; Department of Pediatrics[2], Yinchuan Maternity & Child Health Hospital; Department of Nephrology[3], General Hospital of Ningxia Medical University, Yinchuan, Ningxia, China

# Walnut oil promotes healing of wounds and skin defects in rats *via* regulating the NF-kB pathway

Qiang Ma[1], Yin-Sheng Wu[1,*], Jiang-Yong Shen[1], Zhi-Bin Yang[1], Hong-Xia Shen[2], Miao Yao[1], Chun-Hua Yu[3]

*Received June 24, 2018, accepted July 31, 2018*

**Corresponding author: Prof. Yin-Sheng Wu, Department of Burn and Plastic Surgery, General Hospital of Ningxia Medical University. No. 804 South Shengli Road, Yinchuan 750004, Ningxia, China*
*wysw16@126.com*

*Pharmazie 73: 721–724 (2018)* *doi: 10.1691/ph.2018.8605*

The effects of walnut oil on wound healing and skin injury repair was observed in Sprague-Dawley (SD) rats, and mechanism of action was investigated. Normal SD rats were divided into an experimental group and a control group. Each group was observed at4 time points (day [D]3, D7, D14, and D21). In both groups, a skin wound was created on the back of the rats, with the spine as the central axis. In the experimental group, the wound was covered with walnut oil, and then bandaged and fixed with sterile gauze. In the control group, the wound was bandaged with vaseline gauze. At each corresponding time point, the wound area and wound healing time of each rat were examined. Epithelial cells of the wound tissues were observed using haematoxylin and eosin staining and immunohistochemical analysis,and the numbers of inflammatory cells and capillaries were counted. A western blot method was used to detect the expression of nuclear factor (NF)-κB and epidermal growth factor (EGF) in the wound tissues of both groups. Meanwhile, enzyme-linked immunosorbent analysis (ELISA) was used to detect the expression of transforming growth factor (TGF)-β1 and matrix metalloproteinase (MMP)-1 in rat sera. A total of 48 SD rats completed the experiment. Healing time of residual wounds in the experimental group was 10.0±3.5 days, which was significantly shorter than that in the control group (18.0±6.0 days) ($p<0.05$). The wound healing rates in the experimental group were 54.14 % (D3) and 91.2 3% (D7), whereas those in the control group were 22.12% (D3) and 54.84% (D7 ($p<0.05$).Histological examinations revealed no epithelial cells on D3, D7, D14, and D21 in both the experimental and control groups. However, the number of inflammatory cells decreased significantly and the number of capillaries increased significantly in the experimental group compared to control ($p<0.05$). NF-κB expression was significantly lower, EGF expression significantly higher in the in the experimental group. Conversely, ELISA showed a significant increase in the expression of TGF-β1 and MMP-1 in rat sera in the experimental group. So we conclude that walnut oil has significant effects in promoting the healing of skin defect wounds in SD rats.

## 1. Introduction

Blisters often appear in the early stages of burn wound healing; these can easily rupture and cause complicated infections, thereby forming residual wounds. The termination of sucha phenomenon often requires a longer course of treatment (Li 1995). The healing of residual wounds has long been a time-consuming and arduous problem in the treatment of burn wounds. There are many treatment methods including wound dressing changes, use of various biomaterials to promote wound healing and tissue growth, and use of local anti-infectives (Yu et al. 2009); for long-term refractory wounds, surgical treatment may eventually be indicated (Poulakidas and Kowal-Vern 2008). In particular, among infants, young children, elderly patients, and patients with severe heart and lung diseases who cannot tolerate painful stimuli and surgical treatment, such wounds are difficult to treat and thus are frequently encountered in clinical settings. Improper treatment methods can further aggravate wound infections and cause the formation of granulation wounds, leading to the eventual need for surgical treatment, to chronic non-healing wounds, or to granuloma formation. Such consequences not only increase the patients' treatment risks and pain, but also create additional financial burden and prolong hospitalization. As a result, the treatment process will be painful, time consuming, and strenuous for patients, regardless of the method used. For many years, external application of walnut oil has been used at our department to treat residual burn wounds. This method causes minimal or no pain to the patients, is not costly, provides good wound healing effects, and has achieved relatively satisfactory results, thus providing a novel approach for the treatment of residual burn wounds. However, the mechanism of action of walnut oil remain sunclear. Therefore, in the present study, an animal experiment was conducted to simulate refractory wounds of the human body, such as residual burn wounds, in order to investigate the mechanism of action of walnut oil. In this experiment, self-made walnut oil was used, and SD rats with skin defects and wounds on the back were used as an animal model to simulate residual burn wounds. The experimental group was treated with self-made walnut oil, whereas the control group was treated with vaseline. At different time points, the wound healing time was recorded, the wound healing rate was calculated, histomorphological and cell ultrastructure observations of the wounds were performed, and the expression of epidermal growth factor (EGF) and other growth factors in wound tissue was measured, to provide an experimental basis for clinical application.

## 2. Investigations and results

### *2.1. Comparison of wound healing time and wound healing rates in rats*

Through gross observations of the granulation tissue on the backs of the rats, the wound surfaces were found to be dry with little

exudate and secretion and the wound areas were significantly reduced in size after external application of walnut oil on wounds in the experimental group (Fig. 1A). In the control group, after external application of Vaseline gauze, wound secretion significantly increased but the size of wound areas was not significantly reduced. The wound healing time in the experimental group was 10.0±3.5 days, which was significantly shorter than that in the control group (18.0±6.0 days) ($p<0.05$). The wound healing rates in the experimental group were 54.14 % (D3) and 91.23 % (D7), whereas those in the control group were 22.12 % (D3) and 54.84 % (D7). The wound healing rates in the experimental group were significantly higher than those in the control group ($p<0.05$).The comparison of the wound healing rates showed that the wounds in the experimental group completely healed on D14. The wound healing rates in the experimental group were basically the same on D14 and D21, and the wound healing time in the experimental group was significantly shorter than that in the control group. The wound healing rates in the experimental group were significantly higher than those in the control group, with the differences between the 2 groups being significant (Fig. 1B, C).

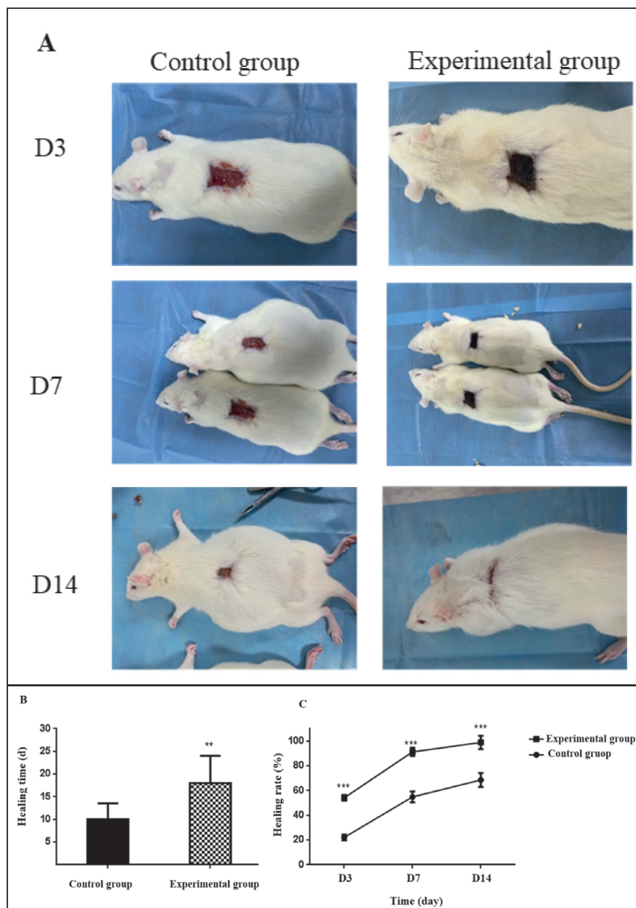


Fig. 1: (A)Gross observations of experimental rats. Comparison of healing time (B) and healing rates (C) between the 2 groups.Magnification: 100×; **, $p<0.01$ compared with the control group;***,$p<0.001$compared with the control group.

## 2.2. Morphological observations of wound tissues

On D7, wound tissues were obtained from sacrificed rats and subjected to HE staining. Microscopic observations revealed that the number of inflammatory cells in the experimental group was significantly lower than that in the control group (Fig. 2A), whereas the number of capillaries was significantly higher (Fig. 2B). No epithelial cells were detected in either group. Therefore, the above experiment shows that walnut oil can significantly inhibit the inflammatory response of wound tissue in rats, and stimulate the regeneration of capillaries in wound tissues to promote wound repair.

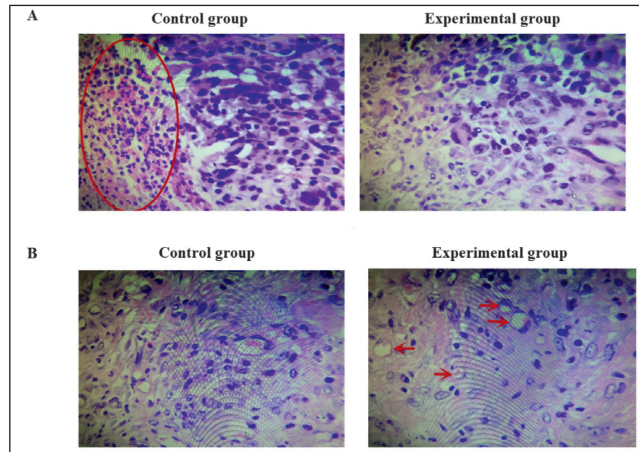


Fig. 2: Haematoxylin and eosin staining of wound tissues on the backs of ratson day 7. (A) Inflammatory cells; (B) capillaries.Magnification: 100×.

## 2.3. Expression of the inflammatory factorsNF-κBand EGF in rat wound tissues

Seven days after the rat model had been established, the western blot test was performed on tissues harvested from the backs of the rats. After treatment with walnut oil for 1 week, the expression of EGF in tissues from the backs of the rats was significantly increased, whereas the expression of inflammatory factor NF-κB was significantly reduced (Fig. 3). Therefore, it can be deduced that walnut oil significantly inhibits the expression of inflammatory factors and promotes the expression of EGF. Therefore, walnut oil can regulate wound tissue inflammation through the regulation of NF-κB and promote the healing of wound tissues through the regulation of EGF.

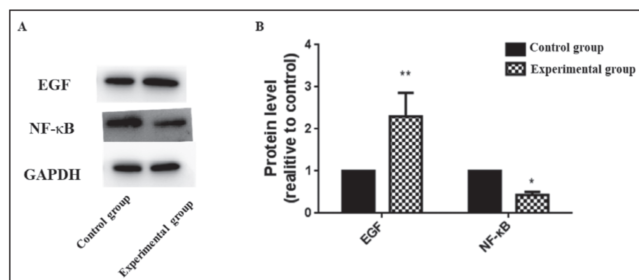


Fig. 3: Western blotdetection of the expression of the inflammatory factorsnuclear factor (NF)-κB and epidermal growth factor (EGF)inwound tissues from the backsof ratssacrificedon day 7.(A) Western blot results; (B)statistical results.*, $p<0.05$compared with the control group; ** $p<0.01$ compared with the control group.

## 2.4. Expression of TGF-b1 and MMP-1 in rat plasma

Seven days after the rat model had been established, the expression of TGF-b1 and MMP-1 in rat sera was detected using ELISA. The expression of TGF-b1 and MMP-1 in blood sample of rats after treatment with walnut oil for 7 days was significantly increased, indicating that the healing effect of walnut oil on rat skin wounds is associated with TGF-b1 and MMP-1 (Fig. 4). TGF-b1 plays an important role in wound healing in humans. Besides promoting collagen synthesis, it also reduces the release of fibroblast growth factor from fibroblasts and ensures rapid wound healing. It can be secreted by many types of cells at the wound site, including platelets, macrophages, fibroblasts, and keratinocytes.

## 3. Discussion

Wound healing is a pathophysiological process involving multiple interconnected and intersecting biological reactions such as inflam-

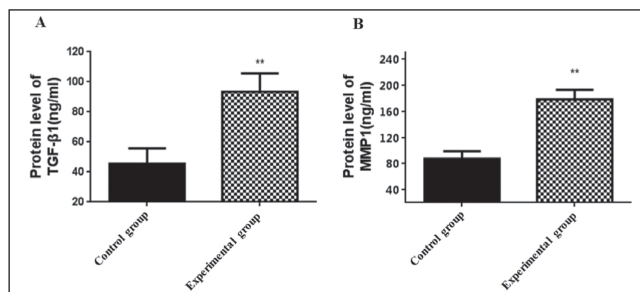


Fig. 4: Expression of transforming growth factor (TGF)-b1 and matrix metalloproteinase (MMP)-1 in rat blood on day7 after model establishment, *,$p$<0.05 compared with the control group, **,$p$<0.01 compared with the control group.

mation, cell proliferation, cell migration, and wound remodelling (Zhang and Yan 2013). The healing of burn wounds starts with an inflammatory phase, progresses to the proliferative phase, which is characterized byre-epithelialization; and ends with the remodelling phase (Kirsner and Eaglstein 1993). During this process, cell proliferation and intercellular matrix formation are essential for tissue repair (Luo et al 2016). At present, refractory wounds pose a challenge in wound treatment, as wound healing is extremely slow and often accompanied by infections and inflammatory reactions.

In the field of Traditional Chinese medicine (TCM), there is a wealth of clinical experience in the promotion of wound healing, and TCM methods usually boast advantages of unique curative effects and little toxic or adverse effects. Self-made walnut oil is a TCM formulation that has a unique curative effect for burns, and its efficacy is especially significant in the treatment of residual burn wounds, as it promotes the healing of refractory wounds and controls wound infections. In this study, walnut oil was applied externally for the treatment of skin defect wounds in SD rats. It was found that walnut oil significantly shortened wound healing time, increased wound healing rate, reduced inflammatory cell proliferation, and promoted capillary proliferation. The wounds of SD rats healed through wound contraction, which led to complete healing of all wounds by D14. Therefore, in the experiment, the expression of various inflammatory factors in the wound tissues of rats was measured at the mid-point of the healing period (i.e., D7). The walnut oil and residue mixture obtained through the grinding of walnut kernels contains most of the nutritional, health-promoting, and pharmacologically active constituents of the seeds. It contains large amounts of unsaturated fatty acids such as linoleic acid and linolenic acid, and its unsaturated fatty acid content is as high as 90% (Qiao et al. 2017). Linoleic acid, the major fatty acid in the epidermis, plays an important role in the maintenance of permeability in the gastrointestinal stratum corneum, maturation of keratinocytes, inhibition of pro-inflammatory cytokine secretion, and promotion of wound healing (McCusker and Grant-Kels 2010). The gadoleic acid in walnut oil promotes the growth of epidermal fibroblasts and helps unclog blood vessels, whereas squalene, sterols, flavonoids, and vitamin E in walnut oil promote vascular circulation (Smith 2000). At the same time, walnut oil activates functional cells in the body and has a wide range of effects, including anti-inflammatory, anti-allergic, cell-suppressing, virus-suppressing, and anti-oxidative effects. In the environment of a refractory wound, the supply of nutrients plays an important role in the regeneration and healing of the wound. Studies have shown that wound healing can be promoted by oral intake of linoleic acid, which mainly acts by inhibiting inflammation during wound healing (Rodriguez et al. 2008) and increasing vascular endothelial growth factor and angiopoietin-levels to induce angiogenesis, thereby promoting wound healing (Niinikoski 2004). Topical application of linoleic acid to wounds also promotes wound healing (McMonnies 2015). Therefore, walnut oil can significantly improve the wound environment in the process of promoting wound healing. Although anti-inflammatory treatment and improvement of local blood circulation are essential, the improvement of nutrition in the local environment of wounds is equally important and may even be an indispensable factor in the acceleration of wound healing.

By measuring the expression of various factors in wound tissues of rats sacrificed after treatment with walnut oil for7 days, we found that walnut oil can significantly inhibit the expression of NF-κB while promoting the expression of EGF, TGF-β1, and MMP-1. This indicates that walnut oil has an anti-inflammatory effect and can promote angiogenesis and re-epithelializationin wound tissues. NF-κB is a biomarker of inflammation and oxidative stress, and is expressed at different levels during cell differentiation, proliferation, apoptosis, and adhesion (Gupta et al. 2010). The NF-κB pathway is activated during wound healing, which results in the activation of multiple cytokines, chemokines, adhesion factors, and enzymes that mediate the production of inflammatory factors (Ghosh et al. 1998). These factors play a protective role in the early stage of pathogen invasion. Therefore, during skin injury, cells in the wound tissues will activate the NF-κB pathway (May and Shosh 1997), which subsequently activates the migration of macrophages and immune cells to the wound tissues (Bonizzi and Karin 2004), thereby reducing the occurrence of inflammation. Conversely, NF-κB also participates in the regulation of cell proliferation (Ben-Neriah and Karin 2011). During wound healing, it promotes the proliferation of epithelial cells, thus facilitating their involvement in re-epithelialization (Schreml et al. 2010; Na et al. 2016). NF-κB also regulates the expression of MMP (Na et al. 2016), promotes the formation of keratinin the skin, and regulates the secretion of cytokines and growth factors to further promote the healing of epidermal wounds (Mirastschijski et al. 2002). Therefore, the present study results indicated that the anti-inflammatory and wound healing effects of walnut oil may be exerted through the NF-κB and EGF pathways.

In summary, walnut oil may play an important role in wound healing and the treatment of refractory wounds. Through the NF-κB and EGF pathways, walnut oil can significantly promote the growth of capillaries, reduce the number of inflammatory cells, improve local blood circulation, provide the nutrients needed for wound healing, and improve the wound healing environment in the skin defect wounds of SD rats. It causes little injury, is cheap, and produces minor or no adverse effects. Therefore, it is a method with great prospects for wide application in the treatment of refractory wounds.

## 4. Experimental

### 4.1. Experimental animals, reagents, and instruments

Clean Sprague-Dawley (SD) rats comprising equal numbers of males and females with body weights of 350±50 g were purchased from the Animal Experimental Center of Ningxia Medical University (licence no.SCXK [Ning] 2015-0001). Rabbit anti-human EGF receptor monoclonal antibody kit was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. nuclear factor (NF)-κB and EGF antibodies were purchased from Abcam PLC. Transforming growth factor (TGF)-β1 and matrix metalloproteinase (MMP)-1 enzyme-linked immunosorbent analysis (ELISA) kits were purchased from R&D Systems. AL204 electronic analytical balance was purchased from Sartorius (Germany). DM4000 B LED optical microscope and camera system were purchased from Leica (Germany).

### 4.2. Preparation of walnut oil

Commercially available walnut kernels were ground and fried in a stainless-steel wok over a low flame until the kernels turned black and oil was released (about 30 min). The contents of the wok were allowed to naturally cool to ambient temperature, and then ground into an oil-residue mixture with a stone mill. After standardized sterilization, the mixture was stored in a refrigerator at 4 °C before use.

### 4.3. Preparation of animal models

The SD rats were randomly divided into two groups, with 24 rats in each group. Each group was divided into 4 time points, namely, days (D)3, 7, 14, and 21, with 6 rats allocated to each time point. In each rat, after intraperitoneal anaesthesia with chloral hydrate (3.25 mg/kg), skin preparation was carried out on the back. After sterilization with iodophor, a skin defect wound was designedn the back, with the spine as the central axis (a scalpel was used to remove full-thickness skin tissue 3cm ´ 3 cm in size, with subcutaneous fat left intact). In the experimental group, the wound was covered with externally applied walnut oil, and subsequently covered, sutured, and fixed with a piece of sterile gauze (size 4 cm × 4 cm). In the control group, the wound was covered with vaseline gauze (manufactured by the Supply Department of the General Hospital of Ningxia Medical University) to provide an unctuous environment

similar to that in the experimental group. Then, the wound was bandaged, sutured, and fixed using sterile gauze of the same size. In both groups, the dressings were changed once a day. The status and time of wound healing were observed and recorded. At each time point (D3, D7, D14, and D21), 6 rats were sacrificed, gross specimens were photographed, and tissue specimens including unhealed tissues were harvested along the edges of the healing wounds and fixed in 10% formaldehyde solution for haematoxylin and eosin (HE) staining and immunohistochemical analysis.

### *4.4. Determination of wound healing time and wound healing rate*

After the animal models were established, the healing time of the wounds were recorded according to the wound healing status during dressing changes. The healed area of each wound was determined on D3, D7, D14, and D21.The wound surface was covered with transparent tracing paper, and the original wound area was traced along the edge of the wound surface. The excess paper was cut, and an electronic analytical balance (precision 1/10,000) was used to obtain the weight of paper having the same area as the wound tissue. The unhealed wound area was traced with tracing paper and also weighed. With the weight ratio being equivalent to the area ratio, the wound healing rate was subsequently calculated using the following equation:
Wound healing rate = (weight of paper with the area of the original wound - weight of paper with the area of the unhealed wound) / weight of paper with the area of the original wound × 100%.

### *4.5. Histological examination*

At each time point, rats were randomly selected and painlessly sacrificed to obtain comparable tissue samples (1 cm × 1 cm close to the wound margin, including normal skin at the wound base and wound margin). The samples were fixed with 10% formaldehyde. Routine HE staining was performed on paraffin-embedded pathological sections for histological examination. Under a high-power magnification of 10×40, 5 fields were selected, and the number of epidermal cells, inflammatory cells, and capillaries were respectively counted using medical graphic digital system software. Average counts were calculated in each group, and the number of epidermal cells, inflammatory cells, and capillaries were compared between the two groups.

### *4.6. Detection of NF-kB and EGF expression in wound tissues by using western blot analysis*

On D7, 3 rats were selected from each group and wound tissues on the backs of the rats were harvested. For each tissue sample, 50 mg of tissue was weighed, 1 ml of protein lysate was added, and the mixture was ground using a tissue grinder. The cells were disrupted in an ultrasonic cell disruptor, allowed to stand for 30 min at 4°C to facilitate lysis, then centrifuged at 12,000 rpm for 15min at 4 °C.The supernatant was transferred to a new Eppendorf tube. The protein concentration of the supernatant was measured using the bicinchoninic acid method and subsequently adjusted to a standard concentration. Then, corresponding volumes of loading buffer and phosphate-buffered saline were added for denaturation at 95 °C for 10 min. After pre-cooling on ice, gel electrophoresis was performed, and the protein was transferred to a membrane and blocked with blocking solution for 1 h at room temperature. The primary antibody (dilution ratio 1:1000) was added for overnight incubation at 4 °C, and the membrane was rapidly washed 3 times using Tris-buffered saline-Tween 20 (TBST), with each wash lasting 5 min. Subsequently, the secondary antibody (dilution ratio 1:5000) was added for incubation for 1 h at room temperature, and the membrane was rapidly washed 3 times using TBST, with each wash lasting 5 min. Lastly, images of the membranes were captured for analysis.

### *4.7. Detection of TGF-b1 and MMP-1 expression in rat blood using ELISA*

On D7 of model establishment, blood samples were collected from the abdominal aorta of rats to obtain sera before sacrificing the rat. ELISA kits were used to measure the expression of TGF-b1 and MMP-1 in rat sera at each time point according to the instructions provided with the kits.

### *4.8. Statistical analysis*

All data were processed using SPSS 17.0. Categorical data and rates were compared using the $\chi^2$ test; quantitative data and mean values were compared using the t-test; and wound healing rates were compared using the Wilcoxon rank sum test.

Acknowledgment: The work was supported by the Science Research Fund of Ningxia Medical Univerity (XM2016070).

Conflict of interest: None declared.

## References

Ben-Neriah Y, Karin M (2011) Inflammation meets cancer, with NF-κB as the matchmaker. Nat Immunol 12: 715-723.
Bonizzi G, Karin M (2004) The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol 25: 280-288.
Ghosh S, May MJ, Kopp EB (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Ann Rev Immunol 16: 225-260.
Gupta SC1, Sundaram C, Reuter S, Aggarwal BB (2010) Inhibiting NF-κB activation by small molecules as a therapeutic strategy. Biochim Biophys Acta 1799: 775-787.
Kirsner RS, Eaglstein WH (1993) The wound healing process. Dermatol Clin 11: 629-640.
Li A (1995) Therapeutics of Burns. 2nd Ed. Beijing: People's Medical Publishing House, pp. 7-14.
Luo X, Lin C, Wang X (2016) Effects of heterologous acellular dermal matrix combined with autologous skin grafts on the residual wounds with titanium mesh in skull defects. J Wenzhou Med Univ 46: 287-288.
May MJ, Ghosh S (1997) Rel/NF-kappaB and IkappaB proteins: an overview. Semin Cancer Biol 8: 63-73.
McCusker MM, Grant-Kels JM (2010) Healing fats of the skin: the structural and immunologic roles of the omega-6 and omega-3 fatty acids. Clin Dermatol 28: 440-451.
McMonnies CW (2015) Hyperbaric oxygen therapy and the possibility of ocular complications or contraindications. Clin Exp Optom 98: 122-125.
Mirastschijski U, Impola U, Karsdal MA, Saarialho-Kere U, Agren MS (2012) Matrix metalloproteinase inhibitor BB-3103 unlike the serine proteinase inhibitor aprotinin abrogates epidermal healing of human skin wounds ex vivo. J Invest Dermatol 118: 55-64.
Na J, Lee K, Na W, Shin JY, Lee MJ, Yune TY, Lee HK, Jung HS, Kim WS, Ju BG (2016) Histone H3K27 demethylase JMJD3 in cooperation with NF-κB regulates keratinocyte wound healing. J Invest Dermatol 136: 847-858.
Niinikoski JH (2004) Clinical hyperbaric oxygen therapy, wound perfusion, and transcutaneous oximetry. World J Surg 28: 307-311.
Poulakidas S, Kowal-Vern A (2008) Facilitating residual wound closure after partial graft loss with vacuum assisted closure therapy. J Burn Care Res 29: 663-665.
Qiao X, Wu ST, Liu LY, Hu CR, He DP, Yang J (2017) Comparison in qualities of different China pecans and China pecan oils. China Oils Fats 42: 139-143.
Rodriguez PG, Felix FN, Woodley DT, Shim EK (2008) The role of oxygen in wound healing: a review of the literature. Dermatol Surg 34: 1159-1169.
Schreml S, Szeimies RM, Prantl L, Landthaler M, Babilas P. Wound healing in the 21st century. J Am Acad Dermatol 63: 866-881.
Smith TJ (2000) Squalene: potential chemopreventive agent. Expert Opin Investig Drugs 9: 1841-1848.
Yu W, Bi Y, Cheng B, Li Q, Yang CH, Liu HW, Zeng D (2009) Expression and effectiveness of connective tissue growth factor in residual wound after skin grafting. Chin J Aesthetic Med 2009: 194-196.
Zhang AJ, Yan ZY (2013) Effect of TGF-β on wound healing and scar formation and the intervention effect of Chinese medicine on TGF-β. Northwest Pharm J 28: 101-105.