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The *in vivo* and *in vitro* diabetic wound healing effects of a 2-herb formula and its mechanisms of action

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ABSTRACT

Ethnopharmacological relevance: The herbs Radix Astragali (RA) and Radix Rehmanniae (RR) have long been used in traditional Chinese Medicine and serve as the principal herbs in treating diabetic foot ulcer.
Aim of study: Diabetic complications, such as foot ulcer, impose major public health burdens worldwide. In our previous clinical studies, two Chinese medicine formulae F1 and F2 have achieved over 80% limb salvage. A simplified 2-herb formula (NF3) comprising of RA and RR in the ratio of 2:1 was used for further study. NF3 was examined for the ulcer healing effect in diabetic rats, and its potential mechanisms of action in fibroblast proliferation, angiogenesis and anti-inflammation *in vitro*.
Materials and methods: A chemically induced diabetic foot ulcer rat model was used for studying the wound healing effect. In the *in vitro* mechanistic studies, human fibroblast cells (Hs27), human umbilical vein endothelial cells (HUVEC) and mouse macrophage cells (RAW264.7) were assessed for tissue regeneration, angiogenesis and anti-inflammatory activities, respectively.
Results: Our *in vivo* results demonstrated a significant reduction of wound area at day 8 in NF3 (0.98 g/kg) group as compared to control ($p < 0.01$). NF3 could significantly stimulate Hs27 proliferation in a dose dependent manner ($p < 0.05$). Besides, NF3 could significantly increase the cell migration and tube formation ($p < 0.05$–0.001) of HUVEC in the angiogenesis study. Furthermore, significant inhibition of nitric oxide production ($p < 0.01$) was found in NF3-treated macrophage cells, suggesting its anti-inflammatory activity.
Conclusions: Our study presents for the first time scientific evidence towards the efficacy of the two-herb formula NF3 in enhancing diabetic wound healing through the actions of tissue regeneration, angiogenesis and anti-inflammation.

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1. Introduction

Diabetes is projected to rise from 171 million to 366 million in 2030 worldwide (World Health Organization, 2010). This major increase in morbidity and mortality of diabetes is due to the development of both macro- and micro-vascular complications, including failure of the wound healing process. Currently, the approved growth factor and cell therapies for diabetic foot ulcers are not routinely used during treatment. Improper wound healing control may result in diabetic foot ulcer or even amputation (Levin, 2002).

Wound healing is a very orderly and highly controlled process characterized by four distinct but overlapping phases: hemostasis, inflammation, proliferation and remodeling (Diegelmann and Evans, 2004). The repair process needs the coordination of various cells, growth factors and cytokines. Inflammation is the initial phase of wound healing which the macrophages mainly take the role. They are responsible for removing non-functional host cells and bacteria. Besides, they coordinate repair through production of a broad spectrum of factors that influence angiogenesis, fibroplasias and extracellular matrix synthesis (DiPietro, 1995). Angiogenesis is sustained until the terminal stages of healing. The proliferation,

Abbreviations: DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; Hs27, normal human skin fibroblasts; HUVEC, human umbilical vein endothelial cells; LPS, lipopolysaccharide; MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NO, nitric oxide; PS, penicillin–streptomycin; RA, Radix Astragali; RR, Radix Rehmanniae; STZ, Streptozotocin; TCM, traditional Chinese medicine.

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migration and remodeling of endothelial cells followed by subsequent tube formation are the crucial process in angiogenesis (Park et al., 2006). In the final stage of wound healing, fibroblast proliferation involves in the restoration of structure and function in the wound site (Clark, 2001). However, many studies showed that high glucose concentration inhibits wound healing process associated with prolonged inflammatory phase (Naguib et al., 2004), defected angiogenesis (Goren et al., 2006) and diminished fibroblast proliferation (Hehenberger and Hansson, 1997).

Traditional Chinese medicine (TCM) is widely practised nowadays and is viewed as alternatives to conventional medicine in different diseases. Based on the positive clinical effects of the two herbal formulae F1 and F2 in diabetic foot ulcer patients, about 85% of unhealed legs were prevented from limb amputation (Wong et al., 2001). The principal component herbs Radix Astragali (RA) and Radix Rehmanniae (RR) were effective in enhancing fibroblast proliferation, which was the main step in wound healing (Lau et al., 2007, 2009a). According to the classical Chinese medicine theory, RA, derived from the dried root of *Astragalus membranaceus* (Fisch.) Bge. in the family Fabaceae (Leguminosae), is one of the important "Qi tonifying" herbs from the Chinese materia medica (Sinclair, 1998). RA can support "Qi" which promotes skin wound healing and muscle regeneration (Zhang et al., 2009). On the other hand, RR is obtained from the root of *Rehmannia glutinosa* Libosch in the family of Scrophulariaceae. RR can reduce heat in blood, nourish "Yin" and promote the production of body fluid (Yen, 1997). Based on the traditional Chinese medicine theory, these two herbs could facilitate the healing of ulcers through the improvement of "Qi" and "Yin". According to literature reviews, RA and RR might be active in the healing of diabetic foot ulcers and were believed to elicit anti-inflammatory effects (Zhang, 1974; Zhang et al., 1984). Besides, RA was shown to alleviate insulin resistance (Lu et al., 1999), while RR had inhibitory effect on the progression of diabetic nephropathy (Yokozawa et al., 2004).

Hence, RA and RR were chosen and formed in our new formula (NF3) in the ratio of 2:1 based on the same ratio of our previous formulae. Selection of the two herbs was based on the scientific evidences of the efficacies in wound healing and the literature support for their bioactivities. In this study, NF3 was assessed for its *in vivo* wound healing effect in diabetic wound induced rats. With regard to the underlying mechanisms in wound healing, the *in vitro* cell models of fibroblast Hs27, HUVEC and RAW 264.7 cells were employed to study the tissue granulation, angiogenesis and anti-inflammatory effects, respectively, under the treatment of NF3.

2. Materials and methods

2.1. Authentication and extracts preparation of RA and RR

The raw herbs of RA and RR were purchased from mainland China. They were authenticated by morphological characterizations and thin layer chromatography in accordance with the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2005). Voucher specimens of RA and RR were deposited in the museum of Institute of Chinese Medicine, The Chinese University of Hong Kong, with voucher specimen numbers: 2008–3201 for RA and 2008–3200 for RR. Besides, chemical fingerprinting study on NF3 was also conducted using HPLC analysis (see Supplementary Information).

For extracts preparation, raw herbs of RA and RR were cut into small pieces and mixed in the ratio of 2:1 (w/w). The ratio of 2:1 was derived from our previously established herbal formulae 1 and 2 (Wong et al., 2001; Lau et al., 2007, 2009a). After being soaked in 10 volumes of distilled water for 30 min, the herbs were boiled under reflux for 1 h twice. The extracts were pooled, filtered and lyophilized into dry powder. The extraction yield was around 34% (w/w).

2.2. Animals

Female albino Wistar rats were used in wound healing and diabetic studies. All animals were supplied by and kept in the Laboratory Animal Service Centre, The Chinese University of Hong Kong. They were housed under the conditions of 22–25 °C and a 12-h light-dark cycle. They were supplied with standard animal chow (PicoLab Rodent Diet 20, PMI Nutrition International, Inc., USA) and tap water was constantly supplied to the animals. All animal studies were performed according to the institutional rules concerning animal experiments (CUHK-AEEC animal experimentation ethics approval nos.: 07/084/MIS and 08/003/MIS).

2.3. In vivo diabetic wound healing study

A previously established chemically induced type II diabetic foot ulcer animal model was employed in this study (Lau et al., 2008). The diabetic syndrome in the n5-STZ model was generated by intraperitoneally injecting streptozotocin (STZ) at 70 mg/kg body weight into Wistar rats on the fifth day after birth (n5 = 5 days after birth). When the rats reached 10 weeks old, blood was collected from tail veins and the plasma glucose levels were determined. For those diabetic rats (plasma glucose levels $\geq$ 250 mg/dl), a standardized wound area (2 mm $\times$ 5 mm skin in full thickness removed) was induced on the dorsal surface of the right hind foot of rats under anaesthetisation with ketamine-xylazine cocktail. One day after wound induction, the wound sizes were measured and sample intervention was started. Water (as negative control) or NF3 (at clinical relevant dose of 0.98 g/kg, based on the human equivalent dose in our diabetic foot ulcer study, according to the FDA convention table, U.S. Department of Health and Human Services, 2005) was force-fed into the rats for 7 consecutive days. On day 8, the plasma glucose levels and wound areas were measured.

2.4. Cell culture and cell viability test

Normal human skin fibroblasts (Hs27 fibroblasts), human umbilical vein endothelial cells (HUVEC) and murine monocyte/macrophage RAW264.7 cells were purchased from American Type Culture Collection (ATCC; USA). Hs27 fibroblasts and RAW264.7 cells were maintained in high-glucose DMEM (D-glucose: 4500 mg/l; GIBCO, USA), 10% fetal bovine serum (FBS; GIBCO, USA) and 1% penicillin–streptomycin (PS; GIBCO, USA). HUVEC were subcultured to confluence in complete Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Life technologies, USA) supplemented with 0.1 mg/ml heparin and 0.03 mg/ml endothelial cell growth supplement. All cells were maintained at 37 °C, 5% CO_2 humidified incubator. Cell viability was determined by MTT assay (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, USA) after 48 h treatment for HUVEC and 24 h incubation for RAW264.7 with various concentrations of NF3. The relative amount of viable cells was determined at optical density at 540 nm and expressed as the percentage of control samples without treatment.

2.5. Fibroblast proliferation assay

Hs27 fibroblasts were seeded at 3000 cells per well in 96-well plate in DMEM with 0.125% FBS. Cells were exposed to different concentrations of NF3. The treatment lasted for 48 h at 37 °C. MTT solution (5 mg/ml) in 1$\times$ PBS was added directly to the medium in each well, and the plate was then incubated at 37 °C for 4 h.

All medium was then aspirated and replaced with DMSO, and the optical density at 540 nm was recorded.

2.6. Migration assay

The migration of HUVEC was examined using the wound healing method (Sato and Rifkin, 1988). HUVEC (1 × 105 cells) were seeded into each well of a 24-well plate and incubated with complete medium at 37 °C and 5% CO_2. After 24 h of incubation, the cells were starved in medium with 0.5% FBS for 24 h. HUVEC were scrapped horizontally and vertically with a P100 pipette tip and two views on the cross were photographed on each well attached to the microscope at 4× magnification. The medium was replaced with fresh medium in the absence or presence of NF3. After 16 h incubation, the second set of images were photographed. To determine the migration of HUVEC, the images were analyzed using Tscratch software (Gebäck et al., 2009). Percentage of the closed area was measured and compared with the value obtained before treatment. An increase of the percentage of closed area indicated the migration of cells.

2.7. Tube formation assay

The effect of NF3 on HUVEC differentiation and vascular formation was assessed by tube formation on Matrigel (Merchan et al., 2003). HUVEC were seeded onto 96-well plates at 1.5 × 104 cells per well over 50 μl Matrigel (BD BioSciences, USA). Fresh media in the absence or presence of NF3 were subsequently added. Tubular structures were photographed after 6 h. The total tube length formation was measured for quantification of angiogenesis by the Image-Pro Plus version 6.0 (Media Cybernetics, USA).

2.8. Nitric oxide (NO) inhibitory assay

Macrophage cells RAW264.7 (4 × 105 per well) were seeded in 24-well plate overnight. With 0.1 μg LPS per ml of medium, NF3 was added (at concentrations ranging from 625 to 5000 μg/ml) and incubated for 24 h. Culture supernatant was added to Griess Reagent in the ratio of 1:1 in a 96-well plate and the plate was incubated in darkness for 10 min. The plates were then read at a wavelength of 540 nm spectrophotometrically. Nitrite standard curve was plotted with standard $NaNO_2$ solution with Griess treatment to determine the concentration of nitrite in the nitric oxide assay.

2.9. Statistical analysis

For multiple group comparison of cell proliferation, cell migration, total tube length formation and NO production, the significance of the differences among the treatment groups and their respective control groups were tested by one-way ANOVA with Dunnett's post test. For animal studies, the differences in rats' characteristics between those in the NF3-treated groups and those in the water treatment group were assessed by Student's *t*-test. Multilevel modeling was used to examine whether NF3 treatment was associated with wound area reduction with adjustments for baseline wound area, body weight and blood glucose across the examination period. The free software *MIXREG* (accessed at http://tigger.uic.edu/~hedeker/mix.html) was used for fitting multilevel models and all the other statistical analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Data were presented as mean ± standard deviation (SD) unless otherwise specified. All statistical tests were two-sided, with $p < 0.05$ as considered statistically significant.

Table 1
Effects of NF3 on wound area, plasma glucose level and body weight of diabetic rats in water treatment and NF3 treatment groups. n5-STZ-induced diabetic rats were induced with a standardized wound on the dorsal surface of the right hind foot. Wound sizes were measured on the day after wound induction. Following by 7 days of water or NF3 intervention, wound areas were measured on day 8. Data are expressed as mean ± 95% CI.

Parameter	Water group (n = 47)	NF3 group (n = 48)
Wound area (mm^2)		
Day 0	20.8 ± 4.9	21.8 ± 4.8
Day 8	8.4 ± 4.3	6.6 ± 3.9**
Plasma glucose (mg/dl)		
Day 0	462.3 ± 180.6	449.5 ± 193.4
Day 8	421.1 ± 171.8	389.7 ± 188.2
Body weight (g)		
Day 0	257.7 ± 40.3	263.1 ± 34.4
Day 8	250.7 ± 35.4	257.2 ± 33.7

** $p < 0.01$ versus control.

3. Results

3.1. Effects of NF3 on wound healing of diabetic rats

As shown in Table 1, the average ulcer area in the water treatment group decreased from 20.8 mm^2 on day 1 to 8.4 mm^2 on day 8. For NF3 treatment group, the average ulcer area decreased from 21.8 mm^2 on day 1 to 6.6 mm^2 on day 8. There was a significant difference on wound area at day 8 between water (control) and NF3 (0.98 g/kg) groups after adjusting for baseline wound area, body weight and plasma glucose across the study period (**$p < 0.01$). The results demonstrated that there was a significant wound healing effect in the NF3 treatment group as compared with water treatment group.

3.2. In vitro fibroblast epithelization study

3.2.1. Effects of NF3 on Hs27 fibroblast proliferation

Fibroblast proliferation is an important step in the wound healing for tissue regeneration. From our results (Fig. 1), after treatment of NF3, significant viability-enhancement effects were observed in Hs27 fibroblasts in 625, 1250 and 2500 μg/ml ($p < 0.01$–0.001) with proliferation rate of 122.7%, 130% and 125.3%, respectively.

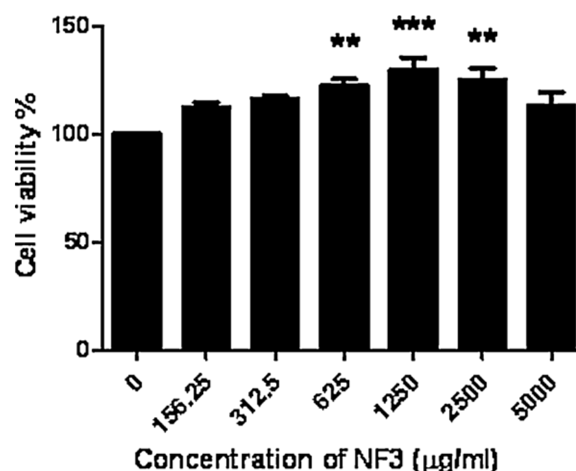

Fig. 1. The effects of NF3 on the viability of Hs27 fibroblasts. Cells were seeded onto 96-well plate with the addition of different concentrations of NF3 for 48 h. The value from the baseline control group was set at 100%. The proliferation effect was estimated by MTT assay and calculated by comparing the values from the NF3 treatment group with the control group. Data are expressed as mean ± SD from three individual experiments. **$p < 0.01$ and ***$p < 0.001$ versus control.

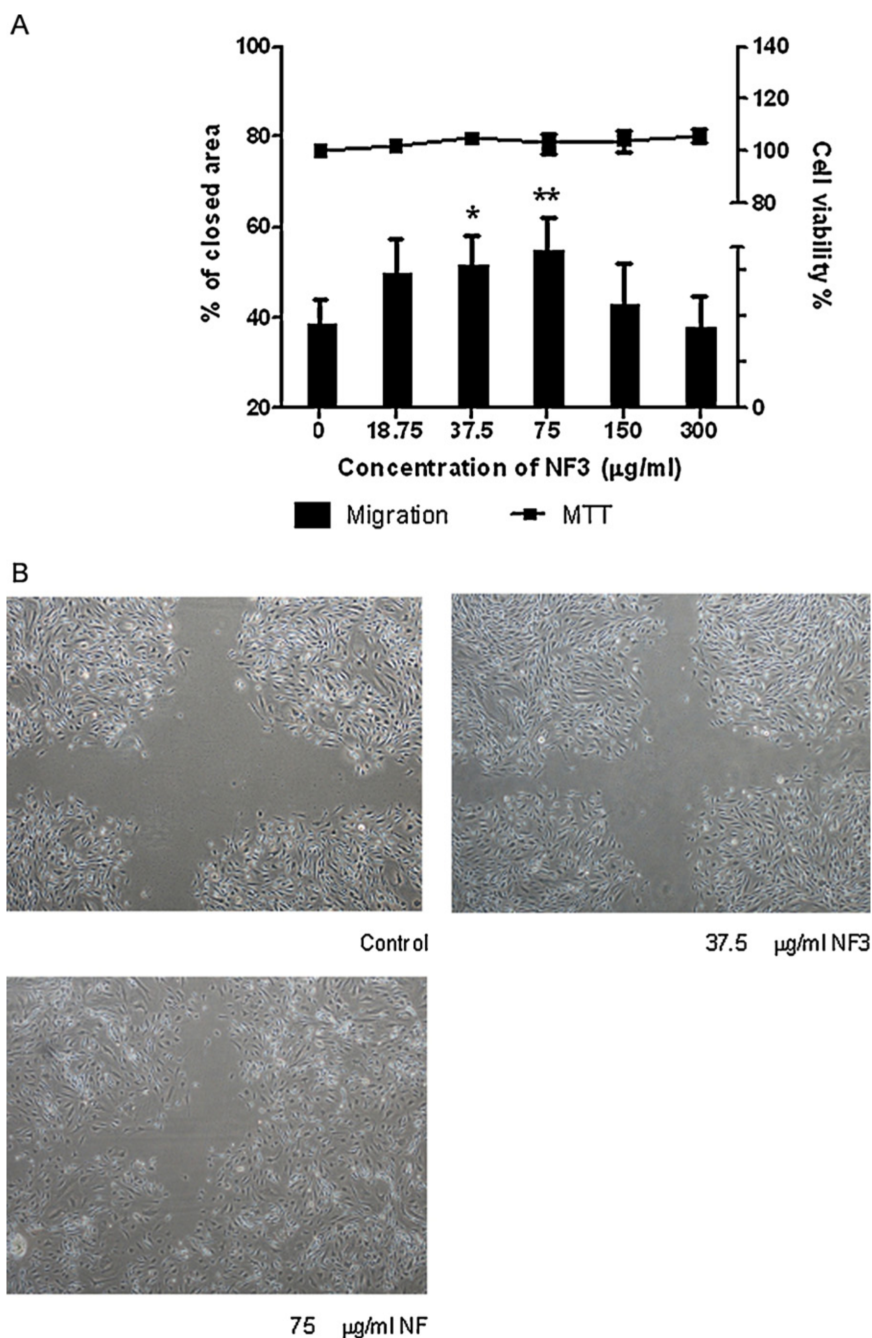

Fig. 2. Effect of NF3 on HUVEC migration. (A) Quantitative analysis of the NF3-induced HUVEC migration (Migration) and cell viability (MTT) were analyzed after the treatment with various concentrations of NF3. (B) NF3-induced HUVEC migration after 16 h were photographed in the wound healing scratched assay. HUVEC were seeded in complete medium and serum starved for 24 h. Cells were scratched horizontally and vertically with P100 pipette tips. Images were captured at 0 h and various concentrations of NF3 were added to the wells. Another set of images were photographed after 16 h incubation of NF3. The migration was quantified by the Tscratch software. Date are expressed as mean ± SD from triplicate experiments for cell viability assay and five individual experiments for migration assay. $^{*}p < 0.05$ and $^{**}p < 0.01$ for differences in percentage of closed area from baseline cultures without treatment.

3.3. In vitro angiogenesis study

3.3.1. Effects of NF3 on migration of HUVEC

The proliferative effects of NF3 extracts on HUVEC were evaluated by the MTT assay. After 24 h of starvation, HUVEC were cultured in medium containing 18.75 to 300 µg/ml of NF3 for 48 h. As shown in Fig. 2A, no significant change was found in the cell viability of endothelial cells treated with different concentrations of NF3, indicating that NF3 was non-toxic and probably did not affect the cellular activities of HUVEC. The effect of NF3 on endothelial cell migration was determined using the wound healing method. As shown in Fig. 2A and B, 37.5 µg/ml NF3 increased migration of HUVEC significantly ($p < 0.05$) by 33.4%. A more significant increase in HUVEC migration was observed at 75 µg/ml NF3 ($p < 0.01$) by 41.7%. Thus, NF3 significantly enhanced HUVEC migration in a dose dependent manner.

3.3.2. Effects of NF3 on tube formation of HUVEC

Angiogenesis is a complex process that involved endothelial cell proliferation and migration, leading to subsequent vascular structure formation (Arnaoutova et al., 2009). Tube formation assay was used to evaluate the ability of endothelial cell differentia-

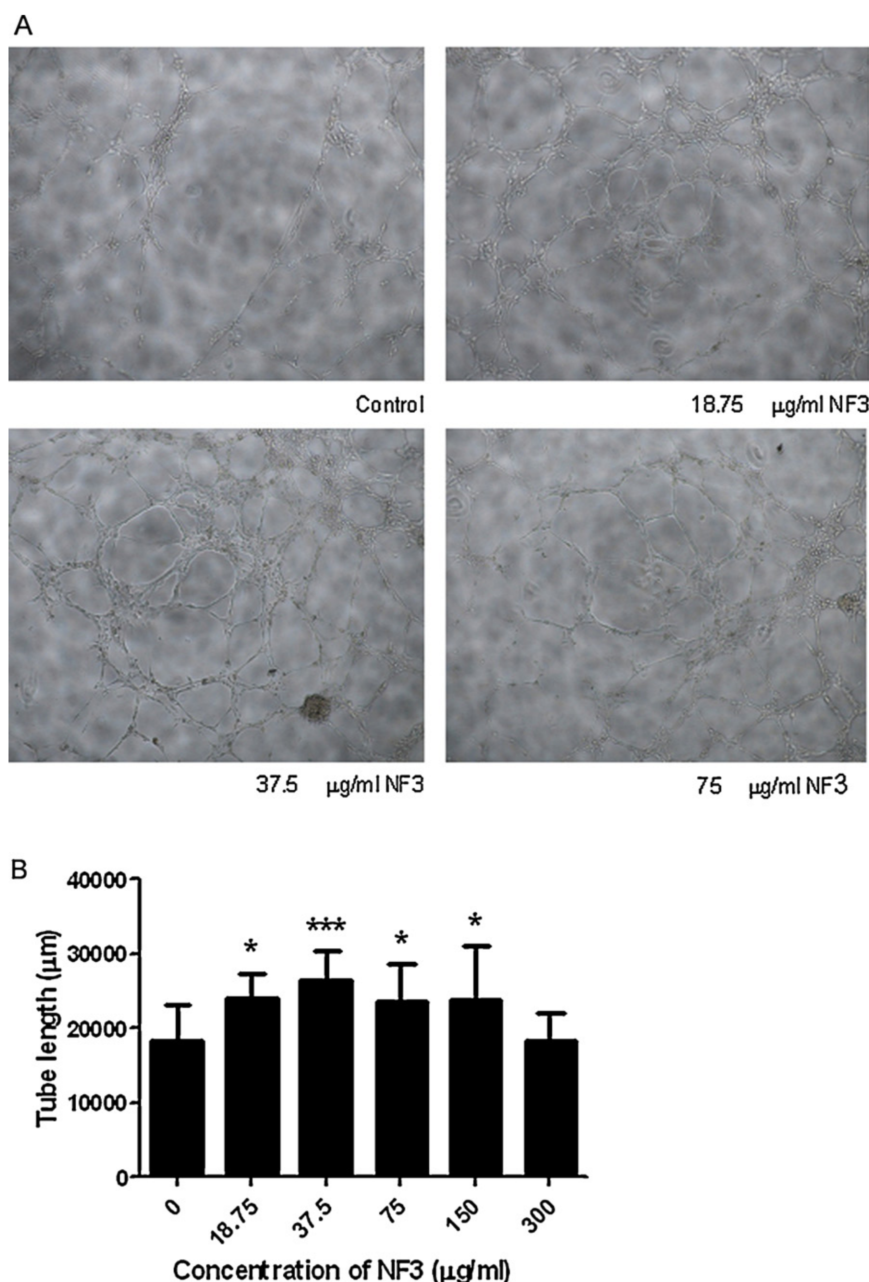

Fig. 3. Effect of tube formation of NF3-treated HUVEC on Matrigel. (A) Morphological changes of HUVEC differentiation in vascular network in three-dimensional Matrigel after treatment of NF3 for 6 h. (B) Quantitative analysis of the total tube length in NF3-treated HUVEC with the use of Image-Pro Plus software. Data are expressed as the mean tube length (μm) ± SD from four individual experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$ versus control.

tion in angiogenesis after treatment of NF3. Our results showed that when HUVEC were cultured on Matrigel without addition of NF3, HUVEC formed little and simple hallow tube-like structure (Fig. 3A). However, after incubation of NF3 for 6 h, a more complex and branched tubular structure was formed. In Fig. 3B, quantification revealed that different concentrations of NF3 from 18.75 to 150 μg/ml significantly enhanced the tube formation ability of HUVEC ($p < 0.05$–0.001).

3.4. *In vitro anti-inflammation study*

3.4.1. *Effects of NF3 on nitric oxide production of RAW264.7 cells*

In Fig. 4, our results demonstrated that NF3 enhanced RAW264.7 cells viability in the concentration up to 5000 μg/ml NF3, revealing that NF3 was non-toxic to the cells and did not affect the normal cellular activity. All concentrations of NF3 (625–5000 μg/ml) strongly suppressed the nitric oxide production in RAW264.7 cells after LPS induction by 13%, 21.7%, 46.3% and 80%, respectively when compared to the baseline control group (100%).

4. Discussion

The treatment of diabetic foot ulcers consists of debridement, offloading and infection control (Cavanagh et al., 2005). Besides, new treatments for diabetic foot ulcer continue to be introduced (Eldor et al., 2004). However, most treatments are effective in mild to moderate wounds and the risk of amputation has not yet been addressed. Integration of the traditional Chinese medicine with conventional treatments is urgently needed in treating diabetic foot ulcers.

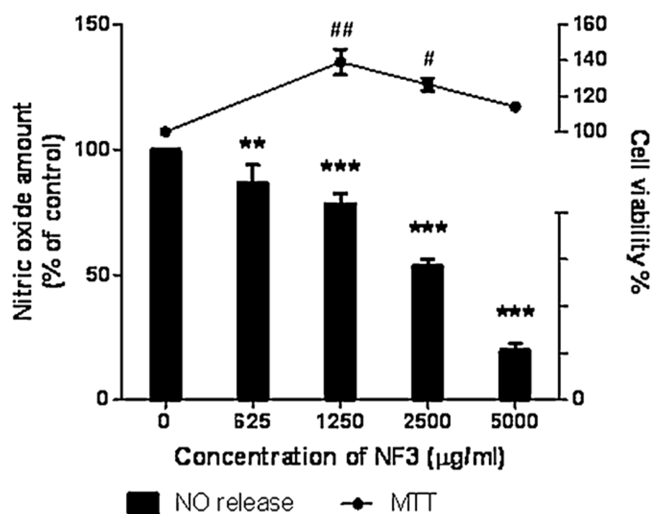

Fig. 4. Effect of NF3 on the NO production in RAW264.7 cells. The levels of NO release and cell viability (MTT) of RAW264.7 cells were assessed after incubation with different concentrations of NF3 for 24 h. NO levels in culture supernatant were determined by Griess assay upon the addition with 0.1 μg/ml LPS. Changes in the level of cell proliferation were analyzed by MTT assay. The value from the baseline control group without treatment was set at 100%. Data are expressed as mean ± SD of three individual experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ for difference in NO levels from control. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ for difference in cell viability from control.

Based on the positive clinical effects of the two herbal formulae F1 and F2 (Wong et al., 2001), with addition to the fibroblast proliferating effects using F1 and F2 formulae and individual herbs of RA and RR (Lau et al., 2007, 2009a), these two herbs (RA and RR) were selected to optimize the formula efficacy. The extracts and constituents of RA and RR have been shown to have various bioactivities. RA extract had previously been reported to alleviate insulin resistance (Lu et al., 1999). Astragaloside IV was shown to improve endothelial cell dysfunction induced by high glucose (Li et al., 2006). Moreover, an isoflavone, calycosin, isolated from RA was revealed to improve impairment of the barrier function induced by hypoxia in endothelial cells (Fan et al., 2003). While RR extract had been demonstrated to improve the peripheral microcirculation of different chronic diseases (Kubo et al., 1994) and have inhibitory effect on the progression of diabetic nephropathy (Yokozawa et al., 2004). Polysaccharides isolated from RR were also shown to accelerate hematopoiesis function (Liu et al., 2009). Besides, catalpol, extracted from RR, enhanced the proliferation, differentiation and matrix mineralization of mouse osteoblast cell line in osteoporosis (Wu et al., 2010). In the present study, a two-herb formula (NF3) comprising of RA and RR in the ratio of 2:1 was derived from the original two herbal formulae and it was evaluated for its *in vivo* diabetic wound healing effect and investigated for the underlying modes of action.

The wound healing effect of NF3 was studied using our previously established diabetic wound induced rat model (Lau et al., 2008), by comparing the wound area size in water (control) and NF3 treatment group. Wound size measurement has long been used to assess the progress of wound healing (Smith et al., 1986; Flanagan, 2003) while fibroblast proliferation has been adopted as an indicator for the assessment of wound healing (Adolphe et al., 1984; Bucalo et al., 1993; Park et al., 2005). Fibroblasts are the major cell type found in the granulation of wound tissues. They play an essential role in wound healing including secretion of a series of growth factors that facilitates angiogenesis, proliferation and matrix deposition (Mansbridge et al., 1999; Park et al., 2005). Fibroblast Hs27 is derived from the normal foreskin of newborn. On the other hand, HUVEC are currently used as *in vitro* model systems for various physiological and pathological processes, especially in angiogenesis research (Park et al., 2006). The proliferation, migration and formation of tubular structure of endothelial cells are the indications for development of new blood vessels from pre-existing vascular bed in angiogenesis (Holash et al., 1999; Lamalice et al., 2007). For *in vitro* anti-inflammation study, the LPS induced RAW 264.7 macrophage cells are widely used as screening platform for anti-NO production activities (Nussler and Billiar, 1993). Bacterial infection or immunological stimuli LPS cause macrophages to produce large amount of NO. High amounts of NO are potentially cytotoxic and capable of destructing the surrounding cells and tissues (Chiou et al., 2000).

In our present study, we showed that the administration of NF3 caused a positive wound healing effect in diabetic rats as indicated by significant wound size reduction after treatment. The wound healing effect of NF3 was similar to our previous findings on the healing effects of RR water extract on the diabetic wounds (Lau et al., 2009b). Our *in vitro* studies demonstrated that NF3 significantly enhanced fibroblast cell proliferation. In addition, NF3 was shown to be potent in promoting angiogenesis as indicated in increased response in migration scratch assay and tube formation assay. We also demonstrated that NF3 was effective in inhibiting the NO production in murine macrophages, suggesting that NF3 was anti-inflammatory.

Wound healing occurs as a cellular response to injury and involves activation of fibroblast, endothelial cells and macrophages. In diabetic ulcers, healing impairment is caused by a number of physiological factors including diminished fibroblast proliferation (Hehenberger and Hansson, 1997), decreased angiogenesis (Galiano et al., 2004) and persistent inflammatory response (Naguib et al., 2004). Hehenberger reported that high glucose concentration inhibited proliferation of primary dermal fibroblast from patients and the addition of insulin-like growth factor-I and epidermal growth factor could not reverse the growth inhibition. Poor vascultures of diabetic wound and increased inflammatory cytokine tumor necrosis factor-α expression were also found in diabetic mice (Galiano et al., 2004; Naguib et al., 2004). Therefore, agents that stimulate fibroblast growth, promote angiogenesis and relieve inflammation may improve diabetic wound healing. Our results demonstrated the efficacy of NF3 in diabetic foot ulcer animal model. NF3 may improve the wound healing process by restoring the normal response of fibroblast proliferation, angiogenesis, as well as inflammation. On the other hand, NF3 exerted little effect on plasma glucose levels in our *in vivo* diabetic rats and *db*/*db* mice (data not shown), which implies that the use of NF3 may cause little interference on blood glucose level. This provides the possibility of the combination of NF3 and conventional anti-diabetic drug treatments in diabetic patients with unhealed foot ulcer by not affecting the conventional glucose regulation in diabetic patients. Various ayurvedic herbal drugs, such as *Abrus precatorious* L., *Aloe vera* L., *Galium aparine* L. and *Rheum emodi* Wall, have been used in treating wound (Kumar et al., 2007). The use of ayurvedic herbal drugs is more popular in Africa and India than that in Asian countries. Among these ayurvedic drugs, the wound healing activities of *Aloe vera* were extensively investigated in the *in vivo* and *in vitro* studies. In the *in vivo* diabetic rats study, Atiba et al. (2010) showed that oral administration of *Aloe vera* aqueous extract to diabetic rats with wound at the back produced a significant wound healing effect ($p < 0.05$). Despite our formula NF3 and *Aloe vera* extract both exhibited wound healing properties, the effective dose of NF3 (980 mg/kg) used was higher than that of *Aloe vera* extract (~100 mg/kg). This might be due to the differences in animal species, wound conditions and severity of diabetic conditions.

In the theory of Chinese medicine, different combinations of herbs can cause systemic therapeutic effects in human subjects. Apart from their individual bioactivities, the herbs may contain

various components that act synergistically to reinforce the bioactivities of others, thereby modulating the therapeutic effects of the herbal medicine (Li et al., 2008). However, the specific interactions between RA and RR in NF3 need to be determined in the future. Future work will focus on the identification and purification of the active component(s) of RA and RR responding to the underlying wound healing mechanisms. In addition, some specific biochemical parameters such as tissue DNA or RNA will also be considered to investigate the pattern of differentially expressed gene of the wound area in the diabetic rats with NF3 treatment.

5. Conclusion

The present study demonstrated for the first time that our two herb formula NF3 was effective in promoting wound healing in diabetic rats. Besides, the *in vitro* data indicated that wound healing effects of NF3 might be due to the regulation and coordination of inflammation, angiogenesis and tissue regeneration. The wound healing effects, however, seems to be independent of blood glucose control. This study gave us good scientific evidence that the herbal formula NF3 is a promising complementary supplement for diabetic patients with wound healing defects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2011.01.032.

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