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Synergistic interaction between Astragali Radix and Rehmanniae Radix in a Chinese herbal formula to promote diabetic wound healing

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ABSTRACT

Ethnopharmacological relevance: Astragali Radix (AR) and Rehmanniae Radix (RR) are two traditional Chinese medicines widely used in China for treating diabetes mellitus and its complications, such as diabetic foot ulcer.

Aim of study: In our previous study, a herbal formula NF3 comprising AR and RR in the ratio of 2:1 was found effective in enhancing diabetic wound healing in rats through the actions of tissue regeneration, angiogenesis promotion and inflammation inhibition. The aims of the present study were to investigate the herb–herb interaction (or the possible synergistic effect) between AR and RR in NF3 to promote diabetic wound healing and to identify the principal herb in the formula by evaluating the potencies of individual AR and RR in different mechanistic studies.

Materials and methods: A chemically induced diabetic foot ulcer rat model was used to examine the wound healing effect of NF3 and its individual herbs AR and RR. For mechanistic studies, murine macrophage cell (RAW 264.7) inflammation, human fibroblast (Hs27) proliferation and human endothelial cell (HMEC-1) migration assays were adopted to investigate the anti-inflammatory, granulation formation and angiogenesis-promoting activities of the herbal extracts, respectively.

Results: In the foot ulcer animal model, neither AR nor RR at clinical relevant dose (0.98 g/kg) promoted diabetic wound healing. However, when they were used in combination as NF3, synergistic interaction was demonstrated, of which NF3 could significantly reduce the wound area of rats when compared to water group ($p < 0.01$). For anti-inflammation and granulation formation, AR was more effective than RR in inhibiting lipopolysaccharide (LPS)-induced nitric oxide production from RAW 264.7 cells and promoting Hs27 fibroblast proliferation. In the aspect of angiogenesis promotion, only NF3 promoted cell migration of HMEC-1 cells.

Conclusions: AR plays a preeminent role in the anti-inflammatory and fibroblast-proliferating activities of NF3. The inclusion of RR, however, is crucial for NF3 to exert its overall wound-healing as well as the underlying angiogenesis-promoting effects. The results of present study justified the combined usage of AR and RR in the ratio of 2:1 as NF3 to treat diabetic foot ulcer and illustrated that AR is the principal herb in this herbal formula.

1. Introduction

Diabetes mellitus is a serious global health problem. The total number of people with diabetes is projected to reach 366 million in 2030, affecting 4.4% of the population worldwide (Wild et al., 2004). Improper control of diabetes mellitus leads to a bundle of ailments, such as cardiovascular diseases, neuropathy, retinopathy and foot ulcer. Among these complications, foot ulceration is associated with an increased risk of death in diabetes,

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and in USA, more than 60% of non-traumatic lower-limb amputations occur in diabetic patients (National Diabetes Fact Sheet, 2011; http://www.cdc.gov/diabetes/pubs/pdf/ndfs_2011.pdf). Diabetic foot ulcer is a difficult clinical problem that cannot be tackled by single means and standard drug has far been available. For diabetic patients suffering from chronic foot ulcer, clinical interventions usually involve conventional antidiabetic treatment, broad-spectrum antibiotics for infection control, daily wound care including antiseptic bath, debridement and toe removal for gangrene when necessary. In spite of various options for coadjuvant therapy for diabetic foot ulcers, a considerable number of patients remain unhealed after 12 weeks of treatment (Vivas et al., 2010). Together with this, the rising costs of associated hospitalization for non-healing diabetic foot ulcers urge the pursuit of new therapeutic agents. On top of those standard wound care practices, herbal remedies receive increasing acceptance and prompt to evidence-based clinical studies (Paocharoen, 2010).

In a randomized, double-blind, placebo-controlled trial, two herbal formulae were demonstrated to be effective in promoting the healing of chronic diabetic ulcers, with limb salvage in 85% of the patients achieved (Leung et al., 2008). With these remarkable clinical outcomes, a series of scientific research works ensued. Among the component herbs, Astragali Radix (AR) and Rehmanniae Radix (RR) were identified as the principal ones. Both of them were the most effective in stimulating the growth of fibroblast cells CRL 7522 as well as fibroblasts isolated from foot ulcer tissues of diabetic patients (Lau et al., 2007, 2009a). Moreover, AR could significantly inhibit glucose uptake from the gut (Chan et al., 2007) and RR at 1.85 g/kg (equivalent to maximum human daily dose) was effective in promoting diabetic foot ulcer healing in rats (Lau et al., 2009b).

Astragali Radix is derived from the dried root of *Astragalus membranaceus* (Fisch.) Bge. (family Leguminosae). It has been used as Chinese medicine for over hundreds of years and traditionally been used to reinforce 'qi', strengthen superficial resistance and promote growth of new tissue (State Pharmacopoeia Commission of P.R. China, 2010). In general, it is used to fortify body vital energy. Scientific reports on its immunomodulatory (Zhang et al., 2009a; Liu et al., 2010b), cardioprotective (Xu et al., 2008; Zhao et al., 2008), as well as insulin-sensitizing (Xu et al., 2009; Hoo et al., 2010; Juan et al., 2011) effects are well documented. In our laboratory, the aqueous crude extract of AR was demonstrated to promote fibroblast proliferation, both in cell line and in primary culture from diabetic foot ulcer patients, which was considered as the crucial step in wound healing (Lau et al., 2007, 2009a). The main constituents of AR include polysaccharides, saponins, flavonoids, amino acids and trace elements (Ma et al., 2002; Yu et al., 2007). Saponin and isoflavone-enriched AR extract could promote angiogenesis in human endothelial cells (Zhang et al., 2009b) and calycosin was the active angiogenesis-promoting isoflavonoid isolated from AR (Tang et al., 2010).

Rehmanniae Radix, the root of *Rehmannia glutinosa* Libosch. (family Scrophulariaceae), is classified as "top grade" (very safe) herb in traditional Chinese medicine. As recorded in Chinese Pharmacopoeia, it can remove pathogenic heat from blood, nourish '*yin*' and promote production of body fluid. Therefore, it is widely prescribed to relieve febrile diseases, diabetes, epistaxis and skin eruption (State Pharmacopoeia Commission of P.R. China, 2010). Recent scientific studies proved that RR and its active principals possessed a wide spectrum of pharmacological actions on the blood system, immune system, endocrine system, cardiovascular system and the nervous system (reviewed by Zhang et al., 2008). More specifically, the aqueous extract of RR could stimulate fibroblast proliferation (Lau et al., 2007, 2009a) and was effective in promoting diabetic foot ulcer healing in rats through the processes of tissue regeneration, angiogenesis and inflammation control (Lau et al., 2008, 2009b). On the other hand, its ethanolic extract and oligosaccharide fraction exhibited hypoglycemic activity in streptozotocin (STZ)-induced diabetic rats and glucose-induced hyperglycemic rats, respectively (Zhang et al., 2004; Waisundara et al., 2008). Moreover, the effect of RR extract in inhibiting the progression of diabetic nephropathy was also reported (Yokozawa et al., 2004). In the aspect of chemical composition, more than 70 compounds including iridoids, saccharides, amino acid, inorganic ions, as well as other trace elements have been found in RR (Zhang et al., 2008).

Based on data from pilot study, AR and RR in the ratio of 2:1 formed an innovative formula named NF3. As shown in our previous study, NF3 enhanced diabetic wound healing in rats through the actions of tissue regeneration, angiogenesis promotion and inflammation inhibition (Tam et al., 2011). In this study, the herb–herb interaction between AR and RR in NF3 to promote diabetic wound healing was investigated. Also, the roles of AR and RR in different mechanisms of action would be discussed. An attempt was made to identify the principal herb in the formula by evaluating the potencies of individual AR and RR in different mechanistic studies. In this regards, LPS-induced RAW 264.7 cell inflammation, human fibroblast (Hs27) proliferation and human endothelial cell (HMEC-1) migration assays were adopted to investigate, respectively, the anti-inflammatory, granulation formation and angiogenesis-promoting activities of the herbal extracts.

2. Materials and methods

2.1. Authentication of raw herbs and extract preparation of NF3, AR and RR

Raw herbs of AR and RR were purchased from mainland China. They were authenticated by morphological characterizations and thin layer chromatography in accordance with the Chinese Pharmacopoeia (State Pharmacopoeia Commission of P.R. China, 2010). Voucher specimens of AR 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 AR and 2008-3200 for RR.

For NF3 extract preparation, raw herbs of AR and RR were cut into small pieces and mixed in the ratio of 2:1 by weight. After being soaked in 10 volumes of distilled water for 30 min, they 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). Individual herbal extracts of AR and RR were prepared in the same way and the extraction yields were 37% (w/w) and 45% (w/w), respectively.

2.2. In vivo diabetic wound healing study

Female albino Wistar rats used in diabetic wound healing study 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. Standard animal chow (PicoLab Rodent Diet 20, PMI Nutrition International, Inc., USA) and tap water were allowed to be accessed *ad libitum*. All animal studies were performed according to the institutional rules governing animal experiments (CUHK-AEEC approval no.: 08/003/MIS).

A previously established chemically induced type II diabetic foot ulcer animal model was employed in this study (Lau et al., 2008; Tam et al., 2011). Diabetes was induced in animals by intraperitoneal injection of streptozotocin (STZ) (Sigma–Aldrich Co., St. Louis, MO, USA) at 70 mg/kg body weight into Wistar rats on their fifth day after birth. When the rats reached 10 weeks old, they were weighed, blood was collected from tail veins and plasma glucose

levels were determined. For those diabetic rats (plasma glucose levels ≥ 250 mg/dl), a standardized wound area (2 mm × 5 mm skin in full thickness removed) was induced on the dorsal surface of the right hind foot of rats under anesthetization with thiopentone sodium (40 mg/kg) (Hospira Inc., Lake Forest, IL, USA). One day after wound induction, the wound sizes were measured and sample interventions were started. Water (as negative control), NF3 (at clinical relevant dose = 0.98 g/kg), AR (0.98 g/kg) or RR (0.98 g/kg) was force-fed into the rats for 7 consecutive days. On Day 8, body weights, plasma glucose levels and wound areas were measured.

2.3. Cell culture

Normal human skin fibroblasts (Hs27), human microvascular endothelial cell (HMEC-1) and murine monocyte/macrophage (RAW 264.7) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Hs27 fibroblasts and RAW 264.7 cells were maintained in high-glucose DMEM (D-glucose: 4500 mg/L) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin (PS) (Invitrogen Co., Carlsbad, CA, USA). HMEC-1 cells were sub-cultured to confluence in complete MCDB 131 medium supplemented with hydrocortisone and 125 ng/ml human epidermal growth factor. All cells were maintained at 37 °C, 5% CO_2 humidified incubator.

2.4. Anti-inflammation – lipopolysaccharide (LPS)-induced inflammation assay

Cell viability of mouse macrophage RAW 264.7 cells was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay after 24 h incubation with various concentrations of NF3, AR or RR. The relative amount of viable cells was determined as optical density at 540 nm. For anti-inflammation assay, RAW 264.7 (4×10^5 per well) were seeded in 24-well plate overnight. With 0.1 μg LPS per ml of medium, NF3, AR or RR at concentrations ranging from 0.625 to 5 mg/ml were added to the cells and incubated at 37 °C for 24 h. Culture supernatant was transferred and 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 plate was then read at 540 nm spectrophotometrically. Nitrite standard curve was plotted with standard $NaNO_2$ solution with Griess treatment to determine the concentration of nitrite in NF3/AR/RR-treated samples.

2.5. Granulation formation – fibroblast proliferation assay

Hs27 fibroblasts were seeded at 3000 cells per well in 96-well plate in DMEM with 0.125% (v/v) FBS. Cells were then incubated in different concentrations of NF3, AR or RR for 48 h at 37 °C. Thirty microliters of MTT solution (5 mg/ml in phosphate buffered saline) was added directly to the medium in each well. After incubation at 37 °C for 3 h, all medium was aspirated and replaced with 150 μl of DMSO. The optical density at 540 nm was measured by a spectrophotometer.

2.6. Angiogenesis – cell migration assay

Cell viability of HMEC-1 cells was determined using MTT assay after 48 h treatment with various concentrations of NF3, AR or RR. The relative amount of viable cells was determined as optical density at 540 nm. The migration of HMEC-1 was examined using the *in vitro* wound healing assay (Sato and Rifkin, 1988). HMEC-1 (1×10^5 cells) were seeded into each well of a 24-well plate and incubated at 37 °C and 5% CO_2. After 24 h of incubation, the cells were starved in medium with 0.5% (v/v) FBS for 24 h. Then, they were scrapped horizontally and vertically with a P100 pipette tip and two views on the cross were photographed on each well by a camera attached to a microscope at 4× magnification. The medium was replaced with fresh medium in the absence or presence of different concentrations of herbal extracts. After 6 h of incubation, a second set of images was photographed. To determine the migration of HMEC-1, 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 cell migration.

2.7. Statistical analysis

For animal studies, interactions of AR and RR by comparing the effects of NF3 against AR and RR based on the same dosage level were modeled. This was to examine whether AR, RR or NF3 treatment was associated with wound area reduction with adjustments for set of experiments, baseline wound area, body weight and plasma glucose level across the examination period. This was a 2-step approach. At first, the effectiveness of NF3 compared with control in the new data sets (on top of those reported in Tam et al., 2011) was re-examined to ensure the effect of NF3 on wound healing was validated. Then, in the second stage, the effect of NF3 with AR and RR was compared, respectively. Given that all three treatments had the same dosage of 0.98 g/kg, our hypotheses were to test whether the effect of NF3 was statistically superior than using either AR or RR alone. Given that the same potency might or might not apply to the effects of AR and RR, this was analogous to test the potency of the three different combinations of the same drug. Note that the basis of an additive effect was to assume one herb (*e.g.* AR) as a more or less potent and acted like a dilute (or concentrate) version of the other herb (*e.g.* RR). Given that NF3 was a mixture of AR and RR, *i.e.* by replacing 1/3 of AR with RR, our hypothesis was to see if the effect of NF3 deviated significantly from the additive effect of AR and RR. If the effect of NF3 was statistically superior to AR and RR, this implied super-additive and hence AR and RR were synergistic in producing the effect. If the effect of NF3 was statistically inferior to AR or RR, this implied sub-additive and hence AR and RR were antagonistic in producing the effect.

To test for synergistic interactions between AR and RR in our *in vitro* studies, similar conceptual underpinning was applied to the three cell culture experiments namely nitric oxide production, cell proliferation and cell migration. For each dosage level, the significance of the differences between treatment groups (AR, RR and NF3) and their corresponding control groups were tested. This was because we did not assume the same relative potency to all levels of effect for the two herbs.

The software SPSS 18.0 was used for conducting all statistical analyses. Data are presented as mean ± standard deviation (SD) unless otherwise specified. All statistical tests were two-sided, with $p < 0.05$ considered as statistically significant.

3. Results

3.1. In vivo diabetic wound healing study

The effects of AR, RR and NF3 on body weights, plasma glucose levels and wound sizes of diabetic rats were shown in Fig. 1. After 7 days of treatment, AR, RR and NF3 did not affect the body weights of rats (Fig. 1A). On the other hand, AR and RR led to a mild decrease (about 10%) in plasma glucose level, but their hypoglycemic effects were insignificant when compared to the water group (Fig. 1B). At clinical relevant dose (0.98 g/kg), NF3 could reduce the wound size from 21.1 ± 5.4 mm^2 at Day 1 to 6.1 ± 3.6 mm^2 at Day 8. There was 70.8% reduction in wound area. After adjusting for set of experiment, baseline wound area, body weight and plasma glucose level

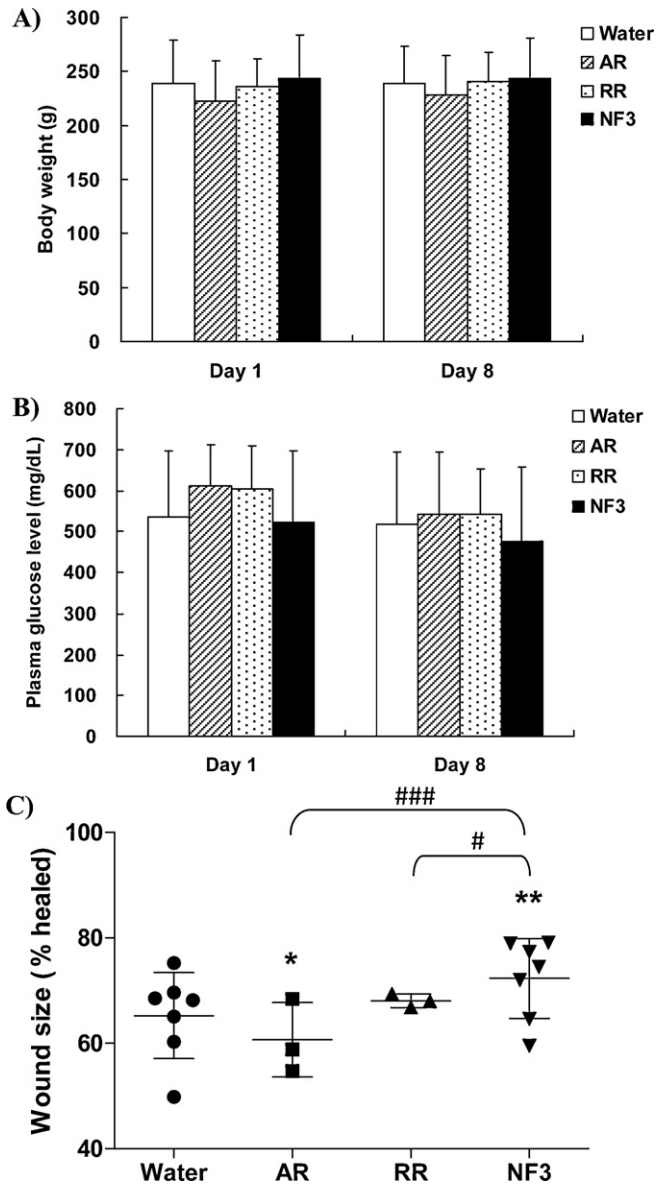

Fig. 1. Effects of AR, RR and NF3 on (A) body weights, (B) plasma glucose levels and (C) wound sizes of rats in *in vivo* diabetic wound healing study. Data are expressed as mean ± SD of at least 35 animals from 3 to 7 separate sets of experiment. Number of animals in water, AR, RR and NF3 groups were 96, 35, 36 and 93, respectively. * denotes $p < 0.05$ and ** denotes $p < 0.01$ when compared to water group; # denotes $p < 0.05$ and ### denotes $p < 0.001$ when compared to NF3 group using MANOVA.

across the examination period, NF3 was found to promote wound closure in diabetic rats significantly when compared to water group ($p = 0.005$) (Fig. 1C). Based on the analysis of comparison, NF3 was 9.7% ($p = 0.045$) more effective than RR and 19.7% ($p < 0.001$) more effective than AR in wound recovery. It was also found that the effect of RR had no statistical difference to control and the effect of AR was statistically lower than control by 9.8% ($p = 0.045$). Therefore, it was concluded that the synergistic effect between AR and RR is statistically significant based on our data.

3.2. Anti-inflammation – LPS-induced inflammation assay

Inflammation takes an initial place in the process of wound healing. Disordered and uncontrollable inflammatory response is always associated with unhealed foot ulcer in diabetic patients. In the present study, LPS was used to induce inflammation in murine

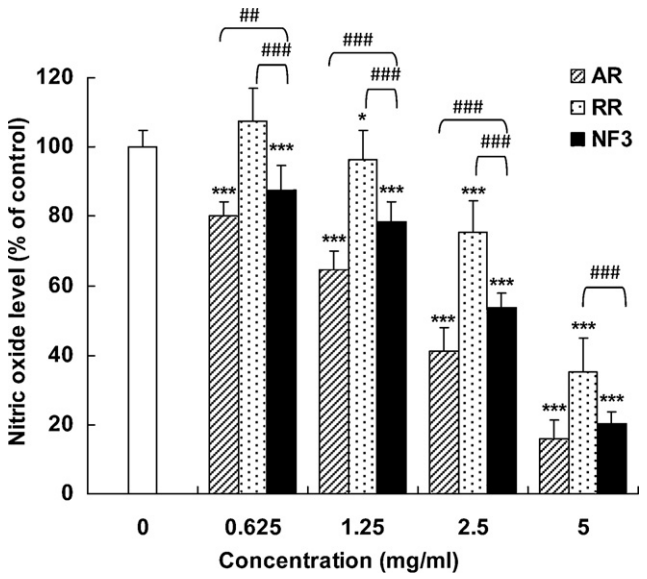

Fig. 2. Effects of AR, RR and NF3 on LPS-induced nitric oxide production in RAW 264.7 cells. Data are expressed as mean ± SD from three to four individual experiments. * denotes $p < 0.05$ and *** denotes $p < 0.001$ when compared to control (0 mg/ml); ## denotes $p < 0.01$ and ### denotes $p < 0.001$ when compared to NF3 group at the same concentration using MANOVA.

macrophage RAW 264.7, of which the severity was reflected by the amount of nitric oxide (NO) produced. As shown in Fig. 2, AR, RR and NF3 could significantly inhibit NO production in a dose-dependent manner ($p < 0.05$ or $p < 0.001$ when compared to control). At 0.625–5 mg/ml (a concentration range that was tested by MTT assay to be non-cytotoxic to the macrophage), AR, RR and NF3 could, respectively, suppress 20–84%, 4–65% and 13–80% of the NO production. Their potencies in significant descending order was AR > NF3 > RR.

3.3. Granulation formation – fibroblast proliferation assay

Fibroblast proliferation is an important step in wound healing for granulation formation. As shown in Fig. 3, AR, RR and NF3 could significantly stimulate the proliferation of Hs27 fibroblast cells in

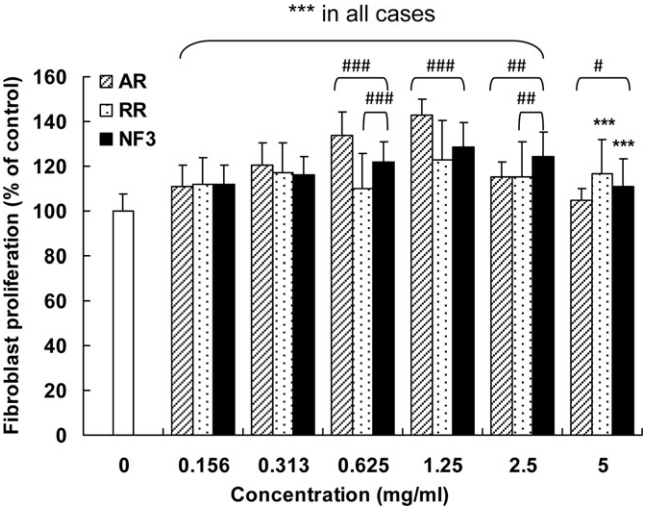

Fig. 3. Effects of AR, RR and NF3 on proliferation of Hs27 fibroblasts. Data are expressed as mean ± SD from three individual experiments. *** denotes $p < 0.001$ when compared to control (0 mg/ml); # denotes $p < 0.05$, ## denotes $p < 0.01$ and ### denotes $p < 0.001$ when compared to NF3 group at the same concentration using MANOVA.

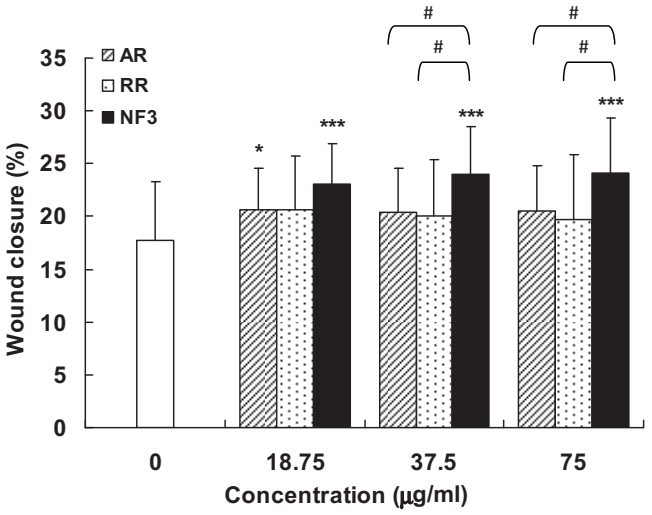

Fig. 4. Effects of AR, RR and NF3 on HMEC-1 cell migration. Data are expressed as mean ± SD from four individual experiments. * denotes $p < 0.05$ and *** denotes $p < 0.001$ when compared to control (0 μg/ml); # denotes $p < 0.05$ when compared to NF3 group at the same concentration using MANOVA.

a dose-dependent manner ($p < 0.001$ when compared to 0 mg/ml). For AR, its effective concentration range was 0.156–2.5 mg/ml. At 1.25 mg/ml, its effect was peaked where 42.8% increase in proliferation rate was achieved. Similar trend was observed in RR or NF3-treated fibroblasts. RR and NF3 could significantly stimulate fibroblast proliferation at 0.156–5 mg/ml, with maximum proliferation (122.9% and 128.5%, respectively) detected at 1.25 mg/ml. At concentrations below 1.25 mg/ml, AR was the most effective among the three samples tested.

3.4. Angiogenesis – cell migration assay

Effects of AR, RR and NF3 on endothelial cell migration were determined using the scratch assay. At 18.75–75 μg/ml (a concentration range of the three samples that was tested by MTT assay to be non-cytotoxic to the endothelial cells), AR could mildly stimulate wound closure (~20%), but its effect was insignificant when compared to control except at the concentration of 18.75 μg/ml (Fig. 4). The potency of RR was similar to that of AR. RR could induce ~20% wound closure, but no statistical significance was achieved. On the contrary, NF3 could significantly promote cell migration of endothelial cells at all concentrations tested ($p < 0.001$ when compared to control). At 37.5 μg/ml and 75 μg/ml, NF3 was found to be significantly more effective than AR and RR ($p < 0.05$).

4. Discussion

Wound healing is a complex, dynamic and orderly controlled process that involves three discriminated but overlapping phases, namely inflammation, proliferation, and wound closure and remodeling (Peppa et al., 2009). Tissue injury triggers acute inflammatory response, in which neutrophils, monocytes and mast cells infiltrate to the site of injury and produce cytokines (Trautmann et al., 2000). The released cytokines then stimulate the proliferation and migration of several kinds of cells such as keratinocytes, endothelial cells and fibroblasts to the wound site. In the last step, extracellular matrix remodeling, angiogenesis and re-epithelialization are responsible for wound closure and scar formation. In diabetic patients, impaired wound healing is resulted from aberrant inflammatory response (Acosta et al., 2008), decreased angiogenesis (Duraisamy et al., 2001) and insufficient fibroblast proliferation (Hehenberger et al., 1998). In view of the fact that impaired wound healing associates with high morbidity and mortality in diabetic patients, an innovative herbal formula NF3 was developed to tackle the problems of recalcitrant diabetic foot ulcer. It was demonstrated that NF3 could promote diabetic wound healing in rats through the action of tissue regeneration, angiogenesis promotion and inflammation inhibition (Tam et al., 2011).

Compound formulae, usually called 'fufang' in Chinese, are combinations of TCM prescribed for treating various diseases in China. They may be as simple as Danggui Buxue Tang that contains only Astragali Radix and Angelicae Sinensis Radix, or as complicated as Pinggan Shuluo Wan that is composed of 43 kinds of TCM (State Pharmacopoeia Commission of P.R. China, 2010). In comparison with individual herbs, compound formulae always exhibit greater efficiency (Dai et al., 2002; Chen et al., 2006; Liu et al., 2010a) and lower toxicity (Wang et al., 1997). The therapeutic potencies of herbs are found additive, or even synergistic, when used as combination (Wang et al., 1995; Gao et al., 2009). Tsutsumi et al. (2003) demonstrated that the water extract of AR did not affect singly but potentiated the anti-hyperglycemic action of an alkaloid isolated from Stephania tetrandra Radix in STZ-diabetic mice. Since NF3 was composed of two Chinese herbs AR and RR, it was of great interest to reveal whether the diabetic wound healing effect of NF3 in rats was attributed to the synergistic action between AR and RR. As shown in present study, individual AR or RR neither promoted diabetic wound healing in rats nor stimulated cell migration of HMEC-1 cells. However, when used in combination as NF3, they could significantly enhance diabetic wound closure (Fig. 1C) and endothelial cell migration (Fig. 4). The synergistic interaction between AR and RR was demonstrated and it could justify the co-usage of these two herbs in the compound formula NF3 for diabetic wound healing.

After revealing the synergistic interaction between AR and RR, the potencies of individual herbs in different mechanistic studies were evaluated so as to identify the principal herb in the formula. Results of mechanistic studies demonstrated that AR and RR could stimulate fibroblast proliferation and inhibit LPS-induced cellular inflammation. In other words, they possessed similar biological actions, with AR being more effective. Although AR exhibited more potent activities *in vitro*, its efficacy in promoting diabetic wound healing in rats was not satisfactory. The discrepancies between *in vitro* and *in vivo* results might be due to the fact that AR was poorly absorbed. Xu et al. (2006) reported that some flavonoids in AR decoction could be absorbed and metabolized by the intestine. Nevertheless, their contents had not been quantified. It was speculated that the desirable effective concentration could not be achieved when AR was used alone. However, in the presence of RR, the effect of AR was potentiated and led to the significant wound healing effect of NF3 in diabetic rats. It was, therefore, postulated that RR might enhance the absorption of AR in animals. Results of present study justified the combined usage of AR and RR in the ratio of 2:1 as NF3 to treat diabetic foot ulcer and illustrated that AR was the principal herb in this herbal formula. The herb–herb interaction between AR and RR in the aspect of pharmacokinetics might need to be explored in the future.

Many compound formulae have already been used for a long period of time in China. Enormous efforts, however, are still needed to pursue scientific evidence, quality assurance, pharmacokinetic information as well as composite modification (Gao et al., 2007; Ye et al., 2009). For example, Liuwei Dihuang Wan (LDW), which is one of the most commonly used 'fufang' (*i.e.* compound formula) in eastern Asia, was created by a TCM practitioner in 1119 AD. Based on the past 900 years of experience, several formulae such as Zhibai Dihuang Wan, Mingmu Dihuang Wan and Qiju Dihuang Wan are derived from the original LDW to relieve different clinical symptoms, but in the meanwhile, keeping the *yin*-nourishing and kidney-tonifying properties (State Pharmacopoeia Commission of

P.R. China, 2010). Although AR and RR have long been used in China for treating diabetes mellitus and its complications, the formula NF3 is rather innovative. The ratio of AR and RR (2:1) in NF3 was derived from our previous herbal drinks designed for diabetic foot ulcer (Wong et al., 2001; Lau et al., 2007). A pilot study using the diabetic wound healing animal model showed that AR and RR in the ratio of 2:1 exerted stronger wound healing effect when compared to the ratio of 1:1 and 1:2 (data not shown). According to Gao et al. (2009), not all combination ratios of herbs could exert synergistic interaction. Therefore, further investigation is needed to optimize the ratio between AR and RR for the pursuit of greatest synergy.

5. Conclusion

A herbal formula NF3 could significantly promote diabetic wound healing in rats and its effect was attributed to the synergistic interaction between its two component herbs, AR and RR. From the results of mechanistic studies, AR, which played a preeminent role in the anti-inflammatory and fibroblast-proliferating activities of NF3, was found to be the principal herb in this compound formula.

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