PHYTOTHERAPY RESEARCH
Phytother. Res. **21**, 938–947 (2007)
Published online 22 June 2007 in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/ptr.2191

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Investigation of the Effects of Chinese Medicine on Fibroblast Viability: Implications in Wound Healing

T. W. Lau[1], Y. W. Chan[2], C. P. Lau[1], C. M. Chan[2], C. B. S. Lau[3], K. P. Fung[1,2], P. C. Leung[1,4] and Y. Y. Ho[2]*

[1]Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China
[2]Department of Biochemistry, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China
[3]School of Pharmacy, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China
[4]Department of Orthopaedics and Traumatology, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China

Diabetes mellitus has been a clinical problem for hundreds of years. Over 194 million people suffer from this disease worldwide. Improper control of diabetes may result in diabetic foot ulcer or even amputation. Granulation formation is an important issue essential for ulcer healing. The CRL-7522 fibroblast cell line and primary fibroblasts from a diabetic foot ulcer patient were used to model the wound healing enhancing activities of two clinically efficacious Chinese herbal formulae, Formula 1 (F1) and Formula 2 (F2) and their component herbs. Results showed that the two formulae and four of their component herbs, Radix Astragali, Radix Rehmanniae, Rhizoma Alismatis and Rhizoma Atractylodis Macrocephalae significantly enhanced CRL-7522 cell viability. However, these component herbs showed compromised effects on the viability of primary fibroblasts cultured from the ulcerous tissue of a diabetic patient. Interestingly, F1 and F2 enhanced the viability of primary cultured fibroblasts from the diabetic patient even in the face of insulin resistance. These results further support the previously reported clinical efficacies of the two formulae on healing diabetic foot ulcers. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: diabetes mellitus; foot ulcer; fibroblast; wound healing; insulin resistance; Chinese medicine.

INTRODUCTION

Diabetes mellitus has been a clinical problem for hundreds of years. Over 194 million people suffer from this disease worldwide. Diabetic complications increase the overall cost of health care and reduce the quality of life for patients. A number of diabetes-associated complications specifically affect the lower limb such as ischaemic ulceration, Charcot's joints and microangiopathy, etc (Mooney and Merriman, 1995). Improper control of these symptoms may result in diabetic foot ulcer or even amputation (Levin, 2002).

Granulation formation is a main step of wound healing and it includes the accumulation of macrophages, ingrowth of fibroblasts, deposition of loose connective tissue, and angiogenesis (Chin *et al*., 2005). Fibroblast proliferation has long been adopted as a model for the assessment of wound healing (Dudnikova, 1979; Adolphe *et al*., 1984; Park *et al*., 2005). For instance, human fibroblast cell lines derived from hypertrophic scar and normal dermal explants were used to investigate the effect of 880 nm low level laser energy on wound healing. The results showed that the treatment had no significant effect on fibroblast proliferation (Webb and Dyson, 2003). Various factors have been studied for their effects on fibroblast proliferation. Tumour necrosis factor (TNF) and interleukin 1 (IL-1) were found to stimulate the proliferation of Tenon's capsule fibroblasts (Cunliffe *et al*., 1995). Fibroblast migration is another essential feature of the wound healing process. It was found that fibroblasts entering from the underlying subcutaneous tissue, instead of from the surrounding dermis were crucial to the healing process (Dale *et al*., 1997). A fibroblast cell line CRL-7522 derived from human connective tissue was adopted in this study to model granulation formation.

A previous report showed that fibroblast proliferation was inhibited under high glucose concentrations (Hehenberger *et al*., 1998). Therefore fibroblast proliferation is also an important model for the study of wound healing under diabetic conditions. Furthermore, primary cultures of fibroblasts from patients with type 2 diabetes mellitus showed a diminished proliferation capacity and altered morphologies compared with diabetic nonlesional and age-matched control fibroblasts (Loots *et al*., 1999). Hence, primary culture fibroblasts from a diabetic foot ulcer patient were included in this study to simulate granulation formation as well.

Based on the positive clinical effects of the two herbal formulae F1 and F2 on the diabetic foot ulcer healing (Wong *et al*., 2001), the components of the formulae were speculated to be effective in promoting wound healing. From literature reviews, the principal component herbs, Radix Astragali and Radix Rehmanniae of the formulae may be active in healing of diabetic foot ulcers. Radix Astragali has been shown to alleviate insulin

* Correspondence to: Dr Yuan-Yuan Ho, MMW 509C, Department of Biochemistry, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, China.
E-mail: yuanyuanho@cuhk.edu.hk
Contract/grant sponsor: University Grants Committee of the Hong Kong Special Administrative Region, China; contract/grant number: AoE/B-10/01.

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Received 5 September 2006
Revised 28 March 2007
Accepted 28 March 2007

resistance (Lu *et al*., 1999) and elicit antiinflammatory effects (Zhang *et al*., 1984). Radix Rehmanniae had anti-hyperglycemic (Miura *et al*., 1997; Li, 2000; Zhang *et al*., 2004a, 2004b) and antiinflammatory effects (Zhang, 1974). In this study, the formulae F1, F2 and their component herbs were tested for their effects on enhancing fibroblast viability.

MATERIALS AND METHODS

All the raw herbs were purchased from an herbal shop in the wholesale market of Sheung Wan, Hong Kong. All 12 raw herbs in the Formulae F1 and F2 have been authenticated by the National Engineering Research Center for Modernization of TCM (Zhuhai, Guangdong, China). To further verify the identity of the herbs, each component herb of the formulae was authenticated by thin layer chromatography using chemical markers or authenticated reference specimens suggested by the Pharmacopoeia of the People's Republic of China (Chinese Pharmacopoeia Commission, 2005). All the chemical markers and reference specimens were provided by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). About 300 g of each experimental species was deposited in the Museum of the Institute of Chinese Medicine, the Chinese University of Hong Kong and their voucher specimen numbers are listed in Table 1. Formulae F1 and F2 individually packed in 5 g packages were produced by the Hong Kong Institute of Biotechnology Limited (Hong Kong, China) under the guideline of Good Manufacturing Practice (Therapeutic Goods Administration in Australia). For extraction of individual herbs, 500 g of the sliced dry herb was boiled twice in 2.5 L of distilled water for 2 h under reflux. The decoction was filtered through cheese cloth, and then frozen and lyophilized into dry powder by freeze-drying (Thermo Savant MODULYO freeze-dryer, E-C Apparatus Corp., Holbrook, NY, USA).

Cells. CRL 7522 fibroblasts were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and incubated at 37 °C for maintenance and for experiments with different treatments including herbal extracts, insulin (positive control) and media control for 5 days. Cells between passage numbers 3 and 10 were used for experiments.

With the aim of studying the diabetic fibroblast viability-enhancing effects of Formulae F1 and F2 and their component herbs, primary cultured fibroblasts from a diabetic patient were used. This male patient had a history of diabetes and was 68 years old when recruited to this study. Metformin was consumed daily to control the diabetic condition. The patient had a non-healing ulcer on the foot, and infection was well controlled. The patient had not received any clinical treatment with the Formulae before tissue collection. The fibroblasts extracted from the granulation tissue of the patient were cultured and used for experiments. All procedures were conducted with patient's consent and ethical approval (ref. no. CRE-8342) from an educational hospital.

Upon tissue collection, the ulcer was first sterilized with necrotic tissue removed by debridement. About 0.1 g of tissue was taken from the newly formed granulation area. The tissue was placed in PBS for

Table 1. Botanical information of the component herbs in Formulae F1 and F2 with voucher specimen numbers provided. All the voucher specimens were authenticated based on their organoleptic characteristics and thin layer chromatography; and deposited at the Museum of the Institute of Chinese Medicine, the Chinese University of Hong Kong, Shatin, Hong Kong.

Name in Chinese medicine	Description of used parts and botanical origins	Raw herb/ formula (w/w %)	Voucher specimen numbers
1. Radix Astragali[1,2]	Dried root of *Astragalus membranaceus* (Fisch.) Bge. in the family Leguminosae	30% of F1 27% of F2	2003–2457
2. Radix Rehmanniae[1,2]	Dried rhizome derived from *Rehmannia glutinosa* Libosch. in the family Scrophulariaceae	14% of F1 17% of F2	2003–2452
3. Rhizoma Atractylodis Macrocephalae[1]	Dried rhizome of *Atractylodes macrocephala* Koidz. in the family Compositae	14% of F1	2003–2458
4. Radix Stephaniae Tetrandrae[1]	Dried root tuber of *Stephania tetrandra* S. Moore in the family Menispermaceae	14% of F1	2004–2526
5. Radix Polygoni Multiflori Preparata[1]	Dried root tuber of *Polygonum multiflorum* Thunb. in the family Polygonaceae	14% of F1	2003–2460
6. Rhizoma Smilacis Chinensis[1]	Dried rhizome of *Smilax china* L. in the family Smilacaceae	14% of F1	2003–2463
7. Poria[2]	Dried sclerotium of the fungus, *Poria cocos* (Schw.) Wolf in the family Polyporaceae	8% of F2	2003–2454
8. Rhizoma Dioscoreae[2]	Dried rhizome of *Dioscorea opposita* Thunb. in the family Dioscoreaceae	12% of F2	2003–2453
9. Fructus Schisandrae Chinensis[2]	Dried fruit of *Schisandra chinensis* (Turcz.) Barll. in the family Magnoliaceae	8% of F2	2003–2462
10. Cortex Moutan[2]	Dried root bark of *Paeonia suffruticosa* Andr. in the family Ranunculaceae	8% of F2	2003–2456
11. Fructus Corni[2]	Dried ripe sarcocarp of *Cornus officinalis* Sieb. et Zucc. in family Cornaceae	12% of F2	2003–2461
12. Rhizoma Alismatis[2]	Dried tuber of *Alisma orientalis* (Sam.) Juzep. in the family Alismataceae	8% of F2	2003–2455

[1] component of F1; [2] component of F2.

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DOI: 10.1002/ptr

temporary storage. Afterwards, the tissue was dipped into 75% ethanol for sterilization, and rinsed with PBS three times. It was then soaked in DMEM and cut into paste by scissors. The tissue was smeared on the surface of a Petri dish for air-drying. A few drops of full culture media were added onto the tissue surface before it became semi-dry. Fibroblast growth was monitored every 2 days. Full culture media was replaced frequently to ensure that the tissue was moist and nourished. After a week of proliferation, the fibroblasts were subcultured for storage and experiments.

Fibroblast viability assays. Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). Insulin was chosen as a positive control for cell viability (Komorov and Fedotov, 1974). In each trial, a baseline control experiment was carried out by incubating the cells with media only. The values from the baseline control wells were set at 100%. The viability enhancing activities were calculated by comparing the values from the herbal treated culture wells with those of the baseline controls. In order to cover a proper range of dosage, 5000 μg/mL of herbal extract was chosen as the highest concentration and other trial concentrations were conduced by two-fold serial dilutions. Any significant enhancement effect found between any two concentrations was further investigated by repeating the experiments at more dosage points within that concentration range. Each condition in the same trial was performed in quadruplicate set up. At least three trials were conducted to confirm the results. A multilevel regression statistical model was employed to assess the treatment effects of Formulae F1 and F2, component herbs and insulin (positive control) against the baseline control. Such a statistical model can be used to account for the clustering nature of the outcome measurements within each trial and hence adjust for the heterogeneity among trials. Raw data were logarithmically transformed to correct skewness, and then fitted to multilevel regression model with a random intercept and a fixed treatment effect. The public domain software MIXREG (accessible at http://tigger.uic.edu/~hedeker/mix.html) was employed to estimate the intercept and treatment effects, and their standard errors. Less than 100% in treatment effects indicated that the treatment had negative effects on cell viability, while more than 100% of treatment effects indicated an enhancement of cell viability when compared with the baseline control. The significant effect of each treatment was assessed by the Wald test. The standard errors of the treatment effects were estimated by Monte Carlo method. All statistical tests involved were two sided, with the significant level determined at $p < 0.05$.

Since the pH value of the cell culture media may change by the addition of herbal extracts, fibroblast activities might be affected (Liu *et al.*, 2002). The pH values of the media with the effective concentration of the Formulae F1 and F2 and their component herbs were monitored by a pH meter.

The sugar content in the herbs may possibly affect the metabolic activities of the studied cells. Hence, the sugar contents of the efficacious herbs in the CRL 7522 fibroblasts viability assays were determined by high performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD) system. The HPLC setup and conditions included auto-sampler (Beckman, System Gold 508), solvent pump (Beckman System Gold 125 Solvent Module), stationary phase (Alltech, Prevail Carbohydrate ES Column, 5 μm, 250 mm × 4.6 mm), mobile phase (acetonitrile–water 7:3, v/v), injection volume (20 μL), flow rate (1 mL/min) and running time (20 min for standards and 30 min for tested solutions). The ELSD setup and conditions included detector (Alltech ELSD 2000), temperature (80 °C) and nitrogen gas flow (2 L/min). Since fructose, glucose and sucrose are sugars commonly found in plants; these three sugars were included for viability assessment. The CRL-7522 fibroblasts were incubated with a mixture of fructose, glucose and sucrose in the amount and ratio equivalent to those of the efficacious concentrations of herbs in the MTT assays.

Cell proliferation assay by direct cell count. The same cell incubation procedures as the fibroblast viability assays were performed. After incubation for 5 days, media were removed and 0.1 mL trypsin was added to each culture well of 24 well plate and incubated at 37 °C for 5 min to dislodge the cells. 10 μL of the solution from each well was transferred to the chambers of a haematocytometer for cell counts. Only the component herbs showing significant effects on the CRL-7522 cell viability assays were repeated for cell counting. Each treatment was conducted with a total of 12 replicates in three trials. The raw counts from each experiment were normalized by the mean of the baseline control values (media only). The normalized data from different experiments were pooled together for statistical analysis. The Mann-Whitney statistical test was employed to assess the significance ($p < 0.05$) difference between the baseline control and herbal treatment groups.

Glucose uptake studies on patient fibroblasts. Patient fibroblasts were grown to a confluent state in 24-well plates. Prior to the uptake assay, the cells were glucose-starved by glucose-free DMEM for 1 h. The cells were divided into a control group (glucose-free DMEM) or an insulin treatment group (1 nM, 10 nM, 100 nM and 1000 nM insulin in glucose free DMEM). The cells were then incubated at 37 °C for 30 min. The experimental media were then removed by washing with PBS for three times. Three hundred microliters radiolabelled 2-DOG mixture (2 μCi/mL 2-deoxy-D-[1-^{3}H]-glucose, 0.1 mM 2-DOG in glucose-free DMEM) was added and the reaction was allowed to proceed for 15 min at 37 °C. The reactions were then stopped by adding 1 mL/well of ice-cold 10 mM 2-DOG solution. Each well was washed by the same ice-cold 10 mM 2-DOG solution twice. Then 200 μL of 0.5 M NaOH was added to lyse the cells, and the plates were put on an orbital shaker for 10 min to ensure complete lysis. An equal amount of 0.5 M HCl was then added to neutralize the cell lysate. Two hundred microliters of the cell lysate was transferred to a scintillation vial and 4 mL of OptiPhase HiSafe 2 scintillation fluid (PerkinElmer, Boston, MA, USA) was added for radioactivity counting by a Packard Tri-Carb® 2900TR liquid scintillation analyser (Perkin Elmer Life Science Inc, Boston, MA, USA). The protein content of samples was determined by bicinchoninic acid (BCA) assay (Smith *et al.*, 1985). The amount of 2-DOG uptake was normalized by the protein amount.

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DOI: 10.1002/ptr

For statistical analysis, two-sided Mann-Whitney tests ($p < 0.05$) were performed for comparisons of 2-DOG uptake between the control and insulin stimulated groups.

RESULTS

Effect of herbal extracts on CRL-7522 fibroblast viability

Formulae F1 and F2 enhanced the viability of CRL-7522 fibroblasts as measured by MTT assays. Shown in Fig. 1a, F1 had a wider effective concentration range (50–400 μg/mL) compared with F2 (100–200 μg/mL). These positive results are consistent with the formulae's clinical efficacy on diabetic foot ulcer healing and led us to further investigate the effects of their component herbs. Four of the twelve component herbs elicited significant dose-dependent enhancement effects on cell viability (Fig. 1b–d). They were Radix Astragali, Radix Rehmanniae, Rhizoma Atractylodis Macrocephalae and Rhizoma Alismatis. It is important to note that some component herbs (Rhizoma Smilacis Chinensis, Cortex Moutan, Radix Stephaniae Tetrandrae and Fructus Corni) elicited negative effects on cell viability even at low concentrations, implying possible cytotoxic effects of these herbs when used individually.

The possibility that the sugar content in herbs may affect cell viability was tested. The amount and composition of simple sugars used for experiments were equivalent to those in each of the corresponding herbal extracts at the specified dosage as determined by HPLC-ELSD. As shown in Table 2, there was almost no enhancement

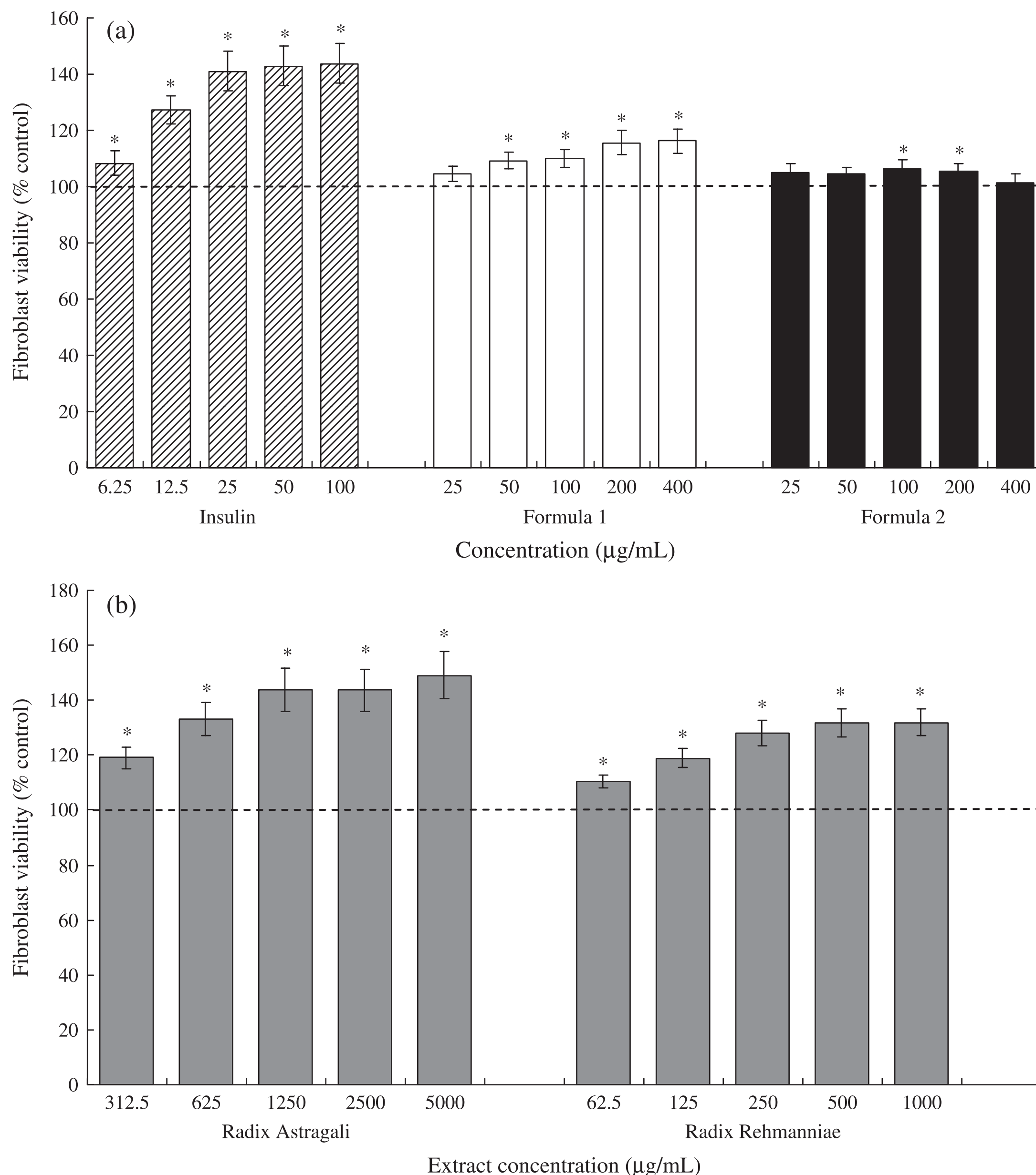

Figure 1. Effects of herbal extracts on CRL-7522 fibroblast viability. Cells were exposed to the indicated treatments for 5 days at 37 °C followed by the determination of viability by MTT assays. The results are presented as mean ± SD in percent changes relative to the media control value (set at 100%). (a) Effects of insulin (positive control), F1 and F2. (b) Effects of principal component herbs in both F1 and F2, Radix Astragali and Radix Rehmanniae. (c) Effects of component herbs in Formula 1. (d) Effects of component herbs in F2. A multilevel regression statistical model and Wald test were employed to test the significance of the treatment effects. * Statistically significant increase ($p < 0.05$); # statistically significant decrease ($p < 0.05$).

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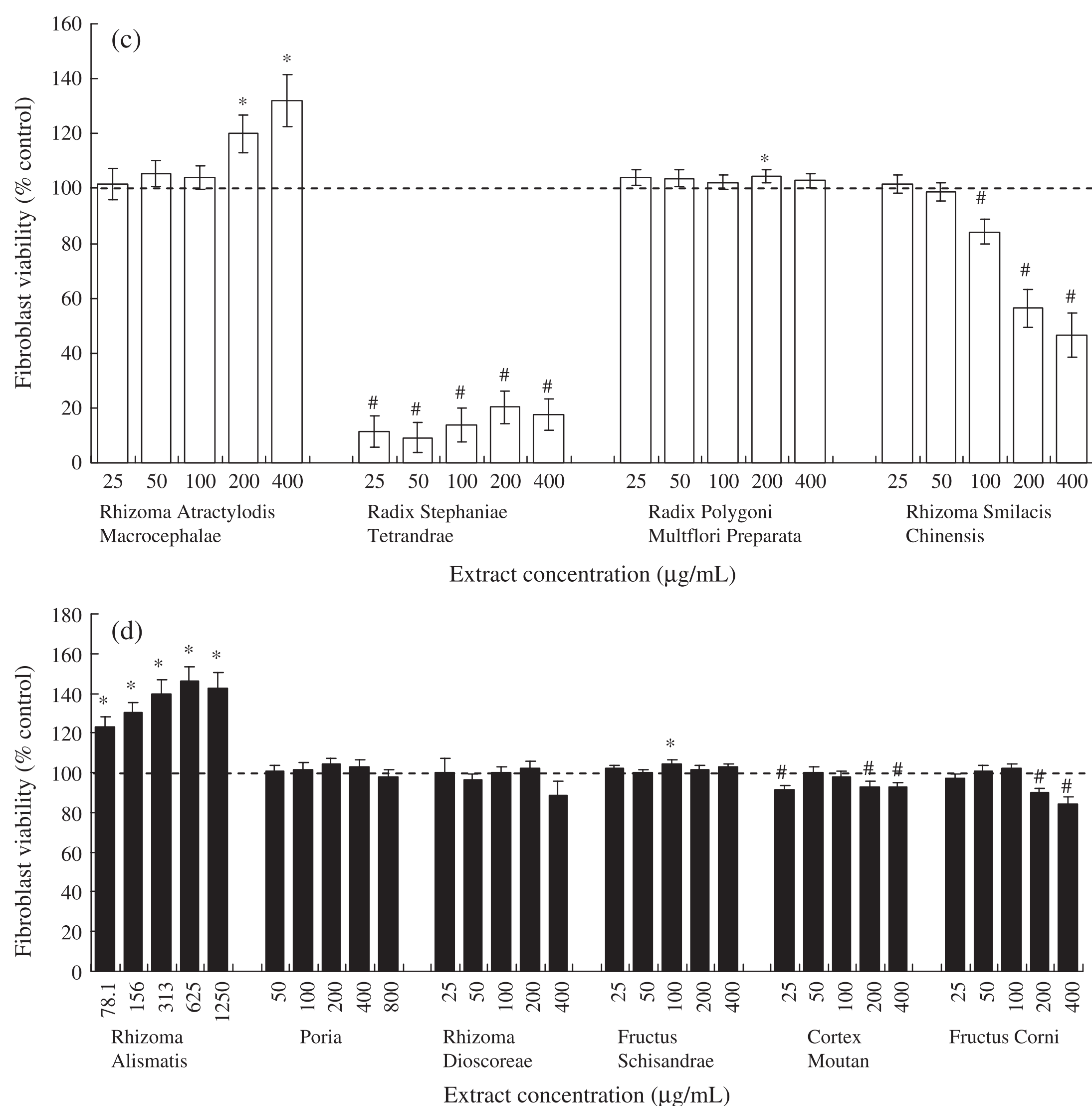

Figure 1. (*Continued*)

Table 2. Effects of simple sugar contents in herbal extracts on the viability of CRL-7522 fibroblasts. Sugar contents of the herbs were determined by HPLC-ELSD method. A mixture of fructose, glucose and sucrose in an amount and ratio equivalent to those in the herbal extracts were incubated with CRL-7522 fibroblasts at 37 °C for 5 days followed by MTT assay for cell viability test. The medium control value was set as the baseline (100%). Cell viability was presented as percentage changes (mean ± SD) relative to the medium control. A multilevel regression statistical model and Wald tests were employed to test the significance of the sugar effects.

Herbal extract concentration (μg/mL)	Sugar content (μg/mL)			Cell viability (% of medium control)
	Fructose	Glucose	Sucrose	
Rhizoma Atractylodis Macrocephalae (200)	21.2	1.3	4.8	100.03 ± 2.86
Rhizoma Atractylodis Macrocephalae (400)	42.4	2.6	9.6	98.73 ± 3.78
Radix Rehmanniae (125)	7.2	6.1	8.9	107.84 ± 3.65*
Radix Rehmanniae (250)	14.5	12.1	17.8	102.76 ± 3.05
Radix Rehmanniae (500)	29.0	24.2	35.6	102.97 ± 3.58
Radix Rehmanniae (1000)	57.9	48.4	71.2	97.75 ± 3.80
Radix Astragali (625)	32.3	13.0	301.8	97.16 ± 3.72
Radix Astragali (1250)	64.5	26.0	603.5	94.82 ± 4.50
Radix Astragali (2500)	129.0	52.0	1207.0	97.07 ± 4.33
Radix Astragali (5000)	258.1	104.0	2414.2	96.84 ± 4.47
Rhizoma Alismatis (156.25)	4.6	3.2	6.3	102.89 ± 3.27
Rhizoma Alismatis (312.5)	9.2	6.4	12.6	99.07 ± 3.59
Rhizoma Alismatis (625)	18.4	12.8	25.2	99.96 ± 3.26
Rhizoma Alismatis (1250)	36.9	25.6	50.4	98.55 ± 3.44

* Statistically significant increase ($p < 0.05$).

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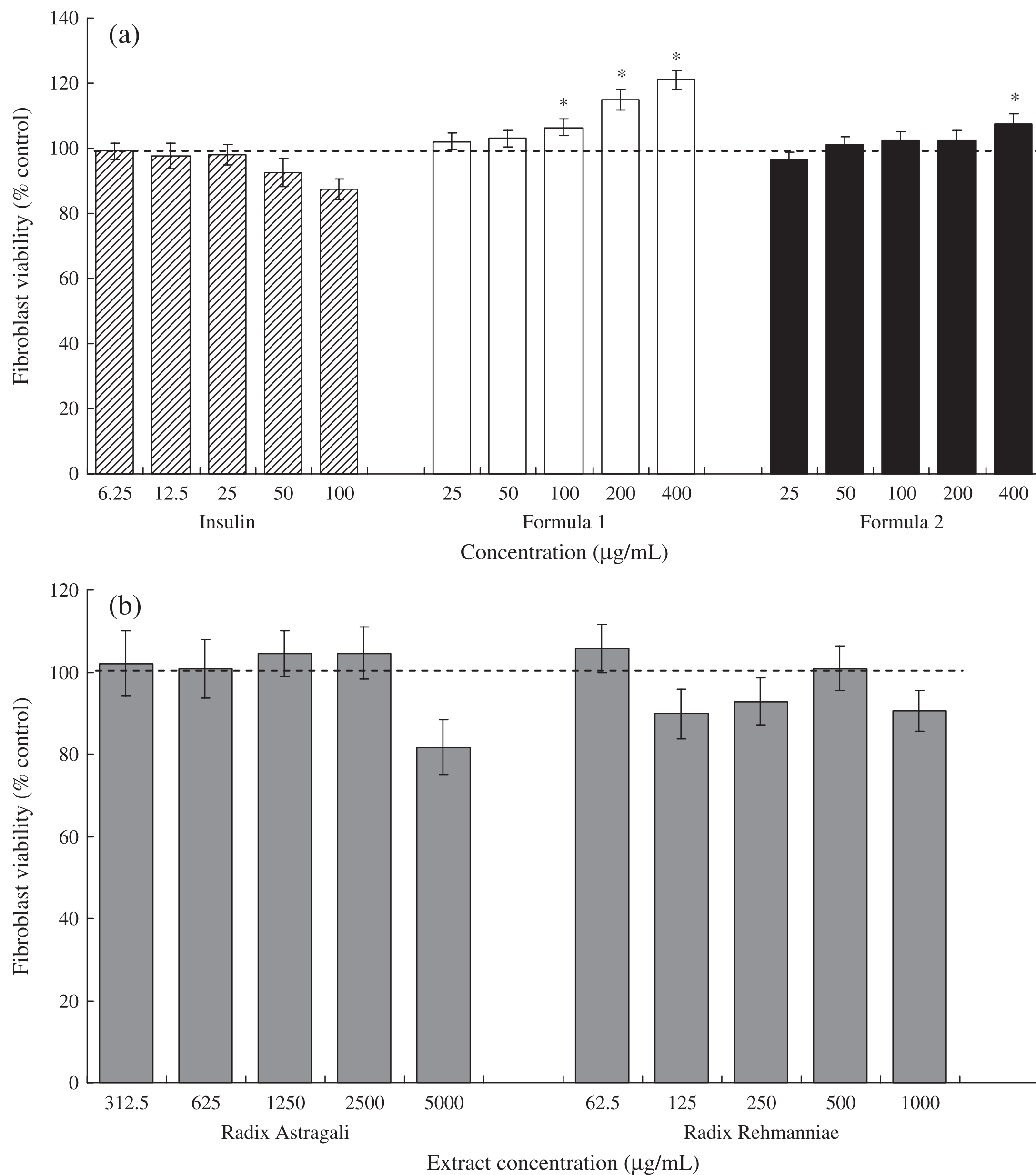

Figure 2. Effects of herbal extracts on the viability of fibroblasts from a diabetic patient. Cells were exposed to the indicated treatments for 5 days at 37 °C followed by the determination of viability by MTT assays. Results are presented as mean ± SD in percent changes relative to the medium control value (set at 100%). (a) Effects of insulin, F1 and F2. (b) Effects of principal component herbs in both F1 and F2. (c) Effects of component herbs in F1. (d) Effects of component herbs in F2. A multilevel regression statistical model and Wald test were employed to assess the treatment effects. * Statistically significant increase ($p < 0.05$); # statistically significant decrease ($p < 0.05$).

of CRL-7522 cell viability with the amount and combination of sugars tested. Only the mixture of 7.2 μg/mL fructose, 6.1 μg/mL glucose and 8.9 μg/mL sucrose (equivalent to 125 μg/mL Radix Rehmanniae) significantly enhanced the viability of the CRL-7522 fibroblasts.

The next question was whether the herbal extract induced cell viability was associated with cell proliferation. In direct cell count experiments, 5000 cells were seeded into each well at the beginning of the experiments. There was an overall increase of cell number in treatment groups and control groups after 5 days. However, no statistical significant change of cell number in response to any of the herbal treatments was detected (data not shown).

Effects of herbal treatment on the viability of fibroblasts from a diabetic patient

The cell viability of the primary fibroblasts cultured from the granulation tissue of a diabetic patient was tested by incubating the cells with F1 and F2 and the extracts of the 12 component herbs (Fig. 2). Insulin had no effect on cell viability even at 100 μg/mL. Such results indicate that the primary fibroblasts from this patient may be insulin resistant. The glucose uptake assay further support the lack of insulin response of these cells (Fig. 3). Interestingly, F1 and F2 significantly enhanced the viability of the patient fibroblasts. F1 showed a range of effective concentrations from 100 μg/

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DOI: 10.1002/ptr

Figure 2. (*Continued*)

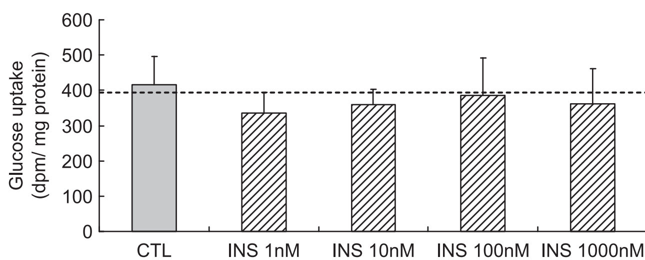

Figure 3. Glucose uptake studies of fibroblasts from a diabetic patient. Primary cultured fibroblasts from the patient were grown to a confluent state in 24-well plates. Prior to the uptake assay, the cells were glucose-starved by glucose-free DMEM for 1 h. Cells were divided into control group (CTL) using glucose-free DMEM or treatment group (INS) using 1 nM, 10 nM, 100 nM and 1000 nM insulin in glucose free DMEM. Results were analysed by Two-sided Mann-Whitney tests with no statistically significant difference found between the control and treatment groups.

mL to 400 μg/mL while F2 had a significant positive effect at 400 μg/mL only. The effects of the formulae on patient fibroblasts were consistent with those observed in the cell line CRL-7522 that F1 was effective at a wider concentration range relative to F2. Since the primary cultured fibroblasts from diabetic patients more closely resemble the tissue samples of the foot ulcer, it was encouraging to have observed that F1 and F2 circumvented insulin resistance, and enhanced the viability of patient fibroblasts. These results further support the effects of the formulae on foot ulcer healing of diabetic patients observed in our clinical trials (Wong *et al*., 2001).

Of all the component herbs tested, only Radix Polygoni Multiflori Preparata showed a minor, but significant, viability enhancement effect on patient fibroblasts. Such observations are quite different from the results

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of the viability assays in CRL-7522 cells (Fig. 1). It is noteworthy that more than one treatment concentration of Radix Stephaniae Tetrandrae and Rhizoma Atractylodis Macrocephalae elicited significant negative effects on the viability of patient fibroblasts. Herbs with negative effects on cell viability are unlikely to facilitate ulcer healing in diabetic patients.

DISCUSSION

Granulation formation is a main stage of wound healing following inflammation. Fibroblasts are the major cell type found in the granulation of wound tissues and play an important role in wound healing (Dudnikova, 1979; Adolphe *et al*., 1984; Park *et al*., 2005). Fibroblasts are responsible for the secretion of a series of growth factors, i.e. VEGF, interleukin and TGF-β to promote wound healing events including angiogenesis, cell proliferation and matrix deposition (Mansbridge *et al.*, 1999).

Fibroblast viability and proliferation are important in the granulation formation that leads to wound healing. In this study, F1, F2 and some of their component herbs were effective in enhancing fibroblast viability under a range of treatment concentrations. Most of the positively effective concentrations were limited up to 1250 μg/mL due to possible cytotoxicity except for Radix Astragali which was found to be effective and not toxic even at 5000 μg/mL.

As the pH values of all the effective herbal treatment concentrations were close to neutral (data not shown), the experimental treatments with F1, F2 and their component herbs were unlikely to be confounded by pH changes. In addition, fibroblasts treated with different concentrations of herbal extracts were inspected under a microscope with normal morphology observed. It is thus reasonable to consider that the osmolarity change associated with the herbal treatments did not have overt effects on the viability of the cells.

There was no significant enhancement of CRL-7522 cell viability with different mixtures of glucose, fructose and sucrose treatments, except for the sugar content equivalent to 125 μg/mL of Radix Rehmanniae extract. These results implied that the sugar effects from the herbal treatment are mostly negligible in our experimental systems. Nevertheless, it is important to note that glucose, sucrose and fructose are not the only sugars found in Chinese medicine. The possibility that the polysaccharides in the tested herbs may affect the viability of cell models used here cannot be excluded.

From the results of the viability assessment of CRL-7522 fibroblasts, significant enhancement effects were found in Formulae F1 and F2 as well as in four component herbs, Radix Astragali, Radix Rehmanniae, Rhizoma Atractylodis Macrocephalae and Rhizoma Alismatis. The principal herbs Radix Astragali and Radix Rehmanniae of the formulae elicited significant enhancement effects on CRL-7522 viability. Interestingly, Rhizoma Alismatis, the component herb of F2 also elicited significant fibroblast viability enhancement effects over a wide range of concentrations. Since the four individual herbs discussed above showed greater fibroblast viability enhancement effects than did the formulae, especially at higher dosages, increasing the amount of these herbs in the formulae may possibly enhance the overall effects of the formulae.

Surprisingly, the herbal extracts effective in enhancing CRL-7522 viability did not promote cell proliferation as no difference in cell number increment was observed in the control vs herbal treatment groups after a 5 day incubation. It is possible that the herbal treatment increased cell viability by stimulating cell migration rather than proliferation for tissue repair (Cornwell *et al*., 2004; Briggs, 2005).

In this study, insulin resistance in terms of viability and glucose uptake were observed in primary fibroblasts of the diabetic patient. The insulin resistance properties of the patient fibroblasts may explain their lack of response to most component herbs. The principal herbs Radix Astragali and Radix Rehmanniae did not enhance the viability of patient fibroblasts. Interestingly, Rhizoma Atractylodis Macrocephalae had inhibitory effects on the viability of patient fibroblasts, which is contradictory to the results from the CRL-7522 fibroblasts. Radix Stephaniae Tetrandrae had a similar cytotoxic effect both in the CRL-7522 fibroblasts and patient fibroblasts, providing evidence to support the adjustment of the dose of this herb in the formulae to minimize possible side effects.

While the responses of CRL-7522 and patient fibroblasts to the treatment of individual herbs varied, both F1 and F2 enhanced the viability of the two cell types. Such an effect was the most potent in F1. Hence, the formulae contain effective agent(s) for activating fibroblasts and possibly improving wound healing under normal as well as diabetic conditions.

The positive effects of the formulae, especially F1 on enhancing patient fibroblast viability indicate that Chinese medicine treatment in the form of formulation may be effective in circumventing insulin resistance in diabetes. Individual component herbs of the formulae, however, seemed to be less effective as compared with the formulae in patient fibroblasts. This observation is consistent with the principle of using Chinese medicine in the form of formulation instead of a single herb for the treatment of diseases. Some of the herbs in the formulae may serve as adjuvant herbs to enhance or complement the effects of the principle herbs. Thus, synergistic effects among different component herbs may exist. Future studies on the interactive effects of the herbal components may further elucidate the pharmacological efficacy of F1 and F2.

The positive effects of the formulae and some of their component herbs on fibroblast viability raised the possibility that these herbal extracts are effective in promoting wound healing under both normal and diabetic conditions. While the enhanced fibroblast viability supports the improvement of granulation formation, it is important to note that the observations obtained in this study do not directly explain the improved granulation, since granulation is a complicated process involving the interactions of fibroblasts, macrophages, blood vessels and loose connective tissues (Mauch *et al*., 1993). In addition, the actual pathway of drug delivery to tissue involves digestion, absorption and assimilation through blood circulation. The results from incubation of cells with the herbal extracts only serves as *in vitro* evidence to support further validation of the effects of the herbs *in vivo*.

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Table 3. Comparison of clinical dosage amount with *in vitro* amount of formulae and each formulae component herbs

	Raw herb amount in formulae (g)	Extraction efficiency (w/w %)	Estimated maximum concentration in plasma based on clinical use (μg/mL)	Dosage range in *in vitro* studies (μg/mL)
Formula F1	65	30%	3333	25–400
Formula F2	74	34%	3333	25–400
Radix Astragali[1,2]	20	29.4	3920	312.5–5000
Radix Rehmanniae[1,2]	9[1] or 12[2]	50	3000–4000	62.5–1000
Rhizoma Atractylodis Macrocephalae[1]	9	43	2580	25–400
Radix Stephaniae Tetrandrae[1]	9	26.3	1578	25–400
Radix Polygoni Multiflori Preparata[1]	9	14.2	852	25–400
Rhizoma Smilacis Chinensis[1]	9	13	780	25–400
Poria[2]	6	1.6	64	50–800
Rhizoma Dioscoreae[2]	9	18	1080	25–400
Fructus Schisandrae Chinensis[2]	6	28.7	1147	25–400
Cortex Moutan[2]	6	24.1	963	25–400
Fructus Corni[2]	9	42.6	2557	25–400
Rhizoma Alismatis[2]	6	10.1	404	78.1–1250

[1] component of F1; [2] component of F2.

In trying to simulate the herbal dosages used in *in vivo* in our *in vitro* studies, since direct comparison is not possible, idealized estimations were made. The maximum plasma concentration of each herbal extract was estimated under the assumption of 100% absorption of the clinical dosage of the formulae using the average human plasma volume of 3 L (Wong *et al*., 2001, Margarson and Soni, 2005). Since it is anticipated that the absorption of any herbal extract is less than 100%, the dosages used in *in vitro* studies should reasonably approximate such an estimated maximum plasma concentration. As shown in Table 3, F1, F2 and the effective concentration ranges of the component herbs were consistent with the clinical dosages. Thus, the fibroblast viability enhancement effects observed *in vitro* support the clinically observed diabetic foot ulcer healing-promoting effects of the formulae.

In conclusion, the Chinese herbal formulae F1 and F2 enhanced the viability of fibroblasts which are important in granulation formation. Our *in vitro* models facilitated the understanding of the mechanisms underlying the clinical efficacies of F1 and F2 on diabetic foot ulcer healing. The positive effects of the two formulae support our continuous efforts to study the pharmacological effects of the formulae and their component herbs in animal and clinical models.

Acknowledgements

We thank Dr K. C. Choi at the Center for Epidemiology and Biostatistics, the Chinese University of Hong Kong for his generous help in statistical analysis, Dr H. Cao at the National Engineering Research Center for Modernization of Traditional Chinese Medicine (Zhuhai, Guangdong, China) for morphological authentication of herbs, and Ms K. L. Choi at the Institute of Chinese Medicine, The Chinese University of Hong Kong for the chemical authentication of herbs by thin layer chromatography.

The work described in this paper was substantially supported by a grant from the University Grants Committee of the Hong Kong Special Administrative Region, China under the Area of Excellence project 'Chinese Medicine Research and Further Development' (Project No. AoE/B-10/01) coordinated by the Institute of Chinese Medicine of the Chinese University of Hong Kong.

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DOI: 10.1002/ptr