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Research Paper

Preclinical evaluations on the efficacy of a topical Chinese herbal formula for swelling control and pain relief

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ABSTRACT

Ethnopharmacological relevance: Patients suffering from musculoskeletal pain and swellings occupy many hospital beds and demand many rehabilitation facilities. Chinese Medicine is offering many alternatives to ameliorate pain and swelling. However, evidence-based scientific publications supporting their efficacy on pain relief are inadequate. The *in vitro* and *in vivo* efficacy of a topical use Chinese herbal bath formula (HB) on anti-inflammation and swelling control was studied.

Materials and methods: The therapeutic mechanisms of HB were studied *in vitro* via anti-inflammatory and pro-angiogenic assays on RAW264.7 and HUVEC cells, respectively. Fibroblast proliferation was also studied with Hs27 cells. The *in vivo* angiogenic effect of HB was also studied using zebrafish model, while its efficacy of *in vivo* anti-Inflammation and swelling control were investigated using rat paw edema model. The affected paw was treated by immersing it in the HB or distilled water as control. The sensation of pain, change in paw thickness and inflammation marker in serum were analyzed.

Results: *In the anti-inflammation assay*, HB significantly inhibited nitrite release from RAW264.7 by 47.6% at 800 μg/ml. In the pro-angiogenic assays, it reduced wound area in HUVEC by 8.2% and increased tube formation of HUVEC by 11.5% at 300 μg/ml. HB also stimulated Hs27 proliferation up to 23.5% at 1200 μg/ml. It showed *in vivo* pro-angiogenic effect by increasing the mean sprout number in the embryos of zebrafish by 2.4 folds. The *in vivo* therapeutic effects of HB on edema was illustrated by the significant longer thermal withdrawal latency and thinner paw thickness compared with control. After 14 days of treatment, HB also reduced the IL-6 concentration in the serum of rat by 20.9% significantly.

Conclusions: This study showed that HB is effective for swelling control and pain relief from edema due to its anti-inflammatory and pro-angiogenic properties.

1. Introduction

Musculoskeletal pain, swellings and limitation of movement resulting from either injuries or chronic mechanical stress are disabling symptoms affecting general public health. According to a report of a joint project led by United States Bone and Joint Decade, 60% to 67% of injuries of all types treated in health care settings were musculoskeletal injuries (United States Bone and Joint Decade, 2011). It can be understood that patients suffering from musculoskeletal pain hold up many beds and rehabilitation facilities in hospital. The loss of work days from these patients also increases the social-economic burden.

In spite of these concerns, there are not many choices of western medications for reduction of swelling resulted from soft tissue injuries provided by clinics. The common clinical practice is issuing of analgesics to ease the pain. On the contrary, facilitating the reduction of swelling and pain is one of the major areas in the "bone sector" of Traditional Chinese Medicine (TCM). TCM offers a wide variety of alternatives to ameliorate pain and swelling. Nonetheless, these alternatives usually consist of multiple herbs ranging from 5 and up to over 20. The historical combinations are based mainly on old records of herbal masters and the personal

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experiences of the users. Most of them are lack of scientific evidences to support their efficacy.

Nowadays, there are many scientific platforms to study the effects of individual herbs on soft tissue injuries. *In vitro* platforms such as fibroblast proliferation assays (Zhang et al., 2003; Tam et al., 2011; Lai et al., 2012) and nitric oxide inhibition assay in macrophage (Tam et al., 2011; Chen and Zhang, 2014; Chen et al., 2014) have been using for the studies of connective tissues formation and anti-inflammation effect, respectively. Stem cell platforms have also been using to study wound healing (Kuo et al., 2011; Wong et al., 2012; Uysal et al., 2014). *In vivo* platforms are also well-developed for the studies of soft tissue inflammation (Eddouks et al., 2012; Murugananthan and Shivalinge Gowda, 2012; Tumen et al., 2011; Trøstrup et al., 2013).

The current pathological knowledge on swelling could also guide us on the selection of herbs for swelling control. Swelling is the outcome of inflammation which may be acute and chronic. Inflammatory response occurs in three distinct phases (Trøstrup et al., 2013): During the first phase, vascular permeability increases resulting in exudation of fluids from the blood into the interstitial space; the second phase involves the infiltrations of leukocytes from the blood into the tissue; in the third phase, granuloma formation and tissue repair start.

Based on these distinct pathological events, we need herbs that would correspondingly help to (1): promote blood circulation to discharge the fluids remaining in the interstitial space to reduce swelling, (2): control inflammation and subsequently result in pain relief and (3): promote tissue healing. Considering that simplification of herbal formula from traditional complicating ones could help researchers to study the pharmaceutical mechanisms of the herbs more concisely and accurately, we simplified our herbal formula that had been studied clinically for swelling control and pain relief (Zhao et al., 2006). A 3-herb formula is the most simplified formulation that can achieve the purposes as mentioned. Thus, the following three herbs were used:

(1) *Carthamus tinctorius* L., flower, dried (Carthami Flos, "Hong-Hua" in Chinese) belongs to the Compositae family. Recent scientific reports indicated that it also exerts anti-inflammatory effect (Tien et al., 2010; Jun et al., 2011). Our previous study demonstrated that it could enhance fracture healing presumably through blood circulation promotion and anti-inflammation (Peng et al., 2010).

(2) *Angelica sinensis* (Oliv.) Diels., root, dried (Angelicae Sinensis Radix, "Dang Gui" in Chinese) belongs to the Umbelliferae family. It is commonly used for all kinds of blood deficiency syndromes (Shi et al., 2014), pain syndromes (Chen et al., 2000; Zhao et al., 2014), and ulcers (Ye et al., 2003) in TCM. One of its active components has been studied for the treatment of chronic inflammatory pain recently (Zhao et al., 2014).

(3) *Achyranthes bidentata* Blume, root, dried (Achyranthis Bidentatae Radix, "Huai Niu Xi" in Chinese) belongs to the Amaranthaceae family. It is used to promote blood circulation (Wang et al., 2013) and promote diuresis (Wang, 2006) in TCM. It can also prevent bone loss during the development of bone necrosis (Kong et al., 2012) and increase fibroblast proliferation in our previous study (Data not reported).

The aim of this study is to provide evidence-based scientific data to verify the anti-inflammatory and pro-angiogenic efficacy of a Chinese herbal bath through *in vitro* and *in vivo* experiments.

2. Materials and methods

2.1. Herbal materials and preparation of the herbal bath

All the herbs were purchased from Guangzhou Zhixin Ltd (Guangzhou, China). The identities of all herbs had been authenticated using thin-layer chromatography with reference to methods recommended by the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010). The herbarium voucher specimens of the tested herbs were deposited in the museum of the Institute of Chinese Medicine, the Chinese University of Hong Kong, with voucher name and numbers as follows: Carthami Flos: 2013–3415; Angelicae Sinensis Radix: 2013–3420; and Achyranthis Bidentatae Radix: 2013–3413.

Herbal extracts for the preparation of the Herbal Bath (HB) were prepared through aqueous extraction and then followed by ethanol extraction. Firstly, the herbs (50 g each) were extracted by reflux using 1 L distilled water for one hour, filtered and the filtrate was collected. Then, the remaining solid herbal residues were further extracted by reflux using 95% ethanol for one hour and then filtered. The aqueous and ethanol extracts were combined and concentrated. The mixture was lyophilized into powder form using a freeze drier (Freezone 12, Labconco, Missouri, USA). Before the *in vitro* studies, the lyophilized herbal powder were dissolved in relative medium at different concentrations to form extract of HB and then filtered by 0.22 μm filter. Before the topical treatment, a herbal bath was formed by dissolving the powder into distilled water in a concentration of 5 g/L. This concentration was determined by following the experience from a previous clinical study (Zhao et al., 2006).

2.2. In vitro studies on anti-inflammation, angiogenesis and tissue proliferation

Cell lines of murine monocyte/macrophage (RAW264.7), Human Umbilical Vein Endothelial Cell (HUVEC) and Human Skin fibroblast cell (Hs27) were purchased from American Type Culture Collection (ATCC; USA). All cells were maintained at 37 °C, in 5% CO_2 humidified incubator.

2.2.1. Anti-inflammatory effect

RAW264.7 (4×10^5 per well) were seeded in 24-well plate in DMEM overnight. The extract of the HB was added at concentrations ranging from 0 (Control) to 800 μg/ml into medium containing 0.1 μg/mL of lipopolysaccharide (LPS) (Product #: L2755, Sigma, St. Louis, MO, USA). The cells were then incubated for 24 h. To measure the nitric oxide (NO) production, culture supernatant was added to Griess Reagent (Sigma, USA) in the ratio of 1:1 in a 96-well plate and the plate was incubated in darkness for 10 min (Tam et al., 2011). 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 nitrite concentration in the culture supernatant.

2.2.2. Pro-angiogenic effect on endothelial cells

2.2.2.1. Cell migration. In wound healing assay (scratch assay), HUVEC cells (1×10^5 per well) were grown in DMEM/F12 supplemented with 0.5% fetal bovine serum (FBS), growth factors and heparin to full confluence in 24-well plates. Then, two crosses on cells were scratched with a pipette tip (p200) and the wells were washed with PBS to remove detached cells. Cells were then incubated with various concentrations of the HB extract or blank medium (0 μg/ml as Control) for 6 h. Photographs were taken before and after treatment by an inverted microscope (Nikon Eclipse TS100). Data were analyzed with the T-Scratch software using the default parameter settings. Four replicates were done in each individual experiment.

2.2.2.2. Tube formation. Each well (96-well plate) was pre-coated with 50 μl of Matrigel and allowed to solidify at 37 °C. HUVEC cells (1.5×10^4 cells per well) were then seeded into the wells with the addition of various concentrations of the HB extract or blank medium (0 μg/ml as Control), and incubated for 2 h. The network of tubes formed in each well was photographed. The total length of tubes per image was analyzed using Image-Pro Plus 6.0 software. Duplicates or triplicates were done in each individual experiment.