EXPERIMENTAL STUDY

Effect of Bushenhuoxue formula on interleukin-1 beta and discoidin domain receptor 2 levels in a rat model of osteoarthritis

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Abstract

OBJECTIVE: To determine the effects of Bushenhuoxue formula (BHF) on interleukin-1 beta (IL-1β), transforming growth factor beta 1 (TGF-β1), discoidin domain receptor 2 (DDR2) and matrix metalloproteinase-1 (MMP-1) levels in a rat model of osteoarthritis (OA).

METHODS: Sprague-Dawley rats were used to establish an OA model and subjected to various treatments over 6 weeks. Rats were treated with BHF, glucosamine sulfate (GS), or starch as a control. Serum levels of IL-1β and MMP-1 and joint fluid levels of IL-1β were determined by means of ELISAs. We used immunohistochemistry to determine DDR2 levels in knee cartilage. Gene expression levels of MMP-1 in joint synovial tissue were assessed using reverse transcription polymerase chain reaction assays.

RESULTS: Serum IL-1β levels were unchanged throughout the study. Levels of IL-1β in joint fluid and MMP-1 in sera from the BHF- and GS-treated groups were significantly reduced. DDR2 levels in knee cartilage were also significantly reduced in the BHF group. Expression of the MMP-1 gene was significantly reduced by BHF treatment.

CONCLUSION: BHF might be beneficial in the inhibition and alleviation of local inflammatory responses and cartilage degeneration in OA.

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Key words: Osteoarthritis; Cytokines; Discoidin receptor; Matrix metalloproteinase 1; Bushenhuoxue formula

INTRODUCTION

Osteoarthritis (OA) is a degenerative disease; its main pathologic findings are denatured and damaged joint cartilage, accompanied by osteophyte formation, synovitis, and narrowing of the joint space. OA is the result of an imbalance in factors between degradation and synthesis of the cartilage matrix. In Traditional Chinese Medicine (TCM), Bushenhuoxue formula (BHF) is able to increase the available energy of the kidney and activate blood circulation. It is prescribed clinically for the treatment of OA at early
and moderate stages of the disease and is effective in 88.9% of cases. The therapeutic efficacy of BHF has been summarized previously.⁴ The molecular mechanisms involved in the inhibition of tumor necrosis factor-α (TNF-α) and interleukin-1 beta (IL-1β) expression in synoviocytes has been partially elucidated.⁴ Based on these previous results, we conducted experiments to determine the influence of BHF on the expression of IL-1β, matrix metalloproteinase-1 (MMP-1) and discoidin domain receptor 2 (DDR2) which is signaling molecules of OA.

**MATERIALS AND METHODS**

**Rat model of OA**

We used 56 (28 male, 28 female) specific pathogen-free Sprague-Dawley rats that were (250 ± 20) g and 3 months old. Rats were raised in the animal center at Shandong University of Traditional Chinese Medicine (Quality Certificate No. SCXK [Lu] 20050015). All animal procedures were performed in accordance with Shandong University’s ethical principles for the care and use of laboratory animals. All rats underwent identical surgeries; a longitudinal incision was made on the medial side of the right hind knee. The medial collateral ligament was transected and the medial meniscus was cut through.⁵ Damage to the articular cartilage was avoided during surgery. The wound was cleaned and rinsed with phosphate-buffered saline (PBS) containing 50 mg/kg ampicillin to prevent local infection. Animals were fed a normal diet over the 6-week period of our study.

**Treatment groups**

Five rats were randomly selected to verify the establishment of the OA model. Of the remaining rats, 33 were chosen and randomly allocated to three groups (n = 11 rats per group): the BHF treatment group; the glucosamine sulfate (GS) treatment group; and the control group. Equal volumes of a concentrated BHF decoction were gavaged or orally administered to rats in the BHF group twice a day. A powder suspension of GS was given to animals in the GS group, and a starch suspension was given to rats in the control group. The treatment period was 6 weeks. We collected tissue samples from animals prior to establishment of the OA model and after the treatment period. Tissue sections were stained with hematoxylin-eosin (HE) and then examined microscopically.

**Dosage conversion**

The BHF decoction comprised Fuzi (Radix Aconiti Lateralis Preparata) 10 g, Dihuang (Radix Rehmanniae) 20 g, Danshen (Radix Salviae Miltiorrhizae) 20 g, Bajitian (Radix Morindae Officinalis) 15 g and Xianmao (Rhizoma Curculiginis) 10 g. All components were refined granules from the Chinese herbal pieces (Cat. # 0905261s; 999 Pharmacological Company, Shenzhen, China). Granules were dissolved in 66.67 mL of water at 100 °C and then kept at room temperature until required. We purchased GS from Zhejiang Hisun Pharmaceutical Co., Ltd., (Cat. # 09051701, Zhejiang, China).

Equivalent surface area dose conversion factors for humans and rats were used to determine the appropriate dose for rats.⁶ We used the following formula:

\[
\text{Drug dose for human (mg/kg)} \times 0.47 \text{ (rat body surface area ratio) } / 0.08 \text{ (human body surface area ratio) } = \text{ required drug dose for rats (mg/kg)}
\]

Based on a clinical regimen of 500 mg three times a day for GS, and 150 mL of the BHF decoction twice a day for a 50-kg adult human, the dose for a 250-g rat was determined to be 44 mg of GS and 9 mL of the BHF decoction per day. The optimal gavage volume is 2 mL for a rat. A 2-mL suspension contained 22 mg of GS, or a 2-mL concentrated BHF decoction derived from a 4.5-mL BHF decoction. Suspensions were given to rats twice a day.

**Measurement of IL-1β levels**

Blood samples were collected 1 h after the last oral administration of GS, BHF or starch for the determination of IL-1β levels in serum. Synovial fluid was extracted from the right hind knee joint following the injection of 200 μL saline at the termination of the experiment.⁷ Animal were sacrificed by suffocation in CO2 box. Cytokine levels were determined using enzyme-linked immunosorbent assays (ELISAs).

**Immunohistochemistry**

For immunohistochemical staining, rat knee joints were harvested at the end of our study. Tissue sections were deparaffinized, hydrated, and quenched for endogenous peroxidase activity. After treatment with chondroitinase ABC (1: 200 dilution; Sigma-Aldrich, St Louis, MO, USA), sections were incubated with an anti-DDR2 polyclonal antibody (1: 200; Abcam, Cambridge, MA, USA). After washing with PBS (Sigma-Aldrich), samples were incubated with a biotinylated secondary antibody ( Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). We used Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) to analyze images. Ten fields of view were selected for each sample, and positive areas accumulated and averaged. The mean optical density (MOD) and integrated optical density (IOD) were calculated and IOD values were subjected to statistical tests.

**Reverse transcription polymerase chain reaction (RT-PCR) assays**

We used RT-PCR assays to determine MMP-1 mRNA expression levels in joint synovial tissues. We extracted RNA from homogenized joint synovial tissue. Reverse transcription was performed using 2 μg of total RNA and random primers. The resulting cDNA was ampli-
fied over 33 cycles with two specific oligonucleotide primers (5′-ACA GTT TCC CCG TGT TTC AG-3′ and 5′-CCC ACA CCT AGG TTT CCT CA-3′). Each cycle involved denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and a 1-min extension step at 68 °C (StepOnePlus™, Life Technologies, Carlsbad, CA, USA). Amplicons were electrophoresed on 2% (w/v) agarose gels and stained with ethidium bromide.

Measurement of MMP-1
MMP-1 levels were determined using ELISAs. Synovial fluid was extracted from the right hind knee joint following the injection of 200 µL of saline at the termination of our study. Synovial fluid samples were diluted (1 : 4) with assay buffer; 100 µL of diluted samples and standards were added to the wells of a microtiter plate pre-coated with an MMP-1 antibody. The plate was incubated at room temperature for 2 h. After five washing steps with wash buffer to remove unbound antigen, secondary antibodies conjugated to peroxidase were added. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added. The color reaction was stopped by the addition of an acid solution and plates read at 450 nm in an EC 312 microplate reader (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA, USA). The concentration of MMP-1 in a sample was determined by interpolation from a standard curve.

Statistical analyses
We used SPSS 17.0 (SPSS Inc., Chicago, IL, USA) for all statistical analyses. Values were expressed as mean ± standard deviation (x ± s). A one-way analysis of variance was used to determine the difference among groups. A P-value less than 0.05 was deemed statistically significant.

RESULTS
Establishment of a rat OA model and pathological analysis
We randomly selected five rats 6 weeks after surgery to evaluate our OA model. Following euthanasia of these rats, right hind knee joints were dissected. The femur and tibial plateau articular surfaces were collected for morphological examination. Macroscopic observations revealed marked articular damage. Microscopic examination of HE-stained sections were performed; compared with normal joints (Figure 1A), the meniscus-rupture model exhibited reduced articular cartilage thickness, roughness of the cartilage surface, a decrease in cartilage cells counts, subchondral osteoporosis, and micro fractures (Figure 1B). Changes were observed between the BHF and control groups. The cartilage was thinner and in some cases ruptured in the control group (Figure 1D). The integrity of cartilage was maintained in the BHF group even though cartilage cells had degenerated (Figure 1C).

IL-1β in serum and synovial fluid
IL-1β levels in synovial joint fluid were significantly reduced after treatment with BHF or GS in comparison with those seen for the control group (P < 0.05). We did not observe a significant difference in IL-1β levels in serum samples from the three treatment groups (Table 1).

Immunohistochemical analysis of DDR2 levels in cartilage
We detected DDR2 in cartilage sections using immunohistochemical methods (Figure 2A). Reduced expression of DDR2 was obvious in the BHF group (Figure 2B). Quantitation of DDR2 expression by IOD showed a significant difference between the BHF and control groups (P < 0.05; Table 1). WE failed to observe statistical differences between the BHF and GS groups, or between the GS and control groups (P < 0.05).

MMP-1 expression in synovial tissue
MMP-1 expression in synovial tissue was significantly
reduced in samples from the BHF-treated group, and slightly reduced in the GS group when compared with levels in the control group (Figure 3).

![Image](Image 97x562 to 295x733)

**Figure 3** Analysis of MMP-1 expression by RT-PCR

A: 1: control group; 2: glucosamine sulfate (GS) treatment group; 3: Bushenhuoxue formula (BHF) treatment group. B: gene expression profile of MMP-1 following treatment with Bushenhuoxue formula and glucosamine sulfate for 6 weeks. Control group was treated with 2 mL of starch suspension gavaged for 6 weeks twice a day; glucosamine sulfate treatment group was treated with 22 mg of glucosamine sulfate suspension gavaged twice a day for 6 weeks; Bushenhuoxue formula treatment group was treated with 2 mL suspension of Bushenhuoxue formula decoction suspension gavaged twice a day for 6 weeks. MMP-1: matrix metalloproteinase-1; RT-PCR: reverse transcription polymerase chain reaction. MMP-1 expression levels in the Bushenhuoxue formula group were significantly decreased compared with those in the control group ($P < 0.05$).

**MMP-1 levels in synovial fluid**

The levels of MMP-1 in synovial joint fluid were significantly reduced following treatment with BHF or GS when compared with those in the control group ($P < 0.05$; Table 1).

**DISCUSSION**

Based on TCM theory and our practical experiences, OA pathogenesis of the knee primarily involves kidney deficiency, followed by blood stasis. The Bushenhuoxue formula was made to treat these symptoms. Aconite tonifies blood and kidney $Yin$, as well as bone marrow. The spicy divergent characteristics of aconite mean that Rehmannia’s tonification is not stagnated. The greasy characteristics of Rehmannia can reduce the dry nature of aconite, ensuring that tonification is not aggressive. A deficiency in kidney $Yin$ and $Yang$ could be evenly tonified with a combination of the two herbs. Salvia was used to promote blood circulation, soothe the nerves, and eliminate carbuncles so swelling and pain in the knee can be relieved. Morinda and Curculigo warms the kidney, strengthens tendons, dispels coldness, and removes humidity. The function of this prescription was to tonify the kidney and promote blood circulation.

Our findings indicate that BHF reduced IL-1$\beta$ and MMP-1 levels in the synovial fluid of joints, inhibited DDR2 expression in chondrocytes, and lowered expression of MMP-1. BHF had no obvious effect on reduction of serum IL-1$\beta$ levels. However, it significantly reduced IL-1$\beta$ and MMP-1 levels in synovial fluid. This would suggest a specific effect of BHF on synovial cells. BHF reduced IL-1$\beta$ levels in synovial fluid, therefore the functions of this cytokine were diminished. This led to down-regulated expression of MMP-1 mRNA, decreased synthesis of chondrocytes and synovial cells, and reduced secretion of PGE2. Lower PGE2 levels ameliorated destruction of cartilage and reduced the generation of collagen fragments (DDR2 ligands). The DDR2 signaling molecule could be inhibited by BHF, and a reduction in DDR2, DDR2 ligand, and IL-1$\beta$ levels could be sequentially reached through BHF treatment. Our RT-PCR results showed that reduced DDR2 and IL-1$\beta$ levels led to reduced expression levels of MMP-1. Pathologically, BHF treatment maintained cartilage integrity and thickness.

Patients with mild OA of the knee are treated through body weight control, physical therapy and oral medica-

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**Table 1 Levels of IL-1$\beta$, MMP-1 and DDR2 in different groups ($n=11$, $\bar{x} \pm s$)**

<table>
<thead>
<tr>
<th>Item</th>
<th>BHF</th>
<th>GS</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 fluid (ng/mL)</td>
<td>4.13±0.21$^*$</td>
<td>4.54±0.69$^*$</td>
<td>5.33±0.17</td>
</tr>
<tr>
<td>IL-1 sera (ng/mL)</td>
<td>7.39±5.35</td>
<td>8.35±5.27</td>
<td>7.89±5.03</td>
</tr>
<tr>
<td>DDR2 (IOD value)</td>
<td>15.24±2.19$^*$</td>
<td>21.90±5.31</td>
<td>27.60±3.87</td>
</tr>
<tr>
<td>MMP-1 protein (µg/mL)</td>
<td>0.53±0.09$^*$</td>
<td>0.63±0.08</td>
<td>1.04±0.11</td>
</tr>
</tbody>
</table>

Notes: control group was treated with 2 mL of starch suspension gavaged for 6 weeks twice a day; glucosamine sulfate treatment group was treated with 22 mg of glucosamine sulfate suspension gavaged twice a day for 6 weeks; Bushenhuoxue formula treatment group was treated with 2 mL suspension of Bushenhuoxue formula decoction suspension gavaged twice a day for 6 weeks. BHF: Bushenhuoxue formula; GS: glucosamine sulfate; IL-1$\beta$: interleukin-1 beta; DDR2: discoidin domain receptor 2; MMP-1: matrix metalloproteinase-1. For synovial fluid, IL-1$\beta$ levels in samples from the BHF and GS treatment groups were significantly lower than those in the control group ($P < 0.01$). There was no significant difference between the BHF and GS groups ($P > 0.05$). For serum IL-1$\beta$ levels, there was no significant difference among the three groups ($P > 0.05$). DDR2 levels in cartilage for the BHF group were significantly different compared with those in the control group ($P = 0.03$). MMP-1 levels in synovial fluid were significantly lower in BHF groups compared with those in the control group ($P < 0.01$). $^*$ $P < 0.01$, $^*P < 0.05$. 

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The most commonly used drugs are cartilage protective agents and non-steroidal anti-inflammatory analgesics to relieve pain. GS has been widely used with a satisfactory clinical efficacy, but its biological mechanism remains unclear. Some researchers believe that the effects of glucosamine hydrochloride are similar to those for GS. Other researchers found that glucosamine hydrochloride should be combined with other drugs to be used clinically for treating OA as no significant effects were observed when it was used alone. Some studies have suggested that GS might affect inducible nitric oxide synthase. According to our results, GS has some effect on reducing IL-1β levels in synovial fluid.

It is difficult to determine which compound(s) in a Chinese herb plays a major pharmacological role in the treatment of pathological disorders. The method of kidney tonifying and blood platelet activation has been reported as an optional regimen in treating OA. However, current prescriptions differ completely from our formulation. Further investigation is required to explore the specific therapeutic component(s) in our formula and its mechanism(s) of action.

REFERENCES