Effect of Bushen Huoxue Decoction on Apoptosis-related Protein and Serum Inflammatory Factors in Rat Model of Knee Osteoarthritis

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Abstract: In order to observe the effect of Bushen Huoxue Decoction on apoptosis-related proteins and serum inflammatory factors in synovial cells of rats with osteoarthritis (KOA). A total of 45 SD adult rats were established with KOA model. After successful, they were randomly divided into model group, Bushen Huoxue Decoction group and glucosamine hydrochloride group, with 15 rats in each group. The model group was given pure water, the Chinese medicine group was given Bushen Huoxue Decoction, and the control (glucosamine hydrochloride) group was administered with glucosamine hydrochloride. After 8 weeks of treatment, all rats were sacrificed. Rat serum and knee cartilage were collected. The expressions of apoptosis-related protein Caspase-3 and Bax were detected by HE staining, ELISA and RT-PCR. Serum interleukin-1β (IL-1β), tumor necrosis factor (TNF-α), inducible nitric oxide synthase (iNOS) levels, and Mankin scores were used to evaluate articular cartilage damage. Resultsly, the appearance and shape of the knee joint cartilage tissue in the model group were severely damaged. The area of the cartilage surface defect was large. The HE staining showed cell disorder and the tidal line was destroyed. The articular cartilage of the Chinese medicine group had a complete shape and structure, but the surface showed slightly rough. HE staining showed that the cells were arranged regularly and the tidal line was relatively complete. The surface of the knee joint of the aminoglycan group was rough and partially defective. The HE staining showed that the chondrocytes were slightly disordered and some tidal lines were destroyed. The Mankin score of the Chinese medicine group was significantly lower than that of the model group and the control group. The levels of serum IL-1β, TNF-α and iNos and the expression levels of caspase-3 and Bax in the Chinese medicine (Bushen Huoxue Decoction) group were significantly lower than those in the model group and the control group and model group. In conclusion, Bushen Huoxue Decoction can reduce the expression of apoptotic proteins, reduce the content of serum inflammatory factors, alleviate the inflammatory infiltration of knee osteoarthritis and reduce the destruction of chondrocytes.

Keywords: KOA; Bushen Huoxue Decoction; apoptosis; inflammatory factors

1. Introduction

Knee osteoarthritis (KOA) is a chronic disease characterized by various pathogenic factors leading to the destruction, erosion, and degeneration of articular cartilage, leading to bone hyperplasia [1]. Previous studies have shown that the level of inflammation and cell apoptosis in the joint are closely related to the occurrence and development of osteoarthritis. Due to the release of apoptotic factors and inflammatory factors in the articular cartilage, synovium, and subchondral areas, they can trigger inflammatory reactions in the joint, thereby promoting the transcription of apoptotic genes in soft bone cells and accelerating the occurrence and development of knee osteoarthritis [2]. Traditional Chinese medicine theory suggests that the onset of KOA is closely related to qi stagnation and blood stasis, as well as local dampness and turbidity obstruction in the joints. Due to factors such as trauma, synovial membrane injury, infection, and pain that stimulate the knee joint synovial sac, a large amount of tissue fluid seeps into the joint cavity, forming joint effusion, leading to related arthritis symptoms [3-4]. Lu Chao, Deputy Chief Physician of the Joint Surgery Department of the Affiliated Red Society Hospital of Xi’an Jiaotong University, improved the formula "Bushen Huoxue Tang" based on the experience of Guo Hanzhang's academic school studio. It is clinically used for the treatment of osteoarthritis, with the...
functions of promoting blood circulation and resolving stasis, promoting dampness and removing obstruction, and tonifying the kidney and strengthening bones [5-6]. Therefore, this study aims to observe the effects of Bushen Huoxue Tang on apoptosis related proteins and serum inflammatory factors in knee joint cells of osteoarthritis model rats.

2. Materials and Methods

2.1. Experimental animals

45 clean grade SD rats (from the Medical Department of Xi'an Jiaotong University), male and female, with an average age of 28-32 weeks and an average weight of about 250-300g, were fed with a compound feed. All animal treatment methods in this experiment strictly followed the "Guiding Opinions on Treating Experimental Animals" [7].

2.2. Experimental drugs and reagents

The composition of Bushen Huoxue Tang is as follows: 30g mulberry parasitic, 24g Codonopsis pilosula, 20g Siesgesia herb, 15g hot dog spine, 15g Huai Niu knee, 12g Duohuo, Danshen, Angelica, frankincense (vinegar), myrrh (vinegar), white peony, 10g Chuanxiong, Eucommia ulmoides, and 6g licorice (Chinese herbal medicine room of Xi'an Honghui Hospital). After decocting the traditional Chinese medicine compound, store it in a refrigerator at 4 ℃ for future use.

Glucosamine Hydrochloride Capsules (Zhejiang Chengyi Pharmaceutical Co., Ltd.), Caspase-3, Bax, IL-1β, TNF-α, INOS antibodies, ELISA, PCR kits (Xi'an Hanyang Biotechnology Co., Ltd.).

2.3. Animal modeling and grouping

(1) By using the improved Hulth method, we prepared 45 adult SD rats as KOA models [8], and then randomly divided them into model group (15 rats), traditional Chinese medicine (Bushen Huoxue Tang) group (15 rats), and control (glucosamine hydrochloride) group (15 rats). (2) Each group of rats was given the same dosage of therapeutic medication according to their body weight: the traditional Chinese medicine treatment group was given Bushen Huoxue Tang suspension 10ml/kg by gavage, the control group was given glucosamine hydrochloride capsules 10ml/kg by gavage, and the model group was given physiological saline 10ml/kg by gavage. The above groups were given one day per time for 8 consecutive weeks. During the 8 weeks of treatment, we monitored diet and water sources daily to record the diet and water consumption of each group of animals. We cleaned the cage every other day and observed the status, activity, fur color and bowel movements of the animals in each group. The above information was recorded in the laboratory notebook.

2.4. Experimental Methods

2.4.1. Histomorphological observation

(1) According to the experimental design and the recording of the experiment, after 8 weeks of treatment, the rats were euthanized, and the skin, subcutaneous fascia, and joint capsule around the knee joint were gently dissected until the knee joint was fully exposed. Then, the knee joint cartilage was removed along the upper and lower parts of the knee joint, extending 1.5cm. After exposure, the knee joint cartilage was cut along the upper and lower parts of the tibial and femoral joint surfaces, 1-1.5cm respectively, obtaining knee joint tissue specimens for external morphology observation. After taking out the knee joint specimen, the specimen was fixed with paraformaldehyde for 24 hours, decalcified with ethylenediaminetetraacetic acid, dehydrated with gradient alcohol, transparent with xylene, and embedded in paraffin. After that, the sections were stained with HE, and the morphological changes of the stained cartilage were observed using an optical microscope.

2.4.2. Mankin score

A general standard for evaluating the degree of KOA cartilage degeneration [9], combined with HE staining observation results, two observers used a double-blind method to perform Mankin score on mouse knee joint cartilage tissue. Each group selected 12 knee joint specimen slices for scoring and conducted statistical analysis. The higher the score, the more severe the KOA lesion. See Table 1.
Table 1: Mankin scoring criteria for articular cartilage

<table>
<thead>
<tr>
<th>Rating (points)</th>
<th>Grade</th>
<th>Performance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>normal</td>
<td>The surface is flat, without gaps, with evenly distributed cells, without clustered chondrocytes, and the tidal line is intact; HE staining is uniform without loss of staining.</td>
</tr>
<tr>
<td>2-7</td>
<td>mild</td>
<td>The surface is slightly uneven with small cracks, and there are a small number of clustered and single hypertrophic chondrocytes in the middle and deep layers. Some specimens have broken or double tidal lines; HE staining shows loss of staining.</td>
</tr>
<tr>
<td>8-12</td>
<td>moderate</td>
<td>The surface is uneven, with cracks reaching to the middle and deep layers. Some of the surface or middle layers have fibrillar degeneration, and the arrangement of cells in the middle and deep layers is disordered, with a large number of clustered chondrocytes, broken tidal lines or double tidal lines; HE staining is uneven, and there is a loss of staining phenomenon in the surface, middle, and deep layers.</td>
</tr>
<tr>
<td>13-14</td>
<td>severe</td>
<td>The surface layer becomes thinner, the cracks extend deep to the subchondral bone, and the cells are arranged in disorder, with a large number of clustered chondrocytes and the tidal line disappears; HE staining is uneven, with significant loss of staining throughout the entire layer.</td>
</tr>
</tbody>
</table>

2.4.3. ELISA method for detecting serum inflammatory factor IL-1β, TNF-α, and iNOS level

(1) Setting of hole columns: column A was the standard hole, column B-G was the sample hole, and column H was the blank hole. (2) Sample addition: We added 50ul of standard sample to the standard well, added 10ul of plasma to be tested and 40ul of sample dilution to the B-G column. We did not add in the H-column, and then added 100ul of antibody to the A-G column. After all reagents were added, sealed and placed in a 37 °C incubator for 60min. (3) Washing plate: After the incubation time was completed, we took out the orifice plate and quickly inverted it. Throw away all liquid and pat it dried on absorbent paper. Then, we injected washing solution into each hole and gently vibrated to wash away any remaining liquid in the hole × 5 times. (4) Color development: After washing the board, we added 50 color development agents A and B to each hole in sequence in column A-H μ. Afterwards, placed it in a dark bag and place it in a 37 °C incubator for 15 minutes. (5) Termination: After the end of incubation, we added 50 to each well in column A-H μ. Terminate the liquid to terminate the color development. (6) Detection: We placed the well plate in an enzyme labeling detector to measure the OD value of each well. We used the concentration value of the standard substance as the X-axis, and the corresponding OD value of each standard substance as the Y-axis. We drew a standard curve, calculated the regression equation, and calculated the actual concentration of each well based on the standard curve equation[10].

2.4.4. Real time PCR detection of apoptosis related proteins Caspase-3 and Bax mRNA expression in knee joint cartilage tissue

(1) Grinding specimen: We removed the knee joint specimen from the test rat in liquid nitrogen, placed it in a pre cooled mortar and quickly ground it to powder, and then placed it in a pre cooled tube. (2) RNA extraction: We added Trizol to each tube to extract total RNA from cartilage tissue. We used agarose gel electrophoresis to detect the purity and integrity of RNA in the cartilage tissue of the knee joint. (3) Reverse transcription: After thawing in warm water, the extracted RNA was reverse transcribed. After completion, the cDNA5 obtained was diluted with deionized water by a factor of 0, and then used for the detection of Caspase-3 and Bax mRNA. (4) Follow the instructions of the kit for the next step of testing: We used cDNA as a template and follow Go Taq® Preparation of qPCR Master Mix Kit Instructions 20 μ L PCR reaction system, PCR reaction conditions: 95 °C, 10 minutes into circulation; 95 °C, 15 seconds, 60 °C, 1 minute, 45 cycles. We used GAPDH as the internal reference, calculated the expression levels of Caspase-3 and Bax mRNA using relative quantification method.

2.5. Statistical processing

We used the SPASS20. 0 statistical software, the experimental data was represented by mean (X ± S) standard deviation( X ± S). If the results met the normality and homogeneity of variance tests,
one-way ANOVA was used for inter group comparison. If the inter group differences were statistically significant, LSD method was further used for pairwise comparison; If normality and homogeneity of variance were not met, non-parametric tests were used, with P<0.05 indicating statistically significant differences.

3. Results

3.1. Observation results of pathological examination of cartilage tissue

(1) Compared with the traditional Chinese medicine group and the model group, the model group showed more severe damage to the appearance and morphology of the knee joint cartilage tissue, with a large area of cartilage surface defects. HE staining showed disordered cell arrangement and a large amount of damage to the tidal line (see Figure a1, b1). (2) Compared with the ammonia sugar group, the traditional Chinese medicine group had a complete overall shape and structure of the joint cartilage, but the surface showed a slight roughness. HE staining showed a more regular cell arrangement. The tidal line is relatively complete. (See Figures b1 and b2) (3) Compared with the traditional Chinese medicine group, the general shape and surface of the knee joint in the ammonia sugar group rats were rough, with some defects. HE staining showed a slightly disordered arrangement of chondrocytes, and some tidal lines were damaged (see Figure 1).

![Figure 1: a1 and a2 model groups; B1b2 traditional Chinese medicine group (Bushen Huoxue Tang); C1 c2: Western medicine group (glucosamine hydrochloride)](image)

3.2. Mankin scoring results

The score of the model group was higher than that of the aminose group and the traditional Chinese medicine group (both P<0.05), while the aminose group scored higher than that of the traditional Chinese medicine group (P<0.05) (see Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>Mankin score (points)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model Group</td>
<td>15</td>
<td>9.16±1.7</td>
</tr>
<tr>
<td>Traditional Chinese</td>
<td>15</td>
<td>4.16±1.1*</td>
</tr>
<tr>
<td>Medicine Group</td>
<td>15</td>
<td>6.50±0.8#</td>
</tr>
<tr>
<td>Aminoglycoside group</td>
<td>15</td>
<td>61.624</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>F(61,624) &lt;0.05</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Compared with the model group, # * P<0.05, and compared with the ammonia group, * P<0.05.

3.3. ELISA method for detecting IL-1 β, TNF-α Expression results of iNOS

Compared with the model group, IL-1 in the serum of the traditional Chinese medicine group and
the aminoglucose group β, TNF-α. The content of iNOS protein and iNOS protein significantly decreased, with a statistically significant difference (P<0.05). Compared with the aminose group, the serum IL-1 levels in the traditional Chinese medicine group β, TNF-α, The iNOS content was significantly lower, and the difference was statistically significant (P<0.05) (see Table 3).

Table 3: Serum inflammatory factor IL-1β, TNF-α, iNOS levels (\(\bar{x} \pm s\))

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>IL-1β(pg/mL)</th>
<th>TNF-α(pg/mL)</th>
<th>iNOS(U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model Group</td>
<td>15</td>
<td>36.76±1.33</td>
<td>211.44±12.35</td>
<td>20.21±0.86</td>
</tr>
<tr>
<td>Traditional Chinese</td>
<td>15</td>
<td>21.98±1.41*</td>
<td>109.47±13.24*</td>
<td>6.91±0.76*</td>
</tr>
<tr>
<td>Aminoglycoside group</td>
<td>15</td>
<td>30.27±1.42#</td>
<td>162.33±11.26#</td>
<td>11.42±0.88#</td>
</tr>
</tbody>
</table>

F 241.873 131.831 104.574
P <0.05 <0.05 <0.05

Note: Compared with the model group, # * P<0.05, and compared with the ammonia group, * P<0.05.

3.4. RT-PCR detection of apoptosis related proteins Caspase-3 and Bax mRNA results

Compared with the model group, the expression of Caspase-3 and Bax mRNA in the traditional Chinese medicine group and ammonia sugar group was significantly reduced, and the difference was statistically significant (P<0.05); Compared with the aminose group, the expression of Caspase-3 and Bax mRNA in the traditional Chinese medicine group was significantly reduced, with a statistically significant difference (P<0.05) (see Table 4).

Table 4: Comparison of apoptosis related proteins Caspase-3 and Bax mRNA levels in cartilage tissues of each group (\(\bar{x} \pm s\))

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>Caspase-3(ng/mL)</th>
<th>Bax(ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model Group</td>
<td>15</td>
<td>3.29±0.22</td>
<td>4.60±0.31</td>
</tr>
<tr>
<td>Traditional Chinese</td>
<td>15</td>
<td>1.67±0.32*</td>
<td>2.39±0.12*</td>
</tr>
<tr>
<td>Aminoglycoside group</td>
<td>15</td>
<td>2.11±0.13#</td>
<td>3.29±0.27#</td>
</tr>
</tbody>
</table>

F 57.981 78.392
P <0.05 <0.05

Note: Compared with the model group, # * P<0.05, and compared with the ammonia group, * P<0.05.

4. Discussion

Traditional Chinese medicine categorizes KOA into the categories of "knee joint obstruction syndrome" and "bone joint obstruction" \[11\]. Traditional Chinese medicine believes that the formation of this disease is mainly due to the deficiency of liver and kidney caused by one year's old and weak body, the loss of nourishment in the muscles and bones of the whole body, and the long-term fatigue and recovery of wind, cold, and dampness pathogenic factors. The three factors rub and block the meridians, causing poor blood circulation and onset. The root cause of its pathological changes is liver and kidney deficiency, and the external cause is wind cold dampness. Therefore, in treatment, it is advisable to use the methods of tonifying the liver and tonifying the kidney, dispelling wind cold, and removing dampness and unblocking obstruction \[12,13\]. Bu Shen Huo Xue Tang was improved based on the experience of Guo Hanzhang's academic school studio. The formula consists of 12 herbs, including mulberry parasite, Codonopsis pilosula, Siegesia sieboldii, Angelica sinensis, Yanhusuo, hot dog spine, Huai Niu Xi, Danshen, Angelica sinensis, frankincense, myrrh, white peony, Chuanxiong, Eucommia ulmoides, etc. Its functions include tonifying the kidney and strengthening bones, promoting blood circulation and resolving blood stasis, and strengthening tendons and bones. In the formula, frankincense, myrrh, angelica sinensis, and salvia miltiorrhiza are used to promote blood circulation and blood stasis, reduce swelling and relieve pain. They are combined with white peony, Chuanxiong, Eucommia ulmoides, and Duhuo to dispel dampness and phlegm, eliminate obstruction and relieve pain, and treat its symptoms. Mulberry parasitism, Herba Siegesia, Rhizoma Corydalis, Rhizoma Cynomolgii, and Rhizoma Achyranthis are used to strengthen tendons and bones, and both specimens are taken into account \[6\]. With the development and utilization of traditional Chinese medicine in recent years, clinical trials have found that Angelica sinensis, Danshen, frankincense, and myrrh have significant anti-inflammatory and analgesic pharmacological effects; White peony and salvia miltiorrhiza can enhance humoral immunity and inhibit connective tissue proliferation, and have
immune regulatory effects. The combination of these drugs has a preventive and improving effect on the symptoms of arthritis. Modern clinical medicine believes that the essence of KOA's pathological changes is mainly due to the degradation of cartilage and various pathogenic factors that damage and erode cartilage, leading to bone hyperplasia of cartilage. Its pathogenesis is closely related to changes in joint cartilage. Clinical treatment often involves administering glucosamine hydrochloride or adding non steroidal anti-inflammatory drugs to repair cartilage, reduce pain, swelling and other symptoms, and improve joint mobility [14].

Previous experimental studies have found that inflammatory factor 1 in cartilage and synovium of the KOA model β (IL-1β), TNF-α, iNOS can bind to cartilage surface receptors, thereby activating signaling pathways such as JNK and NF-kB in chondrocytes [15]. Among them, the apoptosis response mediated by the JNK signaling pathway can be activated through the synovium and chondrocytes, promoting apoptosis signal transduction in the synovium and chondrocytes, further inhibiting chondrocyte differentiation. Inflammatory factor IL-1β can stimulate the generation of special MMPs and cause degradation of synovial and chondrocyte matrix. Stimulate the proliferation and degeneration of synovial cell tissues to form inflammatory mediators. It can promote the excessive secretion of metalloproteinases in synovial cells and disrupt the synthesis and spatial normal structure of extracellular matrix in chondrocytes [16-17]. Traditional Chinese medicine believes that the main pathogenesis of KOA is liver and kidney deficiency, blood stasis and dampness. In combination with modern medicine, it has been found that obstructed blood circulation can lead to an increase in thrombin and coagulation factor complexes in the blood, thereby stimulating the synthesis and release of inflammatory factors by the vascular endothelium [18-19]. The results of this experiment showed that the content of apoptotic protein Bax and Caspase-3 decreased after treatment with Bushen Huoxue Tang, indicating that Bushen Huoxue Tang treatment can inhibit cell apoptosis, and the inflammatory factor IL-1β, TNF-α, The iNOS level was significantly reduced, and the HE staining results combined with the Mankin score were lower, indicating that the treatment of Bushen Huoxue Tang effectively alleviated inflammatory infiltration and cell apoptosis [20].

5. Conclusion

Based on this experiment, we conclude that Bushen Huoxue Tang can better inhibit the production of inflammatory and apoptotic factors in the synovium and cartilage of rat KOA model compared to glucosamine hydrochloride, in order to alleviate inflammatory infiltration, control the damage of apoptotic factors to synovial chondrocytes, and alleviate inflammatory symptoms. Further clinical experiments are needed to observe the clinical effect.

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References