Original Article
The protective effect of Hedyotis diffusa on collagen induced arthritis rats

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Abstract: Objective: To investigate the protective effect of Hedyotis diffusa (HD) on collagen induced arthritis (CIA) rats. Methods: 40 male Wistar rats were randomly divided into four groups: normal group (blank control, n = 10), CIA model group, HD low dose group and HD high dose group. After CIA rats were prepared successfully, HD extract was administrated orally with the dose of 6 g/(kg. d) and 12 g/(kg. d) to low and high dose group at day 14; normal group was administrated with the same volume of saline. The arthritis index was evaluated weekly after treatment. Rats were sacrificed at day 42, their serum IL-1β, TNF-α levels and nitric oxide (NO) concentration in synovial tissues were measured and compared. The annexin I expression in each groups were measured. The active status of PI3K-AKT signal pathway was examined by western blot. Result: The arthritis index of HD low dose group was significantly lower than model group at day 35 and day 42 (P<0.05). The arthritis index of HD high dose group was significantly lower than model group at day 28, day 35 and day 42 (P<0.05). Histological analysis of joints showed that synovitis and osteoclastic bone resorption were attenuated by daily oral administration of HD. The expression of IL-1β and TNF-α in HD treatment groups were significantly lower than model group (P<0.05), as well as NO concentration in cartilage tissue. We observed significantly higher expression of annexin-I in HD treatment groups than model group (P<0.05). Conclusion: HD could reduce the expression of inflammatory cytokines IL-1β and TNF-α and alleviate the inflammatory reaction and bone erosion in CIA rats.

Keywords: Hedyotis diffusa (HD), collagen induced arthritis (CIA), rat, annexin-I, IL-1β, TNF-α

Introduction
Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease with feature of erosive synovitis [1, 2], studies indicated that the pathogenesis of RA is related with genetic, infection, immune, endocrine factors and so on [3-5]. Currently, there is no satisfactory and effective cure for RA, and there are 4 major types of RA therapeutic drugs for clinical use: non-steroidal anti-inflammatory drugs (NSAIDS); drugs which improve the anti-rheumatic disease-modifying (DMARDS); corticosteroids and biological agents (such as TNF) [6, 7].

The occurrence of rheumatoid arthritis (RA) is related with variety of signal transduction pathways [8, 9]. Recent studies have found that phosphate ester phthalate inositol-3 kinase phosphoinositide 3 kinase (PI3K) and protein kinase B protein kinase B (PI3K-Akt) signal pathway [10, 11] is involved in a very fast signal transduction system, this pathway participated from the membrane to the nuclear signal transduction system. And its downstream pathways, Bad-caspase and NF-κB are important molecular pathways effecting RA [12, 13]. Hsieh et al found that PI3K/AKT signaling pathway was activated in early osteoarthritis process.

Traditional Chinese medicine has a long history of treating RA, folk prescription such as Yiqi Jiedu Huayu Decoction and BiZhongxiao decoction etc have effectively relieved the pain of RA patients, and Hedyotis diffusa (HD) was commonly used in these folk prescription. Modern experimental studies indicated that the single herb prescription of HD has related anti-inflammatory, antioxidant and immune regulation effect in treating RA. To further explore the therapeutic mechanism of HD in RA, collagen induced arthritis model in Wistar rat was adopt-
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Ed in this study, HD was administrated orally to collagen induced arthritis (CIA) rats to observe the anti-rheumatic effect of HD in PI3K/AKT pathway.

Materials and methods

Animals

The 40 male Wistar rats, 8 week old, with body weight of 200 ± 10 g, were purchased from the experimental animal research center of Chinese Academy of Medical Sciences. All the experiment protocol follows “Experimental Animal Management Regulations” of China.

After adaptive feeding for 2 weeks, the 40 rats were randomly divided into 4 groups according to related treatment: normal group received distilled water (N = 10); CIA model group received distilled water after induction; HD low dose group and HD high dose group were administrated orally with the HD dose of 6 g/(kg. d) and 12 g/(kg. d) after induction.

Collagen induced arthritis rats’ model

The CIA rat model was established according to the instructions of Chondrex, Inc. (http://www.chondrex.com/protocol/Rat%20CIA.pdf). Briefly, 10 mg of bovine type II collagen (Chondrex, inc., Redmond, WA, USA) was dissolved in 5 ml of acetic acid solution (0.05 mol/L) with the concentration of 2 mg/ml. The prepared bovine type II collagen was diluted by complete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO, USA) to the concentration of 1 mg/ml. A total of 0.2 mL bovine type II collagen was injected subcutaneously to rat tail root; The same concentration bovine type II collagen (1 mg/ml) was diluted by incomplete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO, USA), and was injected again 7 days after first immunization. The CIA model was evaluated 2 weeks after first immunization, unsuccessful model animal was removed. The same dose of saline was injected in normal group.

Administration of medication

According to equivalent dose ratio of converted body surface area, Hedyotis diffusa tablet (approved serial number: Z36020632, Chinese herbal medicine company, Tianjin, China) administrated dose to a 70 kg adult was 30 g per day, the converted dose was 6 g/(kg. d) for rats. Finally, we adopted 6 g/(kg. d) and 12 g/(kg. d) as low and high HD dosage respectively.

14 days after first immunization, gastric administration was performed at 9:00 am in these rats with the medication or saline volume of 1 ml per day. The administration was continued to day 42.

Arthritis index observation

General observation including the mental state, hair, appetite activity, and the growth of body mass was observed and recorded every.

The arthritis index (AI) was evaluated 14 days after first immunization; A1 was evaluated as 5 grades: 0, no arthritis phenomenon; 1, red swelling occurred in the little toe joints; 2, swelling occurred in joint of toe or foot plantar; 3, swelling occurred in lower part of ankle joint; 4, swelling occurred at ankle joint or over part of ankle joint. The AI index was the final sum score of all joints.

Histological analysis

After completing the experiment, mice were sacrificed and paws were fixed in 10% neutral buffered formalin and decalcified with 10% EDTA, pH 7.4. The paws were then embedded in paraffin and cut into 5-μm thick sections. The deparaffinized sections were stained with hematoxylin and eosin and observed under light microscope (ZEISS Co., Germany).

Serum IL-1β and TNFα levels

After administrated for 42 days, rats were fasting for 12 hours and were sacrificed by decollation. Blood was draw and the supernatant was removed by centrifugation The IL-1β and TNF-α level were detected by ELISA kit (Huamei Bio Engine Co., Ltd., Wuhan, China).

Expression of annexin I

Synovial tissue was taken from sacrificed rats, western blot method was used to detect the expression of annexin I. Briefly: the synovial membrane protein was extract by a total protein extraction kit (BsP003, Bio Engine Co., Ltd., Shanghai, China), the sample volume of 50 μg was loaded after measured the concentration, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed.
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at concentration of 10%, the transferred to Polyvinylidene Difluoride (PVDF) membrane. Incubated with Rabbit anti-annexin-1 polyclonal antibody (ZYMED Laboratories Inc. (Cambridge, UK)) at 4°C overnight (t:500), then incubated with AlexaFluor 647 goat anti-rabbit IgG (1:5000, Abcam plc, Cambridge, MA, USA) for 1 h at room temperature. An infrared imaging system for imaging (Odyssey® CLx, LI-COR Biosciences, Lincoln, NE, USA) was used for semi quantitative analysis. Annexin I expression was calculated as gray value in each group.

Pathological examination

After been sacrificed, bilateral knee joints of the rats were selected as the specimens, including the entire posterior jaw with the ankle joint. Specimens were fixed with 10% poly form aldehyde solution and prepared as paraffin sections, then stained by hematoxylin and eosin (HE) staining. The pathological changes were observed under light microscope.

Nitric oxide colorimetric assay

NO is an important biological molecule in the pathological and physiological state, NO can induce the apoptosis of chondrocytes, and is closely related to the pathological process of rheumatoid arthritis [14]. Cartilage tissue was washed repeated in Hanks solution, then was digested by type I collagenase (0.25%), cut and digested at 37°C for an hour.

NO was assessed indirectly by measuring the levels of oxidized forms (nitrites and nitrates) in samples using the Nitric Oxide Colorimetric Assay Kit (ab65328; Abcam, Cambridge, MA, USA). A standard curve was generated to measure levels between 1 and 100 μM of nitrite per well. Within 2 weeks of freezing, tissues were homogenized fully, after lysis, protein was extracted by total protein extraction kit (BsP003, Bio Engine Co., Ltd., Shanghai, China); protein concentration was determined by BCA methods; denatured protein was separated in 10% SDS-PAGE, then protein was transferred to PVDF membrane, block with 5% skim milk for 1 hour. Anti-PI 3 Kinase p110 delta antibody (EPR386, abcam plc, Cambridge, MA, USA), Rabbit anti-AKT-polyclonal antibody and Rabbit anti p-AKT polyclonal antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) were added and cultured at 4°C overnight, after been washed by TBS buffer 3 times, the horseradish peroxidase labeled secondary antibody was added and cultured at 37°C for 2 hours. After rinsed by TBS, the Chemiluminescence method was adopted; related strip was developed and fixed by x ray. The absorbance values of strips were measured by scanning. Expression of PI3K, AKT and p-AKT was calculated as relatively quantitative to β-actin (Zhongshan Gold Bridge Bio Co., Ltd., Beijing, China).

Statistical analysis

The normal distribution data was expressed as mean ± SD, data was analyzed by SPSS 17.0 software (IBM, Chicago, IL, USA). Multiple average values were compared by single factor analysis of variance. P<0.05 was considered as statistically significant.

Results

After immunization, 24 of 30 rats was assessed having CIA, the 24 rats were randomly divided
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Table 1. Comparisons of the AI index (score) in each group

<table>
<thead>
<tr>
<th>Group (N)</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
<th>Day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (10)</td>
<td>5.53±1.09</td>
<td>5.71±0.99</td>
<td>5.84±0.95</td>
<td>5.48±1.05</td>
<td>5.66±1.10</td>
</tr>
<tr>
<td>Model (8)</td>
<td>7.59±1.22</td>
<td>10.42±1.16</td>
<td>10.09±1.12</td>
<td>9.52±1.17</td>
<td>7.33±1.23</td>
</tr>
<tr>
<td>HD low dose (8)</td>
<td>7.56±1.24</td>
<td>9.72±0.98</td>
<td>8.68±0.89</td>
<td>7.44±1.18*</td>
<td>6.15±1.21*</td>
</tr>
<tr>
<td>HD high dose (8)</td>
<td>7.27±1.09</td>
<td>9.13±1.24</td>
<td>7.78±1.15*</td>
<td>6.95±1.28*</td>
<td>5.78±1.04*</td>
</tr>
</tbody>
</table>

*, P<0.05, compared with model group at the same time point.

Table 2. Paw thickness (mm) comparison in HD high dose group with normal and model group

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
<th>Day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5.11±0.19</td>
<td>5.15±0.16</td>
<td>5.19±0.27</td>
<td>5.36±0.24</td>
<td>5.56±0.25</td>
<td>5.81±0.28</td>
<td>6.06±0.23</td>
</tr>
<tr>
<td>Model</td>
<td>5.26±0.37</td>
<td>5.61±0.94**</td>
<td>6.80±0.76**</td>
<td>7.56±0.52**</td>
<td>8.48±0.53**</td>
<td>8.71±0.67**</td>
<td>8.99±0.71**</td>
</tr>
<tr>
<td>HD high dose</td>
<td>5.24±0.40</td>
<td>5.63±0.94**</td>
<td>6.84±0.68**</td>
<td>7.64±0.54**</td>
<td>8.10±0.91**</td>
<td>7.90±0.81**</td>
<td>7.76±0.84**</td>
</tr>
</tbody>
</table>

**, P<0.01, compared with normal group at the same time point; ***, P<0.01, compared with model group at the same time point.

Figure 1. Oral administration of HD extract attenuated severity of arthritis in CIA rats. From left to right was HE staining of ankle joints from each group.

Table 3. Comparisons of serum IL-1β and TNF-α level (pg/ml) in each group

<table>
<thead>
<tr>
<th>Group (N)</th>
<th>IL-1β</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (10)</td>
<td>19.41±0.92</td>
<td>21.81±1.55</td>
</tr>
<tr>
<td>Model (8)</td>
<td>60.30±2.46*</td>
<td>75.21±1.79*</td>
</tr>
<tr>
<td>HD low dose (8)</td>
<td>38.53±3.36*</td>
<td>42.97±1.64*</td>
</tr>
<tr>
<td>HD high dose (8)</td>
<td>29.74±1.03*</td>
<td>33.15±1.19*</td>
</tr>
</tbody>
</table>

*, P<0.05, compared with normal group; *, P<0.05, compared with model group.

As we can see from Table 1, the AI index (score) in HD low dose was significantly lower than those in model group at day 35 and day 42 (P<0.05); the AI index in HD high dose group were significantly lower than those in model group at day 28, day 35 and day 42 (P<0.05). In addition, the paw thickness in HD high dose group was decrease significantly when compared with model group in same time point (Table 2). Histopathologic examination of some of the arthritic joints of rats from the model group revealed extensive cartilage and bone erosions with massive infiltration of mononuclear cells and fibroblasts. In comparison with model group, histological analysis of joints showed that synovitis and osteoclastic bone resorption were attenuated by daily oral administration of HD (Figure 1).

Serum IL-1β and TNF-α levels

The serum IL-1β and TNF-α levels at day 42 was listed in Table 3, serum IL-1β and TNF-α levels in model group and HD treated groups were
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Table 4. Comparisons of annexin I expression (gray value) in synovial tissue of each group

<table>
<thead>
<tr>
<th>Group (N)</th>
<th>Gray value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (10)</td>
<td>1.02±0.18</td>
</tr>
<tr>
<td>Model (8)</td>
<td>1.44±0.29</td>
</tr>
<tr>
<td>HD low dose (8)</td>
<td>0.95±0.26*</td>
</tr>
<tr>
<td>HD high dose (8)</td>
<td>0.77±0.13*</td>
</tr>
</tbody>
</table>

* P<0.05, compared with model group.

Annexin I expression

Annexin I expression in each group was evaluated as gray value (Table 4 and Figure 2), the gray value in model group was higher than normal group with statistical difference, while HD treatment decrease annexin I expression, the gray value in HD low and high dose group were all lower than model group (P<0.05), but have no difference with normal group (P>0.05).

NO level detection

Measurement result showed the concentration of NO in the cartilage tissue of CIA rats was significantly higher than that in the normal group; we observed the concentration of NO was decreased in HD treated group, especially in high dose group (P<0.05, compared with model group, Table 5).

Table 5. NO level comparisons in cartilage tissue of each groups

<table>
<thead>
<tr>
<th>Group (N)</th>
<th>NO (µmoll/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (10)</td>
<td>14.68±2.19*</td>
</tr>
<tr>
<td>Model (8)</td>
<td>19.03±2.56*</td>
</tr>
<tr>
<td>HD low dose (8)</td>
<td>17.88±2.26</td>
</tr>
<tr>
<td>HD high dose (8)</td>
<td>15.19±2.13*</td>
</tr>
</tbody>
</table>

* P<0.05, compared with model group.

Pathological changes

14 days after the first immunization, there was no obvious abnormality in the synovial tissues of the rats, we observed inflammatory cell infiltration in model group with the prolongation time of immunization, large pannus formation could be observed at days 28, with discontinuous epithelial cell; at day 42, vascular proliferation could been seen, with the aggravation of arthritic inflammation, angiogenesis increased, blood vessel dilated, lymphocyte infiltration could be seen with a large number. In HD treated groups, a few angiogenesis and inflammatory cell infiltration could be seen, with the extension of treatment time, the neovascularization reduced gradually, inflammation reduced.

Western blot analysis

PI3K-AKT signaling pathway is considered to play a role in the pathogenesis of RA. Results showed the expression of PI3K, AKT and p-AKT in model group were significantly higher than normal group. While HD treatment reduced expression of PI3K, AKT and p-AKT significantly (Figure 3), our results suggest that at least in part HD act on RA through this pathway.

Discussion

Hedyotis diffusa is one of main decoction in the many traditional Chinese medicines; HD is commonly used in treating various types of inflammation. Literature research has demonstrated the pharmacological activity, chemical constituents and the quality control of HD [15, 16]. Studies have demonstrated HD has anti-tumor, anti-inflammatory, antibacterial activity, and HD could regulate immune response [17].
Compounds in HD include flavonoids, anthraquinone quinones, terpenoids, sterols, polysaccharides and phenolic acids etc. Trenth's research demonstrated type II collagen could produce antigen effect and cause tissue damage, the established collagen induced arthritis (CIA) model is very similar to human RA [18]. Currently, this animal model is commonly used in RA research.

Although the pathogenesis of RA remains unclear, researches have showed IL-1β and TNF-α play critical contributor in RA initiation and progression [19]; as two of important biomarker, levels of IL-1β and TNF-α were often been tested to monitor the therapeutic effect. Previous researches indicated that the mechanism of HD treating RA mainly through regulating immune, lowering inflammatory factors levels, and inhibiting angiogenesis. Zhang et al [20] found HD could inhibit the proliferation of synovial cells in vitro in RA patients. Wang et al [21] indicated that HD could inhibit the tumor necrosis factor (TNF α) and interleukin 1β (IL-1β), thus decrease the inflammation symptoms caused by collagen induction. Gu et al [22] demonstrated that HD could decrease redundant reactive oxygen species (ROS) in cells after oxidative damage, and reduce the oxidative damage in tumor bearing mice.

In our research, the diet and activity of CIA rats were increased after been treated by HD at day 28, their hair was shiny and the arthritis index of lowered when compared with that of the model group. Significantly decreased paw thickness and serum IL-1β, TNF-α revealed HD could reduce the swelling of ankle joint and inflammatory cytokines in CIA rats. The related mechanism might involve in down-regulate IL 1β and TNF-α level, inhibit the adhesion molecules, inhibit vascular endothelial growth factor and other cytokines, promote collagen synthesis, reduce the expression and activity of matrix metalloproteinases, and to alleviate joint inflammation, bone erosion and reduce the role of pannus formation [23-26]. Consistent with Wang' research and others [21]. Our study also found that the expression of membrane linked I protein in HD treated groups are significantly higher than that in model group, HD could upregulate the membrane annexin I expression. The potential reason might be that as a regulator of anti-inflammatory reaction, HD treatment increases the generation of annexin I. Through directly occupying the surface receptors of neutrophils, inhibiting the secretion of interleukin and plasma tumor necrosis factor, and annexin I inhibit adhesion, exudation and infiltration of inflammatory cell [27-29].

AKT is a serine/threonine protein kinase that plays a key role in the process of cell proliferation and apoptosis. Human AKT gene family includes AKT, AKT2 and AKT3. AKT involved in survival pathway and inhibit apoptosis of cells. AKT2 plays important role in insulin receptor signaling pathway, and mainly act in the regulation of glucose transport [30], PI3K/AKT is an important pathway of inhibiting the apoptosis of cartilage [31]. PI3K, a membrane protein which can receive the incoming signal of the receptor of tyrosine kinase receptor on the membrane, and PI3K could directly or indirectly activate the downstream AKT [32]. AKT plays it’s anti apoptotic effect through the following 3 ways: inhibiting the programmed cell death triggered by Bax~Bcl-2~Bim [33]; Isolation the soluble cytoplasm of Bad-14-3-3 [34]; and plays anti-apoptotic effect through XIAP factor [35].

Studies show when the growth factor binding the receptor in cell membrane, which makes the phosphorylation of tyrosine residue, receptor tyrosine kinase tyrosine phosphorylation of R tyrosine kinase (RTK). At this time PI3K by PB5 subunit SH2 (SRC homology 2) interaction and phosphorylation of tyrosine residues of 3 R P110 subunit active and allosteric regulation activated by PI3K catalyzed R activation of D phosphorylation and PIP3 PIP2 who produced all two intracellular second messenger [14, 15]. DPKB is one of the most important the downstream target kinase PI3K signal transduction pathway in the kinase is a protein product of retroviral AKT8 gene V-AKT encoding the so called AKT. AKT by PIP2 and PIP3 mediated transfer to the cell membrane and exposed T-Loop region Thr308 site DPDK1 (3-phosphoinositide-dependent protein kinase > for lipid binding protein kinase, and PIP2 mediated by PIP3 guide and binding to the cell membrane, when the Ser473 site Thr308 site of Pkb/AKT was at the end of the phosphorylation of PDK1 and C regulatory region by PDK2 phosphorylation, Pkb/AKT is activated. To sum up, the AKT in the PI3K/AKT pathway plays a leading role in the core, PI3K only as a medium of signal transmission.
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In this paper, the activity of AKT in rat model of knee osteoarthritis was significantly lower than that in normal group, which indicate that PI3K/AKT signaling pathway was inhibited in rat RA model. PI3K/AKT in HD treated groups and normal group were higher than that in model group, this result showed HD could protect the cartilage cell function in RA rats, and could up-regulate the AKT signal transduction pathway.

In summary, HD can reduce the levels of inflammatory cytokines IL1β and TNF-α, and alleviate the inflammatory reaction in rats with collagen induced arthritis, and HD has protective effect on collagen induced arthritis in rats, the related mechanism might involve the up-regulate PI3K/AKT signal transduction pathway.

Disclosure of conflict of interest

None.

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