Effects of catalpa alcohol from Rehmannia glutinosa on calcium-binding protein, interleukin-1β and galectin-3 in synovial tissues of rats with knee osteoarthritis

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Abstract: We aimed to evaluate the effects of catalpa alcohol from Rehmannia glutinosa on the expressions of calcium-binding protein (S100A12), interleukin-1β (IL-1β) and galectin-3 in the synovium of rats with early knee osteoarthritis (KOA). Fifty-two adult male Wistar rats aged 3-8 weeks were divided into normal control (n=16), model (n=12), low-dose (n=12) and high-dose groups (n=12). On the 10th day after modeling, 6 rats in normal control group and 6 in other three groups were randomly selected. X-ray and three-dimensional CT images of left knee joint were taken under live anesthesia. The joint cavity of sacrificed rats was opened to observe cartilage surface. After 28 consecutive days of administration, the synovial tissue of left knee joint was collected. S100A12, IL-1β and galectin-3 levels in synovial tissue were detected by immunohistochemistry and ELISA. There were articular cartilage defects in left knees. Radiological examination showed significant joint space narrowing and hyperplasia, and 3D CT joint space value decreased (P < 0.05). The Mankins and OARSI scores of synovial histopathology were significantly different (P < 0.05). The S100A12, IL-1β and galectin-3 levels in synovial tissue of model group significantly exceeded those of normal control group (P < 0.01). Compared with model group, such levels of low-dose (P < 0.05) and high-dose groups (P < 0.01) were significantly lower. The S100A12, IL-1β and galectin-3 levels in
synovium tissue decreased with rising concentration of catalpa alcohol from *R. glutinosa*. Therefore, this drug is potentially suitable for inhibiting inflammatory response to delay the progression of KOA.
Effects of catalpa alcohol from *Rehmannia glutinosa* on calcium-binding protein, interleukin-1β and galectin-3 in synovial tissues of rats with knee osteoarthritis

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Abstract

We aimed to evaluate the effects of catalpa alcohol from *Rehmannia glutinosa* on the expressions of calcium-binding protein (S100A12), interleukin-1β (IL-1β) and galectin-3 in the synovium of rats with early knee osteoarthritis (KOA). Fifty-two adult male Wistar rats aged 3-8 weeks were divided into normal control (n=16), model (n=12), low-dose (n=12) and high-dose groups (n=12). On the 10th day after modeling, 6 rats in normal control group and 6 in other three groups were randomly selected. X-ray and three-dimensional CT images of left knee joint were taken under live anesthesia. The joint cavity of sacrificed rats was opened to observe cartilage surface. After 28 consecutive days of administration, the synovial tissue of left knee joint was collected. The S100A12, IL-1β and galectin-3 levels in synovial tissue were detected by immunohistochemistry and ELISA. There were articular cartilage defects in left knees. Radiological examination showed significant joint space narrowing and hyperplasia, and 3D CT joint space value decreased (P < 0.05). The Mankins and OARSI scores of synovial histopathology were significantly different (P < 0.05). The S100A12, IL-1β and galectin-3 levels in synovial tissue of model group significantly exceeded those of normal control group (P < 0.01). Compared with model group, such levels of low-dose (P < 0.05) and high-dose groups (P < 0.01) were significantly lower. The S100A12, IL-1β and galectin-3 levels in synovium tissue decreased with rising concentration of catalpa alcohol from *R. glutinosa*. Therefore, this drug is potentially suitable for inhibiting inflammatory response to delay the progression of KOA.

**Key words:** *Rehmannia glutinosa*; catalpa alcohol; immunohistochemistry; enzyme-linked immunosorbent assay; calcium-binding protein; interleukin-1β; galectin-3
Introduction

Knee osteoarthritis (KOA), as one of the most common forms of arthritis worldwide, is typified by progressive degeneration of articular cartilage, synovial hyperplasia and bone remodeling. In recent years, researchers have endeavored to clarify the pathogenesis of KOA. Synovitis has been associated with the severity of KOA. Interleukin-1β (IL-1β) is an inflammatory cytokine that is widely expressed upon KOA, as a key mediator of cartilage degradation. Proinflammatory factor galectin-3 is a member of the "chimeric" galactoside-binding protein family, which shows proinflammatory effects mostly by enhancing the activation of macrophages, mast cells, natural killer cells, as well as T and B lymphocytes. Calcium-binding protein (S100A12), a member of the S100 family, is a low molecular weight protein that plays a proinflammatory role by activating mast cells and participating in the metastasis of neutrophils to inflammatory sites. Besides, IL-1β can inhibit chondrocyte proliferation and promote the release of inflammatory cytokines, galectin-3 is important for IL-1β production, and S100A12 can cause progressive cartilage damage by degrading the extracellular matrix (ECM). All the three factors play crucial roles in the pathogenesis of KOA.

As an iridoid glycoside extracted from Rehmannia glutinosa, catalpa alcohol has a variety of pharmacological effects, including anti-apoptotic and anti-inflammatory properties. Until now, raw R. glutinosa has been widely used to treat inflammatory diseases, but systematic basic studies remain limited. In this study, the rat model of KOA was constructed by 4% papain combined with 0.03 mol/L cysteine solution, and the effects of different doses of catalpa alcohol from R. glutinosa on S100A12, IL-1β and galectin-3 expressions in the synovial tissues of rats with KOA were assessed, aiming to explore the underlying mechanism.

Materials and Methods
**Animals**

Fifty-two healthy adult male Wistar rats weighing (0.29 ± 0.1) kg were provided by Qinglongshan Animal Breeding Farm (Nanjing, China, License No. SCXK(Jiangsu)2017-0001). All animals were kept in a pathogen-free environment and fed ad lib. The procedures for care and use of animals were approved by the Ethics Committee of our hospital, and all applicable institutional and governmental regulations concerning the ethical use of animals were followed.

**Apparatus and reagents**

ELISA kits for S100A12 (batch No. E20181201A, item No. Ck-e30711r), galectin-3 (batch No. E20181201A, item No. Ck-e94644r) and IL-1β (batch No. E20181201A, item No. ck-e30419r) were used.

Immunohistochemistry was performed by using antibodies against galectin-3 (Proteintech, item No. 14979-i-ap, batch No. 10006432), IL-1β (Proteintech, item No. 66737-i-ig, batch No. 10006432) and S100A12 (Bioworld, item No. BS7539, batch No. XS20181105000).

Other reagents and apparatus included catalpa alcohol from *R. glutinosa* (Xi'an Anacreontic Technology Biological Co., Ltd.), 4% papain (Shanghai Hengyuan Biotechnology Co., Ltd.), hematoxylin (Wuxi Jiangyuan Industrial Technology and Trade Corporation, batch No. 160401100), horseradish peroxidase (Bioworld, item No. BS10950, batch No. CI33171), horseradish peroxidase color development kit (Beyotime Biotechnology Co., Ltd. (Nantong Branch), batch No. 033016160503), Axioplan 2 imaging optical microscope (Zeiss) and ELx800 microplate reader (Boteng Instrument).

**Modeling and success criteria**

According to a previous literature, 13 52 adult male Wistar rats aged 3-8 weeks were selected and divided into a normal control group (n=16), a model group (n=12), a low-dose group
(n=12) and a high-dose group (n=12). The latter three groups were injected with 4% papain and 0.03 mol/L cysteine (0.2 ml) after conventional shaving and disinfection of the left knee joint on days 1, 4 and 7 after the experiment began. The treatment method and injection site of the normal control group were identical, and the same amount of normal saline was injected into the joint cavity with a syringe. Six rats were randomly selected from the normal control group 10 days after modeling, and six rats were selected from the three groups (2 in each group) (Fig. 42). The rats were anesthetized by intraperitoneal injection of 7% chloral hydrate according to the body mass (5 mL/kg). After X-ray and three-dimensional CT examinations of the left knee joint, the rats were sacrificed. The articular cavity of the left knee was opened to observe the cartilage surface, and histopathological examination was conducted to verify the success of modeling.

**Administration method**

The recommended daily dose of adults was converted into the daily dose of experimental rats according to the formula of dose estimation in pharmacology research of traditional Chinese medicine: $dB = dA \times KB / KA$. The high- and low-dose groups were administered with 100 and 10 mg/kg catalpa alcohol respectively, and each rat was given 0.2 ml/kg of the drug through gavage. Normal control and model groups were given the same amount of normal saline by gavage. Intragastric administration was carried out once daily (8:00 am) for 28 consecutive days.

**Detection of indices**

After 28 days of continuous gavage, synovial tissue was collected from the left knee joint. S100A12, IL-1β and galectin-3 in the synovial membrane were detected by immunohistochemistry and ELISA strictly in accordance with the kits’ instructions.

(1) Immunohistochemistry:
After drug intervention, the rats were sacrificed under excessive anesthesia by injection with 7% chloral hydrate into the hearts. After being sacrificed, the rats were immersed in 10% strong disinfectant cold solution for 10 min, placed on an ultra-clean bench, fixed in the inverted position, and routinely sterilized in the surgical field. After the right knee joint cavity was opened on the medial side of the right hind limb, smooth and bright yellowish synovial tissue was found extending upward from the lower margin of the patella. The synovial tissue was completely stripped off and then cut off with a surgical blade. Soft tissues around the articular surface of the medial femoral condyle of the right hind limb were removed, and a 0.3 cm × 0.3 cm articular cartilage was chiseled according to the elasticity. A small amount of subchondral bone was added to the cartilage on the surface of each joint. 1) The removed synovial tissue was washed repeatedly with PBS and marked clearly in a disposable plastic embedding frame; 2) the plastic embedding frame was dehydrated in gradient concentrations of ethanol solutions for 2 h; 3) the tissue was transparentized for 2 h with 50% ethanol-xylene and xylene successively; 4) after immersion and embedding in paraffin, the tissue block was sectioned into 6 μm-thick; 5) the glass slide holder was put in a 60°C oven, baked for about 5 h and stored in a 4°C refrigerator.

The sections were routinely deparaffinized with xylene and gradient concentrations of solutions. Endogenous peroxidase in the sections was inactivated by 3% H₂O₂. The slide holder was placed in 0.01 mol/L citrate buffer (pH 6.0) at 95°C and incubated with 5% normal goat serum prepared by PBS at 37°C for 10 min, and then excess liquid was discarded. According to the instructions, primary and secondary antibodies were diluted with 5% BSA, and 150 μl of primary antibody was added. The sections were incubated at room temperature for 1 h, washed 3 times with PBS (5 min each time), incubated with 150 μl of secondary antibody at room temperature for 1 h and washed with PBS 3 times (5 min each time). The samples were stained brownish yellow under the microscope after 150 μl of DAB solution.
was added. When the degree of staining was appropriate, the sections were rinsed immediately with PBS for 10 min. After routine hematoxylin staining, the sections were dehydrated with gradient concentrations of ethanol solutions, transparentized with xylene, mounted with neutral resin and photographed under the light microscope (×400). The staining results of immunohistochemistry were analyzed by Image J software. Brown staining was determined positive, and IOD/area (average optical density) was evaluated.

(2) ELISA:

On the first day after drug administration, the synovial tissue was collected by using the same method. Soft tissues around the articular surface of the medial femoral condyle of the right hindlimb were removed, and 0.3 cm × 0.3 cm articular cartilage was chiseled according to the elasticity. A small amount of subchondral bone was added to the cartilage on the surface of each joint. The synovial tissue was separated, added 0.9% precooled normal saline according to the weight (g)/volume (ml) ratio of 1:9 and mechanically homogenized into a 10% homogenate. After centrifugation at 3000 rpm for 10 min, the supernatant was collected and stored in a -80°C refrigerator.

ELISA was performed strictly following the kits’ instructions. 1) S100A12, IL-1β and galectin-3 kits were prepared within 24 h before use. The required slats were taken out of the aluminum foil bag after 20 min of equilibrium at room temperature, and the remaining slats were sealed with a self-sealing bag and stored at 4°C; 2) standard wells were added 50 μL of standard at different concentrations, the sample well was added 10 μL of sample and 40 μL of diluent, and blank wells were not added; 3) except for the blank well, 100 μL of horseradish peroxidase-labeled detection antibody was added to the standard and sample wells, and the reaction well was sealed with sealing plate membrane and incubated at 37°C in a water bath or an incubator for 60 min; 4) after the liquid was discarded and the plate was pat-dried by absorbent paper, each well was filled with washing buffer and left still for 1 min, and the
plate was pat-dried by absorbent paper after the washing buffer was shaken off, which were repeated 5 times; 5) substrates A and B were added to each well, followed by incubation at 37°C in dark for 15 min; 6) 50 μl of stopping buffer was added to each well, and OD was measured at 450 nm with the microplate reader within 15 min. In the SPSS worksheet, a linear regression curve of standard was plotted by using the standard concentration as the abscissa and the corresponding OD as the ordinate, and sample concentrations were calculated according to the curve equation.

Statistical analysis

All data were statistically analyzed by SPSS 25.0 software and expressed as mean ± standard deviation (x̄ ± s). One-way ANOVA was used for pairwise comparison between groups. P < 0.05 indicated a statistically significant difference.

Results

General state of rats after articular cavity injection

After three injections, the model group had decreased appetite and activity, obvious claudication, significant swelling of the knee joint at the injection site, reluctance to land on the hind limbs, and evident weight loss. In contrast, appetite, activity or body weight barely changed in the normal control group, and limb movement disorders or lameness occasionally occurred. Slight swelling of the knee joint at the injection site disappeared 1-2 days after each injection.

Gross observation of articular cartilage of left knee

In the normal control group, the articular cartilage was grayish white with bright color, and the cartilage surface was smooth. There were no cracks or damages on the articular surface, subchondral bone exposure, or hyperplasia (Fig. 2A3A). The articular cartilage of the model group was pale yellowish white with dim color, and the cartilage surface was rough and exfoliated, also with subchondral bone exposure and hyperplasia (Fig. 2B3B). Compared
with the model group, the cartilage surfaces of low- and high-dose groups became smooth, and the articular cartilage color became brighter.

**X-ray examination results of left knee joint**

On the 10th day after modeling, photos were taken according to the methods mentioned above. Orthotopic X-ray revealed that the normal control group had normal knee joint space and smooth bone edge, without stenosis or osteophytes (Fig. 3A4A). In the model group, the knee joint space narrowed, and osteophytes formed at the bone margin (Fig. 3B4B). With the left leg extended, X-ray imaging showed that the joint space of the normal control group was significantly larger than that of the model group (Fig. 3C4C). Compared with the model group, the joint spaces of low- and high-dose groups markedly increased.

**Three-dimensional CT results of left knee joint**

The three-dimensional CT images of knee joints of the normal control group showed clear and normal joint space as well as smooth bone edge, without stenosis (Fig. 4A5A). As to the model group, the joint space narrowed significantly, and osteophytes formed clearly. The bone edges and articular cartilage surfaces were rough (Fig. 4B5B). Besides, the joint space values of normal control and model groups were significantly different (P < 0.05). Compared with the model group, the joints of low- and high-dose groups were significantly enlarged (P < 0.05) (Table 1).

**Histopathological observation results**

The Mankin and OARSI scores of the model group were significantly higher than those of the other three groups (P < 0.05), and the scores of low- and high-dose groups significantly exceeded those of the model group (P < 0.05) (Table 2).

**Levels of S100A12, IL-1β and galectin-3 in the synovium of left knee joint detected by immunohistochemistry**
The expressions of S100A12, IL-1β and galectin-3 in the synovium of left knee joint were detected by immunohistochemistry (Table 3). The levels of S100A12, IL-1β and galectin-3 in the synovial membrane of the model group significantly exceeded those of the normal control group (P < 0.01). Compared with the model group, such levels significantly decreased in administration groups, especially in the high-dose group (P < 0.01) (Fig. 56-78).

Levels of S100A12, IL-1β and galectin-3 in the synovium of left knee joint detected by ELISA

The expressions of S100A12, IL-1β, and galectin-3 in the synovial membrane of left knee joint were detected by ELISA (Table 2). The levels of S100A12, IL-1β and galectin-3 in the synovial membrane of the model group surpassed those of the normal control group (P < 0.01). Such levels of the low-dose group (P < 0.05) and high-dose group (P < 0.01) were significantly lower than those of the model group (Table 4 and Fig. 89).

Discussion

KOA is a whole joint disease characterized by progressive degeneration of articular cartilage, synovial hyperplasia and bone remodeling in various joint tissues. The risk factors for KOA include aging, acute or chronic mechanical stress, joint trauma and metabolic disease, which impair the homeostasis between ECM degradation and repair. Synovitis precedes the destruction of articular cartilage, as a predictor for the progression of KOA. In the case of KOA, chondrocytes and other tissues are activated, and homeostasis is altered by exposure to abnormal environmental damage. The release of proinflammatory factors from the synovium begins catabolic activation leading to the net degradation of ECM. KOA is now recognized as a complex syndrome that affects multiple tissues within the synovial joint and involves many sophisticated homeostatic pathways. S100A12, IL-1β and galectin-3 are important factors participating in the degradation and destruction of cartilage matrix in the progression of KOA.
As an inflammatory cytokine, IL-1β dominantly mediates cartilage degradation by inhibiting chondrocyte proliferation and promoting the release of inflammatory cytokines, which is considerably expressed upon KOA. Meanwhile, the proinflammatory cytokine environment can drive the increase of IL-1β level. Long et al. found that the IL-1β level of the arthritis group was significantly higher than that of the normal group, and a higher level suggested that the disease was more severe.

Galectin-3 is associated with the upstream regulation of NF-κB signal transduction in chondrocytes to promote human KOA. Additionally, it is a broad-spectrum upstream effector upon KOA. At the concentration as low as 1-10 μg/ml, galectin-3 functions as a proinflammatory cytokine and an inducer of matrix metalloproteinase expression. Salamanna et al. induced osteoarthritis in 30 male Sprague Dawley rats aged 12 weeks by destabilizing the medial meniscus for 4 weeks, and found that the galectin-3 level in synovial tissue significantly increased. Furthermore, galectin-3 can amplify IL-1β-mediated inflammatory response in cells. Lacobini et al. reported that the expression of IL-1β affected galectin-3, as well as accelerated the loss of trabecular bone reduction of bone strength and progression of KOA.

S100A12 is a low molecular weight protein in the S100 family. It is essentially involved in immune defense and inflammatory response, also regulating cell growth and differentiation while inhibiting apoptosis. Our group has previously reported that S100A12 secreted in the blood and extra-articular tissues of KOA patients promoted synovitis after entering the blood circulation and reaching periarticular tissues. Moreover, the severity of KOA was positively correlated with the expression level of S100A12. The patients with hip osteoarthritis have similar clinical results.

R. glutinosa was first recorded in "Shennong Bencaojing", ranking top of three herbal drugs. It tastes sweet, bitter and cold, being capable of nourishing Yin, tonifying the kidney
and facilitating the secretion of saliva. The rhizoma of *R. glutinosa* has been widely used to treat inflammatory diseases. Catalpa alcohol is an iridoid glycoside extracted from *R. glutinosa*, which was stipulated by The Chinese Pharmacopoeia (2015 Edition) as an index for the quality control of *R. glutinosa*. In this study, immunohistochemistry and ELISA were conducted to evaluate the effects of catalpa alcohol from *R. glutinosa* on the expression levels of IL-1β, galectin-3 and S100A12 in the synovial tissues of rats with early KOA. Such levels significantly increased in the synovial tissues of KOA rats, which were decreased by low- and high-dose catalpa alcohol (P < 0.05, P < 0.01), suggesting that this drug managed to control early KOA. Notably, the controlling effect of high-dose catalpa alcohol was superior to that of low-dose drug. In summary, catalpa alcohol from *R. glutinosa* can be used to slow down the progression of KOA by reducing the contents of inflammatory factors in synovial tissue and relieving inflammatory response. **This study still has limitations. The numbers of rats in low- and high-dose groups are small, so the results may be biased. Further in-depth studies using more rats are ongoing in our group to verify the results.**

**Acknowledgments**

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Figure Legends

**Fig. 1** Flow chart of experimental procedure.

**Fig. 1-2** Drug injection processes to the left knee articular cavity. A: Shaving and disinfection before injection; B: intraarticular drug injection into the left knee; C: after drug injection.

**Fig. 2-3** Gross observation of articular cartilage incision of left knee. A: Normal control group; B: model group.

**Fig. 3-4** X-ray images of extended left knee joint. A: Normal control group; B: model group; C: difference between normal control and model groups.

**Fig. 4-5** Three-dimensional CT images of left knee joint. A: Normal control group; B: model group.

**Fig. 5-6** S100A12 in synovial tissue observed by microscopy. A: Normal control group; B: model group; C: low-dose group; D: high-dose group.

**Fig. 6-7** IL-1β in synovial tissue observed by microscopy. A: Normal control group; B: model group; C: low-dose group; D: high-dose group.

**Fig. 7-8** Galectin-3 in synovial tissue observed by microscopy. A: Normal control group; B: model group; C: low-dose group; D: high-dose group.

**Fig. 8-9** IL-1β, galectin-3 and S100A12 levels in the synovium measured by ELISA.
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### Table 1 Joint space values of normal control and model groups

| Group            | n   | Joint space (mm) |
|------------------|-----|------------------|
| Normal control group | 6   | 0.68±0.016       |
| Model group      | 6   | 0.58±0.034       |
| Low-dose group   | 6   | 0.64±0.011       |
| High-dose group  | 6   | 0.67±0.012       |

Compared with normal control group, P < 0.05.
**Table 2** Mankin and OARSI scores of cartilage injury (x ± s, n=4)

| Group                | Mankin score | OARSI score |
|----------------------|--------------|-------------|
| Normal control group | 1.53±0.71    | 0.36±0.42   |
| Model group          | 8.23±2.12a   | 2.15±0.78a  |
| Low-dose group       | 4.45±0.72b   | 1.10±0.21b  |
| High-dose group      | 2.34±0.22b   | 0.45±0.18b  |

Compared with normal control group, \(^a\)P < 0.05; compared with model group, \(^b\)P < 0.05.
Table 3  IL-1β, galectin-3 and S100A12 levels in synovial tissues detected by immunohistochemistry (average OD)

| Group              | IL-1β    | Galectin-3 | S100A12   |
|--------------------|----------|------------|-----------|
| Normal control group | 8.50±0.84| 8.75±6.50  | 7.62±1.23 |
| Model group        | 33.93±1.63## | 52.94±2.24## | 30.6±1.34## |
| Low-dose group     | 28.84±0.56** | 26.62±1.70** | 26.99±2.89 |
| High-dose group    | 6.95±0.78** | 25.26±1.33** | 24.29±1.65** |

Comparison between model and normal control groups, and comparison between administration and model groups, *P < 0.05, **P < 0.01.
Table 4 Expression levels of IL-1β, galectin-3 and S100A12 in synovial tissues detected by ELISA (x̅ ± s, ng/ml)

| Group                  | n  | IL-1β      | Galectin-3 | S100A12  |
|------------------------|----|------------|------------|----------|
| Normal control group   | 10 | 896.42±190.92 | 1.69±0.33 | 7.61±1.54 |
| model group            | 10 | 3170.97±168.18 | 6.97±0.35 | 32.78±2.25 |
| low-dose group         | 10 | 2359.97±213.83 | 3.77±0.46 | 28.13±1.26 |
| high-dose group        | 10 | 1328.03±143.57 | 2.46±0.47 | 10.32±2.22 |

Comparison between model and normal control groups, P < 0.01; comparison between low-dose and model groups, P < 0.05; comparison between high-dose and model groups, P < 0.01.
Figure 1

Male Wistar rats

Normal control group

Normal rats

Model group

KOA rats

Low-dose group

High-dose group

KOA rats

X-ray and three-dimensional CT images of left knee joint were taken under live anesthesia

Detection of S100A12, IL-1β and galectin-3 levels in synovial tissue by immunohistochemistry and ELISA

Administration for 28 d
医学伦理委员会声明

我院与南京市第一医院合作申报的2017年度江苏省卫生和计划生育委员会项目，项目名称《地黄锌钙对膝骨性关节炎滑膜中S100A12、IL-1β、galectin-3作用研究》，关于使用研究对象大鼠滑膜组织内容，涉及医学伦理学范畴，我们严格按照医学相关规定对其进行医学伦理学审查，并在我单位伦理委员会监督下开展相关研究工作，确保研究内容符合伦理委员会相关规定。

特此声明。

南京市六合区人民医院医学伦理委员会
2017年5月25日