Hu Qian Wan Decoction Promotes Tendon-bone Healing of the Rotator Cuff in Rats by Inducing the Release of Exosomes Containing Interleukin-1 Receptor Antagonists

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Research

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Abstract

Background: Hu Qian Wan decoction (HQWD) is a traditional Chinese medicine used to treat many orthopedic diseases, including osteoporosis and osteoarthritis. This study aims to explore the mechanism by which HQWD promotes tendon-bone healing in the rotator cuff.

Methods: Tendon-bone healing of the rotator cuff, serum exosome expression, and changes in interleukin-1 receptor antagonist (IL-1RA) in exosomes were analyzed after intragastric administration of HQWD in the rotator cuff reconstruction model of rats. The effects of Hu Qian Wan (HQW) on exosomes, IL-1RA, and Nod-like receptor protein 3 (NLRP3) secretion were verified in rat bone marrow mesenchymal stem cells (BMSCs) and macrophage cells, and the effects of HQW on inflammasomes were observed.

Results: HQWD intragastric administration increased proteoglycan and collagen α (Col α) expression in the tendon-bone interface, improved the tendon-bone interface area, and augmented the maximum breaking load and stiffness of the rotator cuff. HQWD intragastric administration also increased rat exosome and IL-1RA expression in vivo. HQW promoted exosome and IL-1RA expression and suppressed the activation of NLRP3 inflammasomes in rat BMSCs and macrophage cells in vitro.

Conclusions: HQWD inhibited inflammasome-related inflammation by inducing exosome and IL-1RA secretion in rats, thus, promoting tendon-bone healing of the rotator cuff.

Background

Rotator cuff tears (RCTs) are the most common cause of shoulder pain and dysfunction [1]. RCTs often require surgical treatment to relieve pain and restore normal functioning of the rotator cuff [2]. Despite the advances in surgical techniques for rotator cuff repairs [3], the failure rate of reconstruction is 30–94% [4, 5] due to the mechanical stress and poor healing of the tendon-bone interface [6]. Promoting tendon-bone healing is crucial in treating a rotator cuff tear and preventing a re-tear after reconstruction. Tendon-bone healing is associated with many factors, including cytokines, blood supply, and the extracellular matrix [7–9]. Among these, cytokines are vital for the regulation of tendon-bone healing [10], but the regulation of cytokine release has not been well studied.

The exosomes of extracellular vesicles act as paracrine and autocrine regulators, conduct information transfer between cells, and regulate cytokines, immune responses, and tissue metabolism [11, 12]. Exosomes contain large amounts of protein, liposomes, RNA, and DNA and play a crucial role in diseases such as cancer, diabetes, and cardiovascular diseases[13]. However, the function of exosomes in tendon-bone healing is unclear. Mesenchymal stem cells (MSCs) secrete a variety of substances, including exosomes, that regulate the tissue microenvironment and promote tissue repair and reconstruction [14]. MSCs can secrete at least three types of exosomes that play an important role in intercellular signal transduction [15]. Therefore, MSCs can potentially regulate the tendon-bone healing of rotator cuffs [16]. However, studies on the mechanism of exosomes from MSCs in tendon-bone healing are deficient.

Among the factors affecting tendon-bone healing is osteoporosis (OP), a cause that cannot be ignored. Studies have shown that OP is an independent factor affecting tendon-bone healing in rotator cuff injuries, and a significant negative correlation exists between bone mineral density in patients and the success rate of arthroscopic surgical repair [17]. In the reconstructive animal model of rotator cuff injury, the intervention of sclerostin monoclonal antibody, which is used to treat OP, enhances tendon-bone healing in the animal model [18]. Therefore, we hypothesized that it is feasible to search for drugs among the osteoporosis medications to promote tendon-bone healing.

Traditional Chinese medicine is widely used in the treatment of OP, and the effect is remarkable [19]. HQWD is a classic traditional Chinese herbal formula composed of *Phellodendron chinense* Schneid, *Chinemys reevesii*, *Anemarrhena asphodeloides* Bge, *Rehmannia glutinosa* Libosch, *Citrus reticulata* Blanco, *Paeonia lactiflora* Pall, *Cynomorium songaricum* Rupr, *Canis familiaris* L, and *Zingiber ojjicinale* Rosc. For decades, HQWD has been widely used in the treatment of OP and osteoarthritis, and it has achieved favorable clinical efficacy [20]. However, the mechanism of HQWD is unclear. In addition, HQWD can promote the proliferation and differentiation of bone marrow mesenchymal stem cells (BMSCs) [21]. Therefore, exploring the mechanism and the potential clinical indications of HQWD is crucial. Based on previous studies, we speculated that there is a potential role between HQWD and exosomes, and that HQWD can regulate tendon-bone healing through exosomes. This study aimed to explore the changes in the expression of exosomes induced by HQWD and the mechanism of HQWD in promoting tendon-bone healing.

Methods

HQWD preparation

HQWD was provided by the Department of Pharmacy in the Affiliated Hospital of Nanjing University of Chinese Medicine. HQWD is composed of crude herbs, including *Phellodendron chinense* Schneid, *Chinemys reevesii*, *Anemarrhena asphodeloides* Bge, *Rehmannia glutinosa* Libosch, *Citrus reticulata* Blanco, *Paeonia lactiflora* Pall, *Cynomorium songaricum* Rupr, *Canis familiaris* L, and *Zingiber ojjicinale* Rosc (dosages shown in Table 1). The medicinal herb mixture was extracted in boiling water, and the aqueous extracts were vacuum-dried at 60 °C to obtain a powder. The powder was then stored at -20 °C. The content of crude herbs was determined and used for experiments by dissolution in pure water at desired concentrations (0.5 mg/ml). The fingerprint of HQWD was determined by high-performance liquid chromatography (Fig. S1, Supporting Information).

Animal experiments

All the experimental procedures were performed with the approval of the Experimental Research Institute of Nanjing University of Chinese Medicine and followed the guidelines of the Institutional Animal Care and Use Committee. A total of 72 rats (four-week-old Sprague-Dawley (SD) rats, male, 70–100 g, purchased from Beijing Charles River Company) were used for in vivo experiments. Rats were housed in an airconditioned room at 25°C with a 12 h light/dark cycle. Nine rats were used in BMSCs extraction and

63 rats were used in rotator cuff reconstruction model building. Rotator cuff reconstruction model included two groups: control group and HQWD group, and each group included 18 rats. Eighteen rats were used in tissue section analysis (Control group, 9; HQWD group, 9). Eighteen rats were used in biomechanical test analysis (Control group, 9; HQWD group, 9). Twenty-seven rats were used in interleukin-1 β (IL-1 β) and IL-1RA experiments (Control group, 9; IL-1 β group, 9; IL-1RA group, 9). The rats were sacrificed after anesthesia of isoflurane, and tissues were obtained.

RCT and reconstruction model building: After successful anesthesia with isoflurane, we incised the shoulder joint laterally and pulled the trapezius muscle away. The insertion of the supraspinatus to the humeral head was exposed. The supraspinatus insertion was resected. Approximately 2 mm of the distal tendon of the supraspinatus was cut off. Then, the skin was sutured. Four weeks later, a 3-0 nonabsorbable Propathene line was used to fix the supraspinatus tendon to the humeral head through bone tunnels. The subjects were randomly divided into two groups: equal amounts of either normal saline (NS) or HQWD. The intragastric administration of the treatments to the rats was performed after rotator cuff reconstruction.

The interleukin-1 β (IL-1 β) and IL-1RA drugs were administered intravenously into the rat tail at 1 and 10 $\mu\text{g}/\text{kg}$, respectively, once a day after rotator cuff reconstruction.

Extraction and culture of rat BMSCs

BMSCs inside their femurs and tibiae were obtained after anesthesia, according to previous literature [22]. Isolated BMSCs were incubated with standard media comprising DMEM (Gibco) supplemented with 10% FBS and 1% double-antibiotics (streptomycin + penicillin; Gibco). After 3–5 days of incubation (at 80% confluence), cells were re-plated in 10 cm Petri dishes and maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

Extraction of exosomes

Blood was collected from rat hearts after administering anesthesia, and the serum was extracted after centrifugation. Supercentrifugal extraction was adopted, and the cell debris was removed from the serum with 300 $\times$ g centrifugation for 10 min, followed by 29,500 $\times$ g centrifugation for 20 min. The supernatant was filtered through a 0.22 μm disposable sterile filter and centrifuged at 120,000 $\times$ g for 90 min. The resulting supernatant was discarded. The precipitate was dissolved in 100 μL of PBS and stored at –80 °C after packaging.

After BMSCs had grown to about 80% fusion, the culture medium was replaced by exosome-depleted FBS-containing medium (EXO-FBS-250A-1; System Biosciences, Mountain View, CA, USA). The cells were then cultured for a further 48 h. The medium was collected and centrifuged at 4 °C at 300 $\times$ g for 10 min and at 2000 $\times$ g for 10 min. Supercentrifugal extraction was adopted, and the cell debris was removed from the serum with 300 $\times$ g centrifugation for 10 min, followed by 29,500 $\times$ g centrifugation for 20 min. The supernatant was filtered through a 0.22 $\times$ μm disposable sterile filter and centrifuged at 120,000 $\times$ g

for 90 min. The resulting supernatant was discarded. The precipitate was dissolved in 100 μ L of PBS and stored at -80°C after packaging. The protein content of the BMSC exosomes was measured by the bicinchoninic acid assay (BCA; Thermo Fisher, Waltham, MA, USA). A microplate reader (ELx800, BioTek, USA) was used to measure the absorbance at a wavelength of 562 nm.

Grouping of drug and experimental animals

HQWD was provided by the Department of Pharmacy in the Affiliated Hospital of Nanjing University of Chinese Medicine. The drug composition was as follows: *Phellodendron chinense* Schneid, *Chinemys reevesii*, *Anemarrhena asphodeloides* Bge, *Rehmannia glutinosa* Libosch, *Citrus reticulata* Blanco, *Paeonia lactiflora* Pall, *Cynomorium songaricum* Rupr, *Canis familiaris* L, and *Zingiber ojjicinale* Rosc (Table 1). The water decoction of HQWD contained 0.5 mg/ml of crude drug. Rats were randomly assigned to the control group or the HQWD group after reconstructing the supraspinatus muscle. Normal saline was administered to the control group intragastrically. The HQWD group received HQWD decoction 5 g/kg/day intragastrically.

Immunohistochemistry

For the immunohistochemistry assays, the paraffin sections were de-waxed, dehydrated, and incubated overnight at 4°C with anti-Col α , anti-NLRP3, and anti-IL-1RA (diluted 1:100; Abcam). After the primary antibody was removed, the secondary antibody (diluted 1:100, Thermo Fisher) was added, and the sections were incubated for 1 h at room temperature. The stained cells were developed with diaminobenzidine and counterstained with hematoxylin.

Safranin and fast green staining

After the shoulder joint was fixed and decalcified, the shoulder was embedded in paraffin, sliced at 5 μ m in the coronal position, and hydrated after dewaxing. For fast green staining, the slices were placed in the fast green dye liquid for 5–10 min. Excess dye was washed with water until the cartilage became colorless. The slices were then slightly soaked in the differentiation fluid and then washed with tap water. For staining with safranin, the slices were placed in safranin dye liquid for 15–30 s and then quickly dehydrated with three cylinders of anhydrous ethanol. For the transparent seal, sections were cleared with xylene for 5 min and then sealed with a neutral gum seal. The cartilage stained red or orange with a green background.

Western blot analysis

Cells and tissues were placed on ice immediately following treatment and washed with ice-cold Hank's balanced salt solution. All the wash buffers and final resuspension buffer included a 1 $\times$ protease inhibitor cocktail (Pierce, Rockford, IL, USA), NaF (5 mM), and Na₃VO₄ (200 mM). The protein concentration of the lysate was measured using the BCA protein assay kit (Thermo Fischer). Nuclear or total cell proteins were resolved on 8% to 12% SDS-PAGE and transferred by electroblotting to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% bovine serum albumin reagent (Beyotime,

Jiangsu, China) and incubated overnight at 4 °C with primary antibody dilution buffer (the dilution followed the specification; Abcam) and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000) for 2 h. Afterward, the membranes were developed using the enhanced chemiluminescence substrate LumiGLO (Millipore, Bedford, MA, USA) and exposed to X-ray film. The bands were analyzed with Gel-Pro Analyzer 4.0 (Bio-Rad, Hercules, CA, USA).

Biomechanical test

Biomechanical tests were performed 4 weeks after the surgical reconstruction. Mutilation was performed at the lower end of the humerus. The supraspinatus and its insertion were carefully retained; the other muscles, except for the supraspinatus, were removed. The supraspinatus-humerus structure was obtained. The specimen was immediately placed in 4% paraformaldehyde solution, and the shoulder joint tissue was firmly fixed on an INSTRON biomechanical tester (Instron, Boston, MA, USA). The instrument was loaded, and a displacement velocity of 5 mm/min was applied to test the maximum tensile load of the supraspinatus tissue. The loading load when the supraspinatus tendon was broken was observed and recorded in detail as the maximum breaking load (N). The linear slope of the load-displacement curve was taken as the stiffness.

Enzyme-linked immunosorbent assay

IL-1RA was determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA set (Sagon Biotech, Shanghai, USA). ELISA was performed according to the manufacturer's instructions. All samples and standards were measured in duplicate.

Statistical analysis

SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. The measurements are presented as the mean $\pm$ standard deviation. The data were analyzed using a one-way analysis of variance followed by Bonferroni's post-hoc test for multiple comparisons ($p < 0.05$ was considered to indicate a statistically significant difference).

Results

HQWD improved the maximum breaking load and stiffness of the rotator cuff in rats

Four weeks after the supraspinatus reconstruction, the maximum breaking load and stiffness of the supraspinatus-humerus construction were examined (Fig. 1a). The breaking load and stiffness in the HQWD group were higher than in the control group (Fig. 1b, c), indicating that HQWD improved the tendon-bone healing of the rotator cuff in rats.

HQWD promoted the tendon-bone interface healing of the rotator cuff in rats

Based on the differences in breaking load and stiffness, we speculated that HQWD could promote tendon-bone healing of the rotator cuff in rats. The key to tendon-bone healing lies in the tendon-bone interface. The tendon-bone interface area and proteoglycans are crucial indicators of tendon-bone healing. Safranin/fast green staining showed that in the HQWD group, the tendon-bone interface area was larger, and the expression of proteoglycans was stronger than in the control group (Fig. 2a). These results were statistically significant (Fig. 2b). Therefore, HQWD promoted the growth and healing of the tendon-bone interface.

Tendons are composed mainly of collagen, 95% of which is Col α . Immunohistochemistry (IHC) showed that HQWD increased the expression level of Col α , confirming that the tendon-bone healing in the HQWD group was superior to that in the control group (Fig. 2c, d, e, and f). Western blot (WB) analysis was performed on the 3 mm tendinous portion of the distal supraspinatus and showed that HQWD promoted the expression of Col 1 (Fig. 2e, f). These results showed that HQWD promoted the tendon-bone healing of the rotator cuff in rats.

Effects of HQWD on the secretion of exosomes and IL-1RA in rats in vivo

Since exosomes have a strong regulatory effect on tissue metabolism, we then observed the effect of HQWD on exosomes in rats. Briefly, 48 h after the rats underwent intragastric administration of HQWD, serum exosomes were extracted and confirmed by electron microscopy and WB (Fig. 3a, b). The expression of the surface markers of exosomes, such as CD63, CD9, and CD81, increased, indicating that HQWD promoted the secretion of exosomes in rats in vivo (Fig. 3b, c). Based on the immune and metabolic regulation of exosomes on cells and the inhibitory effect of inflammation on tendon-bone healing, we speculated that exosomes carry proteins that regulate inflammatory factors. IL-1RA, which regulates IL-1, was expressed in the exosomes of the HQWD group. WB confirmed increased expression of IL-1RA in exosomes in the HQWD group compared with the control group (Fig. 3d, e). IL-1RA was also detected through IHC in the shoulder joint at 4 weeks after reconstruction. The expression of IL-1RA in the tendon-bone interface of the HQWD group was increased compared with that of the control group; the difference was statistically significant (Fig. 3f, g).

HQW induced BMSCs of rats to release exosomes carrying IL-1RA

Based on the close correlation between BMSCs and tendon-bone healing, we speculated that HQW would induce BMSCs to release exosomes. When HQW was added to the medium of BMSCs, IL-1RA and exosomes were increased in the culture supernatant of BMSCs (Fig. 4a, b and c), and IL-1RA in the exosomes was increased as determined by WB (Fig. 4d, e), confirming that HQW promoted BMSCs to secrete exosomes containing IL-1RA.

IL-1 β and IL-1RA regulated the tendon-bone healing of the rotator cuff in rats

HQWD increased the secretion of exosomes and IL-1RA and promoted tendon-bone healing in the rotator cuff in rats. To verify the effects of IL-1 β and IL-1RA on tendon-bone healing, we applied IL-1 β and IL-1RA

in rats after rotator cuff reconstruction. Consequently, IL-1 β reduced the rotator cuff maximum breaking load and stiffness, restrained the tendon-bone healing, and decreased the expression of Col I (Fig. 5a, b, c, and d). Conversely, IL-1RA improved the maximum breaking load and stiffness of the rotator cuff and promoted tendon-bone healing and the expression of Col I (Fig. 5a, b, c, and d).

HQW inhibited the activation of the NLRP3 inflammasome

The NLRP3 inflammasome consists of NLRP3, ASC, and caspase-1. IL-1RA inhibits the formation of the interleukin-1 receptor-induced NLRP3 inflammasome, and NLRP3 inflammasome activation promotes the release of IL-1 β . We speculated that HQW could inhibit the activation of the NLRP3 inflammasome, and thus, inhibit IL-1 β expression. It was found in macrophages that HQW could antagonize the expression of lipopolysaccharide (LPS)-induced NLRP3 inflammasome component proteins and downstream effector proteins (ASC, pro-caspase-1, caspase-1, pro-IL-1 β , and IL-1 β), which confirmed that HQW can inhibit the activation of the NLRP3 inflammasome (Fig. 6a, b). We observed the effect of HQWD on the expression of NLRP3 in the rotator cuff reconstruction site in rats and confirmed that HQWD inhibited the expression of NLRP3 (Fig. 6c, d).

Discussion

This study aimed to find a treatment for rotator cuff tears using the traditional Chinese medicines, and to explore the relationship and internal mechanism between HQWD and tendon-bone healing. Our study has confirmed that HQWD can induce the release of exosomes containing IL-1RA and inhibit the expression of NLRP3 inflammasomes in rats, thus, promoting tendon-bone healing of the rotator cuff. Meanwhile, HQW stimulated BMSCs to secrete exosomes carrying IL-1RA, and inhibited the activation of NLRP3 inflammasomes in macrophages. Notably, our work demonstrated that HQWD acted by activating IL-1RA to improve tendon-bone healing. As a result, tendon-bone interface regeneration and functional recovery in the rotator cuff were improved.

According to the reconstruction model of the rat rotator cuff, HQWD improved the supraspinatus maximum breaking load and stiffness, thereby promoting tendon-bone healing. Safranin/fast green staining verified the increased tendon-bone interface area and proteoglycan expression. Furthermore, HQWD promoted the growth and healing of the supraspinatus tendon. In addition, 95% of the tendon collagen was Col I, which is thick and closely spaced and is the main component of tendon resistance [23]. Therefore, we investigated the expression of Col I. IHC and WB verified the increased expression of Col I in the tendon-bone interface of the rotator cuff in the HQWD group. Thus, HQWD promoted tendon-bone healing.

Based on the activity of exosomes to regulate the immune and metabolic effects of various cells, we analyzed whether or not HQWD could promote tendon-bone healing through the release of exosomes in rats. Indeed, HQWD increased the level of exosomes in rats. Exosomes may carry substances that promote tendon-bone healing. IL-1 β is a crucial cause of tendon degeneration and tear, and it is an important negative regulator of tendon-bone healing [24]. IL-1 β also induces the generation of matrix

metalloproteinases (MMPs), the main cause for the degeneration in cartilage proteoglycan and tendon collagen [25]. Therefore, regulation of IL-1 β is crucial for tendon-bone healing. IL-1/IL-1R in organisms maintains a dynamic state of balance. IL-1RA can competitively bind to IL-1R, leading to the inactivation of IL-1R and the restrained effect of IL-1 β [26]. IL-1RA restrains the release of MMPs and reduces the degradation of proteoglycan and collagen, thereby retarding the destruction of bone and cartilage [27]. Therefore, improving the expression of IL-1RA could promote tendon-bone healing. Hence, we hypothesized that HQWD induced IL-1RA secretion and promoted tendon-bone healing of the rotator cuff. WB detection of rat exosomes verified that IL-1RA expression was increased in the HQWD group and was detected in the shoulder joint, thereby verifying that the expression of IL-1RA in the tendon-bone interface of the HQWD group was increased compared with that of the control group. Furthermore, the high expression of IL-1RA in the tendon-bone interface of the rotator cuff indicated that the area of the tendon-bone interface would be large and regulated.

BMSCs can differentiate into various types of cells, including tendon cells and bone cells, and they can promote tendon-bone healing of the rotator cuff [16, 28]. HQWD promotes the proliferation and differentiation of BMSCs (Wang et al.,2016).. Therefore, we hypothesized that exosomes in our research originated from BMSCs. Our study verified that HQWD promoted the secretion of exosomes and IL-1RA from BMSCs. In order to verify the role of IL-1RA in promoting tendon-bone healing, we applied IL-1 β and IL-1RA in rats and found that IL-1 β inhibited tendon-bone healing, while IL-1RA promoted tendon-bone healing. IL-1RA has been found to inhibit the activation of NLRP3 inflammasomes, a class of polymeric protein complexes that play an important regulatory role in inflammation and immune response [29]. Upon activation of NLRP3, a key component that mediates the formation of inflammasomes, the adaptor protein ASC is induced to bind pro-caspase-1 to form an inflammatory complex that activates caspase-1. ACS also shears and promotes the maturation and secretion of IL-1 β and IL-18, thus, promoting the inflammatory response [30]. In recent years, NLRP3 has been found to be involved in the regulation and inhibition of tendon-bone healing and bone injury repair [31, 32]. Therefore, we speculated that exosomes coated with IL-1RA could inhibit IL-1R/NLRP3-mediated inflammasome formation and promote tendon-bone healing by acting on macrophages. We verified the inhibitory effect of HQWD on the NLRP3 inflammasome in macrophages and at the tendon-bone interface of the rotator cuff after reconstruction in rats and confirmed that HQWD can inhibit the activation of the NLRP3 inflammasome.

To date, no effective prevention or treatment has been developed for re-tears after rotator cuff reconstruction. Studies on the treatment of rotator cuff tears by traditional Chinese medicine and on the role of exosomes and IL-1RA in this condition are limited. The present study is the first to reveal that HQWD promotes tendon-bone healing of the rotator cuff. This finding is of positive significance for treating rotator cuff tears with traditional Chinese medicine. We preliminarily discussed the mechanism of promoting tendon-bone healing of the rotator cuff by inducing exosomes and the secretion of IL-1RA in response to HQWD. We also confirmed for the first time that exosomes promote tendon-bone healing of the rotator cuff through IL-1RA, demonstrating the crucial role of exosomes in tendon-bone healing. A limitation of this study is that the mechanism by which HQWD induces the expression of exosomes and IL-1RA was not fully elucidated. In addition, exosomes are extremely active and possess various

functions, so whether the inhibition of NLRP3 inflammasome activation by IL-1RA is the most vital factor in promoting tendon-bone healing needs further study, as does the mechanism of tendon-bone healing induced by exosomes and IL-1RA. Nevertheless, the present study provides a new concept for exploring the effects of traditional Chinese medicine on exosomes and IL-1RA in tendon-bone healing of the rotator cuff.

Conclusions

By inducing the secretion of exosomes containing IL-1RA, HQWD inhibits NLRP3 inflammasome-induced inflammation and promotes tendon-bone healing of the rotator cuff.

Abbreviations

IL-1RA: Interleukin-1 receptor antagonist; NLRP3: Nod-like receptor protein 3; BMSCs: bone marrow mesenchymal stem cells; MSCs: Mesenchymal stem cells; Col I: collagen 1; IL-1: interleukin-1; RCTs: Rotator cuff tears; OP: osteoporosis; SD: Sprague-Dawley; FBS: fetal bovine serum; WB: Western Blotting; ELISA: enzyme-linked immunosorbent assay; IHC: Immunohistochemistry; LPS: lipopolysaccharide; MMPs: matrix metalloproteinases

Declarations

Authors' contributions

LNS and YH contributed to the conception and design of the study, collection and assembly of data, data analysis and interpretation, and manuscript writing. LW, BH, and BY performed animal experiments and were responsible for animal handling. LW, BH, HS, FCZ, and BY contributed to the experimentation. All authors read and approved the final version of the manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Table

Table 1

Contents of Hu-Qian-Wan

Chinese name	Botanical name	Common name	Weight (g)	Part used
Huang Bai	<i>Phellodendron chinense</i> Schneid.	Amur Cork-tree Bark	240	Bark
Gui Jia	<i>Chinemys reevesii</i> .	Tortose's Carapae and Plastron	120	Shell
Zhi Mu	<i>Anemarrhena asphodeloides</i> Bge.	Common Anemarrhena Rhizome	60	Rhizome
Shu Di Huang	<i>Rehmannia glutinosa</i> Libosch.	Radix Rehmanniae Preparata	60	Radix
Chen Pi	<i>Citrus reticulata</i> Blanco.	Tangerine Peel	60	Peel
Bai Shao	<i>Paeonia lactiflora</i> Pall.	White Paeony Root	60	Root
Suo Yang	<i>Cynomorium songaricum</i> Rupr.	Herba Cynomorii	45	Stem
Gou Gu	<i>Canis familiaris</i> L.	Dog's Bone	30	Bone
Gan Jiang	<i>Zingiber ojjicinale</i> Rosc.	Rhizoma Zingiberis	15	Rhizoma

Figures

Figure 1

Effects of HQWD on the breaking load and stiffness of the rotator cuff in rats. (A) Biomechanical test after reconstruction of the rotator cuff (supraspinatus). (B, C) Statistical analysis of the maximum breaking load and stiffness at 4 weeks after reconstruction.

Figure 2

Effects of HQWD on the tendon-bone interface in rats. (A) Expression of proteoglycan in the control group and HQWD group as detected by Safranin/fast green staining. (B) Statistical analysis of proteoglycan expression and tendon-bone interface area of the control group and HQWD group. (C) Expression of Col I in tendon as detected by IHC in the control group and HQWD group. (D) Statistical analysis of Col I expression of Figure C. (E) Expression levels of Col I in the tendon in the control group and HQWD group as detected by WB. (F) Statistical analysis of Col I expression of Figure E. (Original magnification $\times 50$)

Figure 3

Effects of HQWD on the secretion of exosomes and IL-1RA in rats. (A) Electron microscope pictures of exosomes. (B) WB detection of the expression of surface markers of serum exosomes after NS or HQWD intragastric administration for 48 h. (C) Statistical analysis of WB detection of the exosomes in the control group and HQWD group. (D) WB detection of IL-1RA expression in the exosomes of the control group and HQWD group. (E) Statistical analysis of the WB results of IL-1RA expression of Figure D. (F) IL-1RA expression as detected by IHC in the control group and HQWD group. (G) Statistical analysis of IL-1RA expression of Figure F. (Original magnification ×50)

Figure 4

Effects of HQWD on BMSCs in vitro. (A) IL-1RA in the culture supernatant of BMSCs was detected by ELISA. (B, C) Exosomes in the supernatant of BMSCs were detected by WB. (D, E) After HQW was added to the medium for 24 h, WB was used to detect the content of IL-1RA contained in the exosomes in the culture supernatant.

Figure 5

IL-1 β and IL-1RA regulated tendon-bone healing in the rotator cuff in rats. (A, B) Biomechanical test of the rotator cuff in the control, IL-1 β , and IL-1RA groups. (C) Safranin/fast green staining and IHC (Col I) of the rat shoulder in the control, IL-1 β , and IL-1RA groups. (D) Tendon Col I expression in the control, IL-1 β , and IL-1RA groups, as detected by WB. (Original magnification $\times 50$)

Figure 6

Effects of HQW on the NLRP3 inflammasome signaling pathway. (A, B) Effects of HQW on the expression of NLRP3 inflammasome signaling pathway related proteins expression according to WB analysis. (C, D) NLRP3 expression and statistical analysis in rat shoulders 4 weeks after reconstruction. (Original magnification $\times 85$)

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