

Original Article

Cartilage protective and anti-edema effects of JTF in osteoarthritis via inhibiting NCOA4-HMGB1-driven ferroptosis and aquaporin dysregulation

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ABSTRACT

Background: Preventing joint edema is crucial in halting osteoarthritis (OA) progression. Growing clinical evidence indicate that Jianpi-Tongluo Formula (JTF) may have a promising anti-edema effect. However, the therapeutic properties of JTF and the underlying mechanisms remains unclear.

Materials and methods: An OA rat model was established and employed to evaluate pharmacological effects of JTF in vivo based on dynamic histopathologic assessments and micro-CT observations. Then, OA-related genes and potential targets of JTF were identified through clinical transcriptomic data analysis and "disease gene-drug target" network analysis, which were verified by a series of in vivo experiments.

Results: JTF administration effectively reduced pain and joint edema, inhibited matrix degradation, chondrocyte apoptosis, and aquaporin expression in OA rats. Notably, JTF dose-dependently reversed damage-associated molecular patterns and inflammatory factor upregulation. Mechanically, our "disease gene-drug target" network analysis indicated that the NCOA4-HMGB1-GSK3B-AQPs axis, implicated in ferroptosis and aquaporin dysregulation, may be potentially served as a target of JTF against OA. Accordingly, JTF mitigated NCOA4, HMGB1, and GSK3B expression, oxidative stress, and iron metabolism aberrations in OA rats. Furthermore, JTF treatment significantly attenuated the aberrant upregulation of AQP1, AQP3, and AQP4 proteins observed in cartilage tissues of OA rats.

Conclusion: Our data reveal for the first time that JTF may exert cartilage protective and anti-edema effects in osteoarthritis therapy by inhibiting NCOA4-HMGB1-driven ferroptosis and aquaporin dysregulation.

Introduction

Osteoarthritis (OA) is a prevalent chronic joint condition distinguished by the degradation and inflammation of articular cartilage (Martel-Pelletier et al., 2016). Edema, characterized by tissue fluid accumulation, is a common clinical manifestation of OA, leading to pain,

impaired knee function, and reduced muscle activity (Sari et al., 2019). Persistent edema often impedes recovery and contributes to complications such as pain and stiffness (Villico, 2012). Epidemiological data from 2020 indicate that OA affected 595 million individuals globally, marking a 132.2 % increase since 1990. Projections suggest that OA prevalence will soar to 74.9 % by 2050 (Hawker and King, 2022). While

Abbreviations: 4-HNE, 4-hydroxynonenal; ADAMTS5, ADAM metalloproteinase with thrombospondin type 1 motif 5; AQP1, aquaporin 1; AQP3, aquaporin 3; AQP4, aquaporin 4; BMD, bone mineral density; BS, bone surface; BV, bone volume; CXB, celecoxib; DAMP, damage-associated molecular patterns; ELISA, enzyme-linked immunosorbent assay; GSEA, gene set enrichment analysis; GSH, glutathione; GSK3 β , glycogen synthase kinase 3 beta; GSSG, oxidized glutathione; H&E, hematoxylin and eosin; H₂O₂, hydrogen peroxide; HMGB1, high mobility group box 1; HO-1, heme oxygenase 1; IHC, immunohistochemical staining; IL-1 β , interleukin 1 beta; IL-6, interleukin 6; JTF, Jianpi-Tongluo Formula; MDA, malonaldehyde; MMP13, matrix metalloproteinase 13; MMP3, matrix metalloproteinase 3; NCOA4, nuclear receptor coactivator 4; NFE2L2, NFE2 like BZIP transcription factor 2; OA, osteoarthritis; PGE2, prostaglandin E receptor 2; qPCR, quantitative real-time PCR; ROS, oxygen species; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TCM, traditional Chinese medicine; TF, transferrin; TFR, transferrin receptor; TNF- α , tumor necrosis factor; TV, tissue volume.

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various pharmacological interventions including non-steroidal anti-inflammatory drugs, opioids, and hormone therapies aim to alleviate swelling and pain in OA, they may also induce adverse effects like gastrointestinal bleeding, cardiovascular issues, osteoporosis, and immunosuppression (Grennan and Wang, 2019). Consequently, the quest for more effective and safer therapeutics remains a critical pursuit.

Traditional Chinese medicine (TCM) offers distinct advantages in managing orthopedic conditions, notably in treating OA (Li et al., 2023). The Jianpi-Tongluo formula (JTF) is a TCM herbal prescription, including *Poria* (Fuling), *Cinnamomi ramulus* (Guizhi), *Atractylodis Rhizoma* (Cangzhu), *Phellodendri Chinensis Cortex* (Huangbo), *Stephaniae Tetrandrae Radix* (Fangji), *Polyporus* (Zhuling), *Drynariae Rhizoma* (Gusuibu), *Corydalis Rhizoma* (Yanhusuo), and *Cyathulae Radix* (Chuanniuxi). Clinically, JTF has shown efficacy in treating early-stage OA characterized by significant pain and swelling. However, the precise therapeutic properties and mechanisms of JTF remain unclear, which may hinder its rational clinical application.

To address this issue, we established an OA rat model to evaluate the cartilage protective and anti-edema effects of JTF through dynamic histopathological and micro-CT observations in vivo. After that, we identified OA-related genes and potential targets of JTF using clinical transcriptomic data, followed by the construction of their interaction network. Candidate targets of JTF against OA were subsequently screened and validated through a series of experiments.

Materials and methods

Preparation of JTF

JTF was prepared by Anguo Changda Co., Ltd. (Hebei, China) according to the methods provided in our previous study (Li et al., 2022) (Supplementary Section 1). The acquired JTF was stored at -20°C for further processing.

Chemical profiling of JTF

Ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-Q-TOF-MS) was used to identify chemical compounds containing JTF. Detailed information on the instrument parameters and the gradient elution program are provided in Supplementary Section 2. Raw data was processed using Waters Masslynx v4.1 software.

Animals

Male Sprague-Dawley rats (180 ± 20 g, $n = 60$) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (SCXK 2021-0006, Beijing, China). The rats were housed under controlled conditions in a pathogen-free environment at a constant temperature of 25°C , with ad libitum access to standard rodent chow and water. All experimental procedures were approved by the Experimental Animal Ethics Committee of the Institute of Chinese Materia Medica (Ethics Approval Number: 2021B029, Approval Date: 2021-03-01).

Grouping, OA modeling and treatment

All rats were randomly allocated to six groups: (1) the sham surgery group ($n = 10$), (2) the OA model group ($n = 10$), (3) the JTF low-dose (L-JTF, 3.47 g/kg, $0.5 \times$ clinical equivalent dose, $n = 10$) group, (4) the JTF medium-dose (M-JTF, 6.94 g/kg, clinical equivalent dose, $n = 10$) group, (5) the JTF high-dose (H-JTF, 13.88 g/kg, $2 \times$ clinical equivalent dose, $n = 10$) group, and (6) the celecoxib (CXB, 21.00 g/kg, clinical equivalent dose, $n = 10$) group.

OA model was established based on the enzyme papain (Jia et al., 2022) (Supplementary Section 3). Administration of all treatments via the intragastric route commenced the day after modeling and continued

for a duration of 4 weeks. On the 28nd day post-modeling, all rats were anesthetized via intraperitoneal injection of pentobarbital. Blood samples were collected and subjected to centrifugation to obtain serum samples, which were subsequently stored at -20°C for further analysis. The joints were surgically excised and then fixed in 4 % para-formaldehyde solution in phosphate-buffered saline for 48 h to prepare for subsequent experiments.

Assessment of arthritis severity

Joint edema was assessed based on the degree of joint swelling while pain thresholds were evaluated by inducing hyperalgesia using mechanical stimuli and acetone (Li et al., 2022). Additionally, the disparity in weight bearing between the hindlimbs was measured as previously described. Histopathological analyses, including hematoxylin and eosin (H&E) staining, Masson trichrome staining, safranin O-fast green staining (Solarbio, Beijing, China), and terminal dUTP nick-end labeling (TUNEL) staining (Thermo Scientific, USA), were employed to assess the pathological characteristics of cartilage tissues (Wang et al., 2022). To quantitatively assess defects of the subchondral bone, the specimens underwent scanning using a micro-CT apparatus (GE Healthcare, USA). Furthermore, to evaluate the inflammatory status in OA rats, the concentrations of interleukin 1 beta (IL-1 β), tumor necrosis factor (TNF- α), prostaglandin E receptor 2 (PGE2), and interleukin 6 (IL-6) were quantified in the serum using enzyme-linked immunosorbent assay (ELISA) (mlbio, Shanghai, China).

Target prediction of JTF and OA

Putative targets of JTF were collected from the ETCM v2.0 (www.tcmip.cn/ETCM2/front/#/) (Zhang et al., 2023b), and analyzed using the TCMIP v2.0 database (www.tcmip.cn/TCMIP/index.php/Home).

The primary symptoms associated with OA, including encompassing pain or discomfort, stiffness, reduced range of motion or flexibility, swelling, tenderness, the presence of bone spurs, bony expansion, and the absence of detectable warmth, were gathered from the HPO database (hpo.jax.org/app/).

Network construction and analysis

The JTF-OA network was constructed using the STRING (string-db.org). Topological characteristics of network node genes were computed utilizing cytoHubba, identifying hub genes with higher MCC, degree, closeness, and betweenness values than respective median values. Subsequently, ClueGO utilized to perform enrichment analysis based on the KEGG (www.kegg.jp). Biological function modules associated with pathways were categorized using KEGG Pathway Identifiers to elucidate the pharmacological mechanisms of JTF against OA.

Clinical transcriptomics data analysis

Clinical transcriptome microarray data based on cartilage tissues of OA patients were retrieved from GEO (www.ncbi.nlm.nih.gov/geo). Expression matrices was analyzed using the limma R package (v3.18). Gene set enrichment analysis (GSEA) was performed using the enrichplot (v1.22.0) and clusterProfiler (v4.10.0) R packages. Both co-expression correlation analysis and subsequent visualization were facilitated by the corplot and ggplot2 R packages (v3.4.4).

Fe²⁺, TF, ferritin, MDA, 4-HNE assay

Fe²⁺ levels, transferrin (TF) and ferritin in OA rats' cartilage of different groups was measured using the Fe²⁺ assay kit and ELISA, respectively. Serum concentrations of 4-hydroxynonenal (4-HNE), Malonaldehyde (MDA), glutathione (GSH)/oxidized glutathione (GSSG), and heme oxygenase 1 (HO-1) were determined by ELISA. All

measurements were conducted following the manufacturers' protocols (mlbio, Shanghai, China).

Quantitative real-time PCR (qPCR)

The mRNA levels of Collagen II, aggrecan, and nuclear receptor coactivator 4 (NCOA4) expression in the cartilage tissues of different groups were determined using qPCR. Standard procedures for RNA isolation, reverse transcription, and PCR amplification were conducted following our previous descriptions (Liu et al., 2022). The primer sequence is shown in Supplementary Table S1.

Immunohistochemical staining (IHC)

To assess how JTF influences the localization and expression levels of candidate targets, including matrix metalloproteinase 3 (MMP3), matrix metalloproteinase 13 (MMP13), NFE2 like BZIP transcription factor 2 (NFE2L2), transferrin receptor (TRFC), NCOA4, high mobility group box 1 (HMGB1), ADAM metalloproteinase with thrombospondin type 1 motif 5 (ADAMTS5), aquaporin 1 (AQP1), aquaporin 3 (AQP3), and aquaporin 4 (AQP4) in the cartilage tissues, IHC was performed according to the standard procedures using kit (Boster., Wuhan, China). IHC scores were evaluated according to our previous studies (Mao et al., 2022). Supplementary Table S2 provides the detailed information of antibodies using for IHC detection.

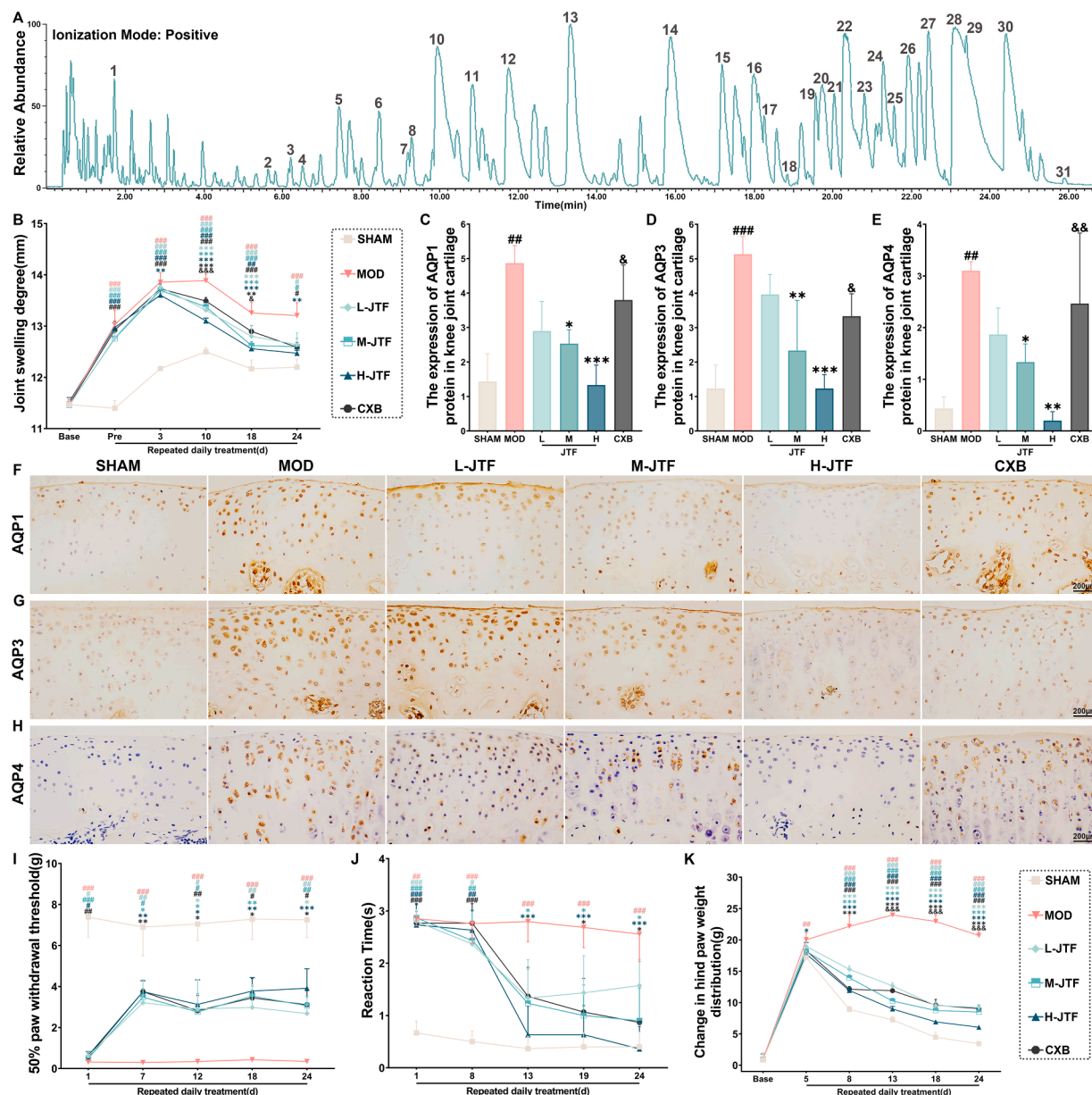


Fig. 1. JTF administration effectively reduces joint edema and increases pain thresholds in OA rats. (A) Representative LC-MS chromatograms of JTF (positive mode). (B) Joint swelling degree of different groups ($n = 10$ per group). (C-E) The expression of AQP1, AQP3, and AQP4 proteins in knee joint cartilage tissues of different groups ($n = 3$ per group). (F-H) Representative IHC images of AQP1, AQP3, and AQP4 protein expression in knee joint cartilage tissues of different groups. (I) The pain thresholds of mechanical-induced hyperalgesia in different groups ($n = 10$ per group). (J) The pain thresholds of acetone-induced hyperalgesia in different groups ($n = 10$ per group). (K) The weight distribution between the left and right hindlimbs of different groups ($n = 10$ per group). Data are expressed as mean $\pm$ SD (post-hoc Tukey test and Dunn's test). ## $p < 0.01$, ### $p < 0.001$ comparison with the sham group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, comparison with the model group, & $p < 0.05$, && $p < 0.01$, comparison with the H-JTF group.

Western blot analyses

Expression levels of glycogen synthase kinase 3 beta (GSK3β) and Phospho-GSK3β-S9 proteins in the cartilage tissues of rats were detected by western blot analysis according to our previous descriptions (Ma et al., 2023). Supplementary Table S3 provides detailed information regarding the antibodies utilized for Western blot analysis.

Statistical analyses

Data analysis was conducted using GraphPad Prism (v8.0.2). Parametric or non-parametric statistical analyses were employed based on the normal distribution testing results. Genes' co-expression correlation analysis utilized the Pearson correlation coefficient. All experimental data underwent analysis using a one-way ANOVA with a post-hoc Tukey test (or a Kruskal-Wallis test with a post-hoc Dunn's test).

Results

JTF administration effectively reduces joint edema and increases pain thresholds of OA rats

Chemical profiling of JTF was identified using UPLC-Q-TOF-MS, generating the base peak intensity chromatogram (Fig. 1A). Following the chemical analysis by Masslynx v4.1, a total of 31 chemical components were identified to be contained in JTF, such as berberine, tetrahydropalmatine, fordianoside, fangchinoline (Table 1).

In addition, JTF administration significantly reduced joint swelling

(Model vs. Sham, all $p < 0.001$, H-JTF vs. Model, $p < 0.01$, Fig. 1B) and the elevated levels of AQP1, AQP3, and AQP4 in the cartilage tissues of OA rats (Model vs. Sham, all $p < 0.01$, H-JTF vs. Model, all $p < 0.01$, Fig. 1C~H). Moreover, it elevated pain thresholds for mechanical and cold pain induced by acetone (Model vs. Sham, all $p < 0.01$, H-JTF vs. Model, all $p < 0.05$, Fig. 1I~J), and reduced weight-bearing asymmetry between the left and right hindlimbs of OA rats (Model vs. Sham, all $p < 0.01$, H-JTF vs. Model, $p < 0.01$, Fig. 1K). These effects were consistently observed across all JTF dose groups, showing a discernible dose-effect relationship. Notably, the reduced effects of CXB administration in joint swelling and AQPs expression were weaker than that of JTF administration (H-JTF vs. CXB, all $p < 0.05$, Fig. 1B~E).

Cartilage protective effects of JTF administration in OA rats

To assess the cartilage protective effects of JTF administration *in vivo*, cartilage tissues collected after 28 days of JTF treatment underwent H&E staining, safranin O fast, Masson trichrome staining, and TUNEL staining. Results revealed that JTF administration markedly reversed pathological alterations in cartilage tissues, including partial or complete loss of cartilage, disrupted chondrocyte arrangement, and near absence of the tidemark (Model vs. Sham, $p < 0.001$, JTF-H vs. Model, $p < 0.01$, Fig. 2A, E). Accordingly, a significant increase in the Masson, TUNEL staining positive area and a substantial reduction in the safranin O positive area within the articular cartilage of OA rats (Model vs. Sham, all $p < 0.01$, JTF-H vs. Model, all $p < 0.01$, Fig. 2B~D, F~H), which displayed the breakdown of the extracellular matrix, the deterioration of the cartilage structure, and the apoptosis of chondrocytes. Conversely,

Table 1
Qualitative analysis of chemical components in JTF.

ID	T _R / min	Identification	Molecular Formula	$m/z[M + H]^+$	MS ²
1	1.72	Candicine	C ₁₁ H ₁₇ NO	180.1408	121.0643, 103.0542, 93.0702
2	5.63	Scoulerine	C ₁₉ H ₂₁ NO ₄	328.1519	178.0867, 163.0610
3	6.21	Coclaurine	C ₂₃ H ₂₉ NO ₈	448.1975	286.1445, 269.1190
4	6.55	Atractyloside A	C ₂₁ H ₃₆ O ₁₀	471.2234	291.1563, 203.0512
5	7.43	3,4-Dihydro-1-[(4-Hydroxyphenyl)Methyl]-7-Methoxy-2-Methyl-6-Isoquinolinol	C ₁₈ H ₁₉ NO ₃	298.1474	283.1216, 254.1175, 190.0876, 177.0781, 107.0481
6	8.45	Fordianoside	C ₂₄ H ₃₂ NO ₈ ⁺	462.2117	254.0950, 237.0897, 175.0742
7	9.19	Phellodendrine	C ₂₀ H ₂₄ NO ₄ ⁺	342.1681	192.1022, 177.0781, 148.0757
8	9.28	Lotusine	C ₂₅ H ₃₃ NO ₈	476.2266	314.1729, 269.1190, 237.0897, 175.0742, 107.0481
9	9.93	2,10-Dimethoxy-7-Methyl-6,8,13,13a-Tetrahydro-5H-Isoquinolino[2,1-B]Isoquinolin-7-Ium-3,9-Diol	C ₂₀ H ₂₄ NO ₄ ⁺	342.1681	192.1022, 177.0781, 148.0757
10	10.55	Coumarin	C ₉ H ₆ O ₂	307.1742	119.04850, 91.05348
11	10.83	N-Methylhigenamine-7-O-Glucopyranoside	C ₂₃ H ₂₉ NO ₈	448.1975	286.1445, 255.1004, 178.0867, 107.0481
12	11.75	Magnoflorine	C ₂₀ H ₂₄ NO ₄ ⁺	342.1746	191.0838, 179.0840
13	13.33	Oblongine	C ₁₉ H ₂₄ NO ₃ ⁺	314.1792	269.1190, 209.0949, 143.0474, 107.0481
14	15.88	D-Lirioferine	C ₂₀ H ₂₃ NO ₄	356.1849	192.1022, 176.0724, 148.0757
15	17.19	Tetrahydrocolumbamine	C ₂₀ H ₂₃ NO ₄	342.1746	178.0867, 163.0610, 135.0697
16	18.11	Tetrahydropalmatine	C ₂₁ H ₂₅ NO ₄	356.1916	192.1022, 165.0834
17	18.27	N-Methyltetrahydrojatrorrhizine	C ₂₁ H ₂₆ NO ₄ ⁺	356.1849	192.1022, 177.0781
18	18.58	8-Oxyberberine	C ₂₀ H ₁₈ NO ₅ ⁺	352.1174	337.0949, 308.0911, 294.0768, 292.0941
19	19.58	Fangchinoline	C ₃₇ H ₄₀ N ₂ O ₆	609.3055	578.2513
20	19.74	Stylopine	C ₁₉ H ₁₇ NO ₄	324.1214	176.0724, 149.0583
21	19.99	Isoboldine	C ₁₉ H ₂₁ NO ₄	328.1902	283.1334
22	20.32	Glaucine	C ₂₁ H ₂₅ NO ₄	356.1916	325.1426, 279.1023
23	20.82	Columbamine	C ₂₀ H ₂₀ NO ₄	338.1428	323.1145
24	21.08	Tetrahydroberberine	C ₂₀ H ₂₁ NO ₄	340.1524	176.0724, 161.0490, 149.0583
25	21.60	Taxilamine	C ₂₀ H ₁₉ NO ₆	370.1648	190.0876
26	21.93	Corydaline	C ₂₂ H ₂₇ NO ₄	370.2054	192.1022, 177.0781
27	22.46	Dehydrocorybulbine	C ₂₁ H ₂₂ NO ₄	352.1571	337.1273, 321.0092
28	23.13	Berberine	C ₂₀ H ₁₈ NO ₄ ⁺	336.1262	320.0907, 306.1139, 292.1001, 278.0799
29	23.40	13-Methyldehydrocorydalmine	C ₂₁ H ₂₂ NO ₄	352.1571	337.1273, 321.0992
30	24.42	13-Methylberberine	C ₂₁ H ₂₀ NO ₄ ⁺	350.1792	334.1091, 306.1139, 292.1001
31	25.9	2-Methoxycinnamaldehyde	C ₁₀ H ₁₀ O ₂	294.1492	145.0669, 135.0820, 107.0481

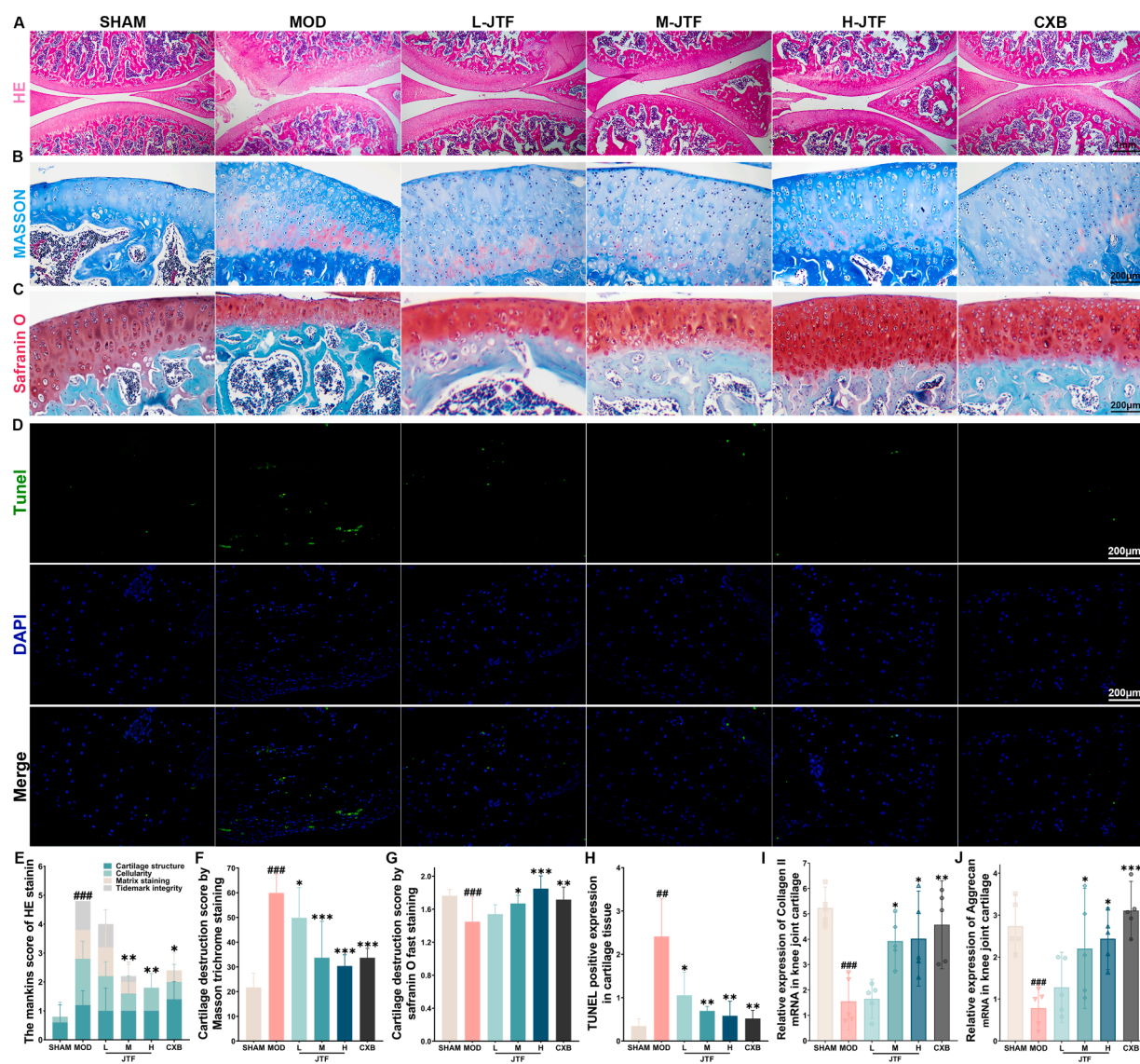


Fig. 2. Cartilage protective effect of JTF administration in OA rats. (A) Representative H&E staining images of knee joints in different groups. (B) Representative Masson trichrome staining images of knee joint cartilage tissues in different groups. (C) Representative safranin O fast staining images of knee joint cartilage tissues in different groups. (D) TUNEL positive expression in cartilage tissues of different groups ($n = 3$ per group). (E) The Mankins scores of HE staining in different groups ($n = 5$ per group). (F) Cartilage destruction scores by Masson trichrome staining in different groups ($n = 5$ per group). (G) Cartilage destruction scores by safranin O fast staining in different groups ($n = 5$ per group). (H) Representative TUNEL staining images of knee joint cartilage tissues in different groups. (I) Relative expression of collagen II mRNA in knee joint cartilage tissues of different groups ($n = 5$ per group). (J) Relative expression of Aggrecan mRNA in knee joint cartilage tissues of different groups ($n = 5$ per group). Data are expressed as mean $\pm$ SD (post-hoc Tukey test). # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ comparison with the sham group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, comparison with the model group.

JTF administration effectively reversed these pathological changes, aligning with statistical data on the expression of cartilage structure markers (collagen II and aggrecan) in cartilage tissues (Model vs. Sham, all $p < 0.001$, JTF-H vs. Model, all $p < 0.05$, Fig. 2I~J).

JTF administration effectively alleviates subchondral bone injury, cartilage matrix degradation and inflammation

Micro-CT scanning was performed on the site of the subchondral bone injury after a 28-day treatment with JTF to evaluate the pharmacological effect of JTF administration on subchondral bone injury repair. In OA rats, the exterior general view of the subchondral bone exhibited a rugged surface with obvious depression and broken edges. Quantitative analysis revealed a significant decrease bone mineral density (BMD), trabecular thickness (Tb.Th), and bone volume/tissue volume (BV/TV) (Model vs. Sham, all $p < 0.01$, JTF-H vs. Model, all $p < 0.05$, Fig. 3E~G).

Conversely, marked increases in trabecular separation (Tb.Sp) and bone surface/bone volume (BS/BV) were observed in OA rats (Model vs. Sham, all $p < 0.05$, JTF-H vs. Model, all $p < 0.05$, Fig. 3H~I). Administration of JTF effectively reversed these changes, similar to CXB administration. Furthermore, the increased expression levels of matrix degradation markers (MMP3, MMP13 and ADAMTS5) in the cartilage tissues (Model vs. Sham, all $p < 0.05$, Fig. 3B~D, J~L) and the levels of inflammatory factors (IL-1 β , TNF α , IL-6 and PGE2) (Model vs. Sham, all $p < 0.001$, Fig. 3M~P) were all reversed by the administration of JTF (all $p < 0.05$, Fig. 3B~D, J~P).

NCOA4-HMGB1-driven ferroptosis might be a candidate target of JTF against OA

A total of 520 putative targets of JTF were collected from the ETCM v2.0 (Supplementary Table S4), while 3679 genes associated with OA

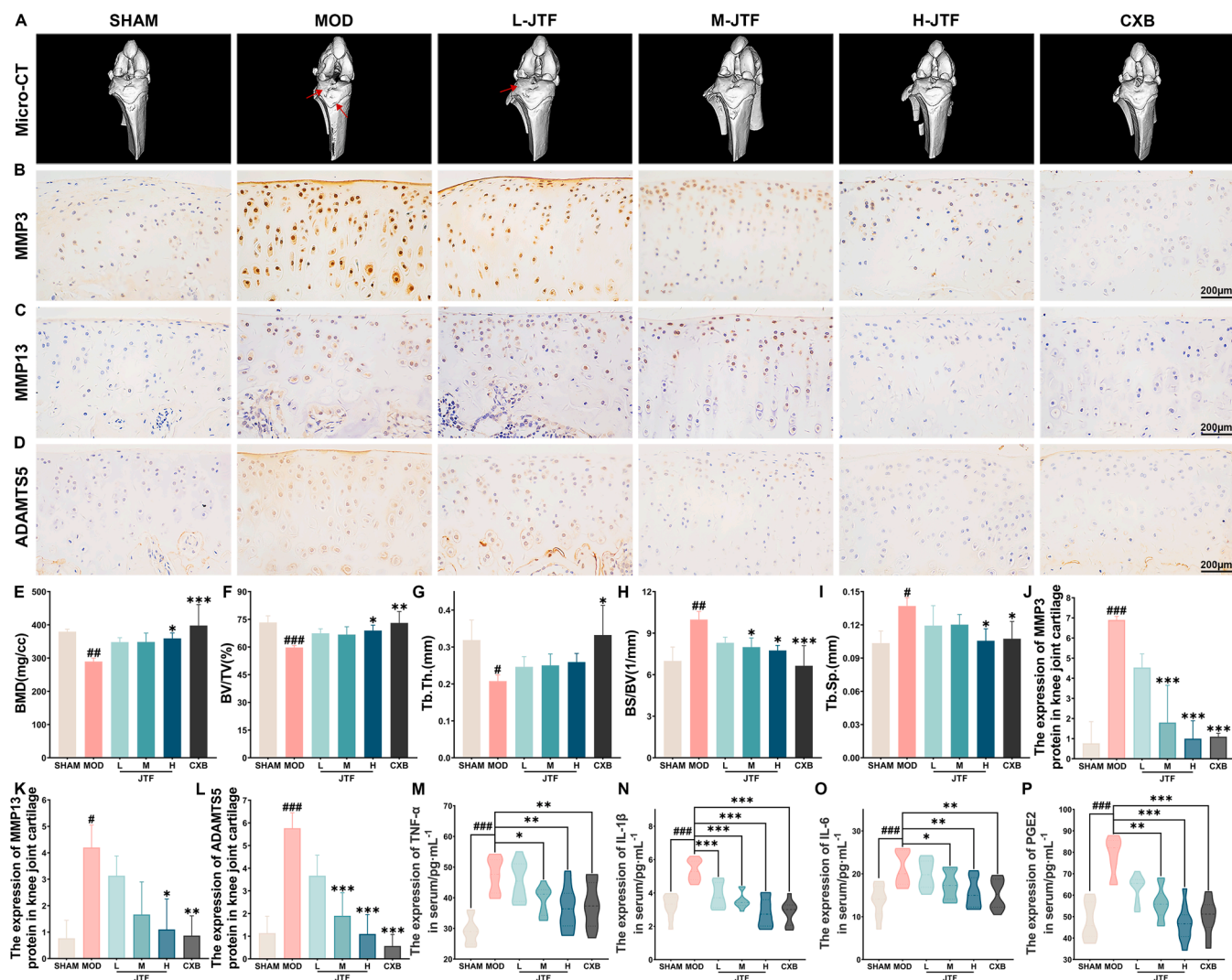


Fig. 3. JTF administration effectively reduces subchondral bone injury, matrix degradation and inflammation. (A) Representative micro-CT images of subchondral bone in different groups. (B–D) Representative IHC images of MMP3, MMP13, and ADAMTS5 protein expression in knee joint cartilage tissues of different groups. (E–I) micro-CT analysis of BMD, BS/BV, BV/TV, Tb.Th, and Tb.Sp of different groups ($n = 4$ per group). (J–L) The expression of MMP3, MMP13, and ADAMTS5 proteins in knee joint cartilage tissues of different groups ($n = 3$ per group). (M–P) Serum levels of TNF- α , IL-1 β , IL-6, and PEG2 in different groups ($n = 10$ per group). Data are expressed as mean $\pm$ SD (post-hoc Tukey test). # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ comparison with the sham group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, comparison with the model group.

symptoms were obtained from the HPO database (Supplementary Table S5). Analysis of the "JTF putative target-OA-related gene" interaction network revealed 1253 hub genes, including 227 JTF candidate targets and 1134 OA-related genes. These hub genes were enriched in various regulatory pathways, related to cellular function, inflammation and immunity, metabolism and endocrinology, neurological regulation, and signal transduction (Fig. 4A). Particularly, ferroptosis and autophagy-related pathways emerged as the top enriched class, closely associated with OA development and progression (Tong et al., 2022). Moreover, the intersection of hub genes, JTF putative targets, and ferroptosis-associated genes from FerrDB identified 7 genes (Fig. 4B). Notably, HMGB1 may lead to pain, osteoclast activation, inflammation in the nervous system and OA (Aulin et al., 2020), and induce chondrocyte cell apoptosis via the activation of GSK3B (Shu et al., 2020). Additionally, GSEA based on the OA-related clinical transcriptomics data (GEO Accession No. GSE114007) displayed the elevated expression levels of components participated into the ferroptosis pathway in OA group, especially for NCOA4 ($p < 0.001$, Fig. 4C), which is a marker for ferritinophagy and implicated in HMGB1 release. Interestingly, both NCOA4 and HMGB1 showed higher expression levels in the cartilage of

OA patients compared to healthy cohorts (Fig. 4D), correlating significantly ($r = 0.41$, $p < 0.01$, Fig. 4E). Furthermore, MMP3, MMP13, ADAMTS5, PTEGR2, AQP1 and TF showed positive co-expression with NCOA4 and HMGB1 (Fig. 4E). These findings suggest the NCOA4-HMGB1-GSK3B-AQPs signaling axis may play a pivotal role in ferroptosis-induced OA pathogenesis (Fig. 5).

JTF may inhibit ferroptosis of chondrocytes by regulating NCOA4-HMGB1-GSK3B-AQPs signaling

In the cartilage tissues of OA rats, experimental analysis revealed significantly elevated levels of Fe²⁺, MDA, and 4-HNE compared to the sham group (Model vs. Sham, all $p < 0.01$, Fig. 6A–C). Conversely, the contents of GSH/GSSG, NFE2L2 and HO-1 were reduced in the OA group (Model vs. Sham, all $p < 0.01$, Fig. 6D–F, and K), indicating oxidative stress in chondrocytes during OA progression. In addition, the expression levels of TF, TFR2, ferritin, and NCOA4 were significantly increased in OA rats compared to the sham group (Model vs. Sham, all $p < 0.01$, Fig. 6G–J, L, M, O). Notably, both JTF and CXB administration effectively reversed the aberrant levels of these molecules (all $p < 0.05$,

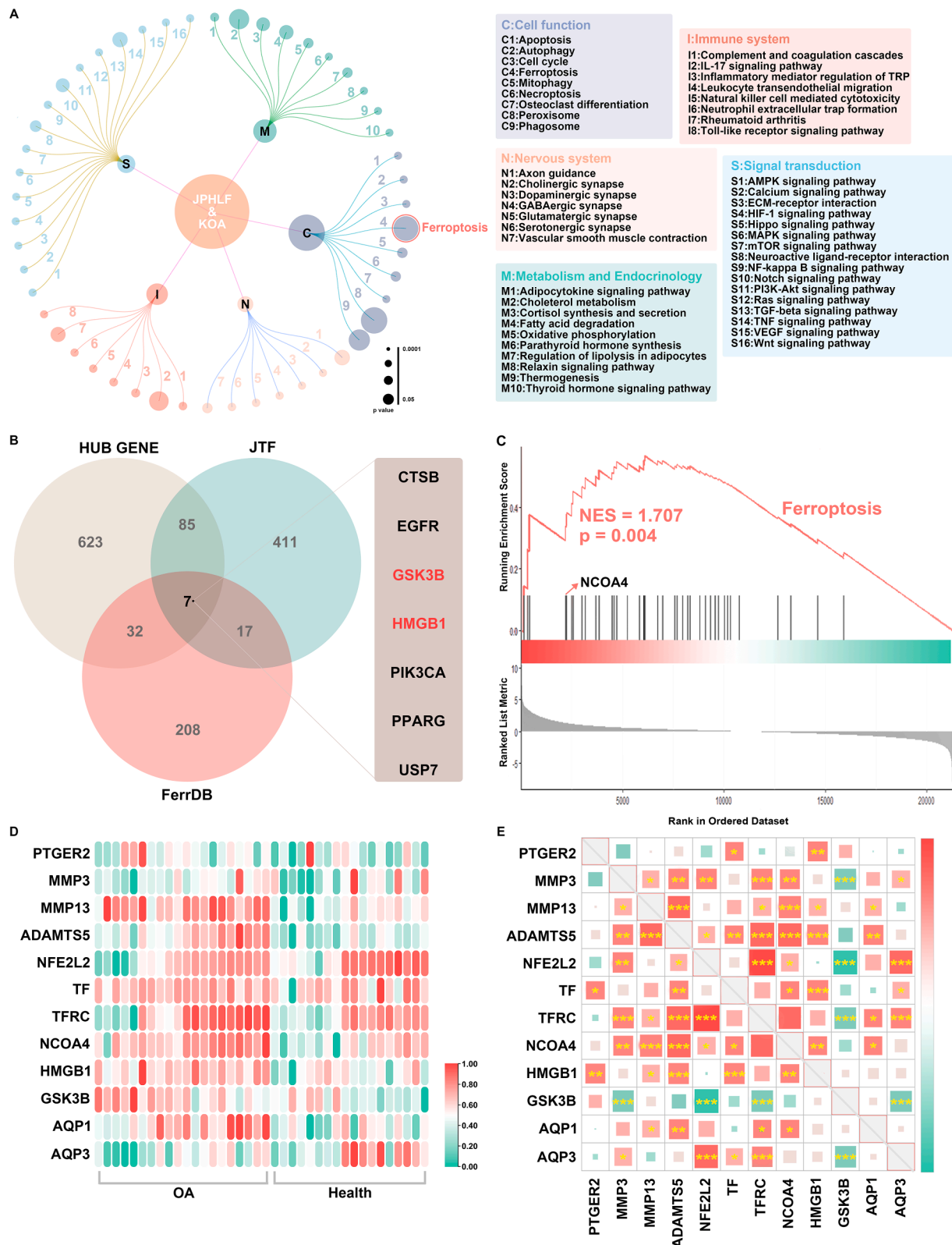


Fig. 4. Targets screening of JTF administration OA. (A) Classification of biological functional modules of “JTF putative target-OA-related gene” interaction network by KEGG. (B) Venn map of hub genes, JTF putative targets, and ferroptosis-association genes from FerrDB. (C) GSEA analysis results of ferroptosis pathway and NCOA4. (D) Heat map of candidate targets expression. (E) Co-expression correlations of candidate targets (Pearson correlation coefficient).

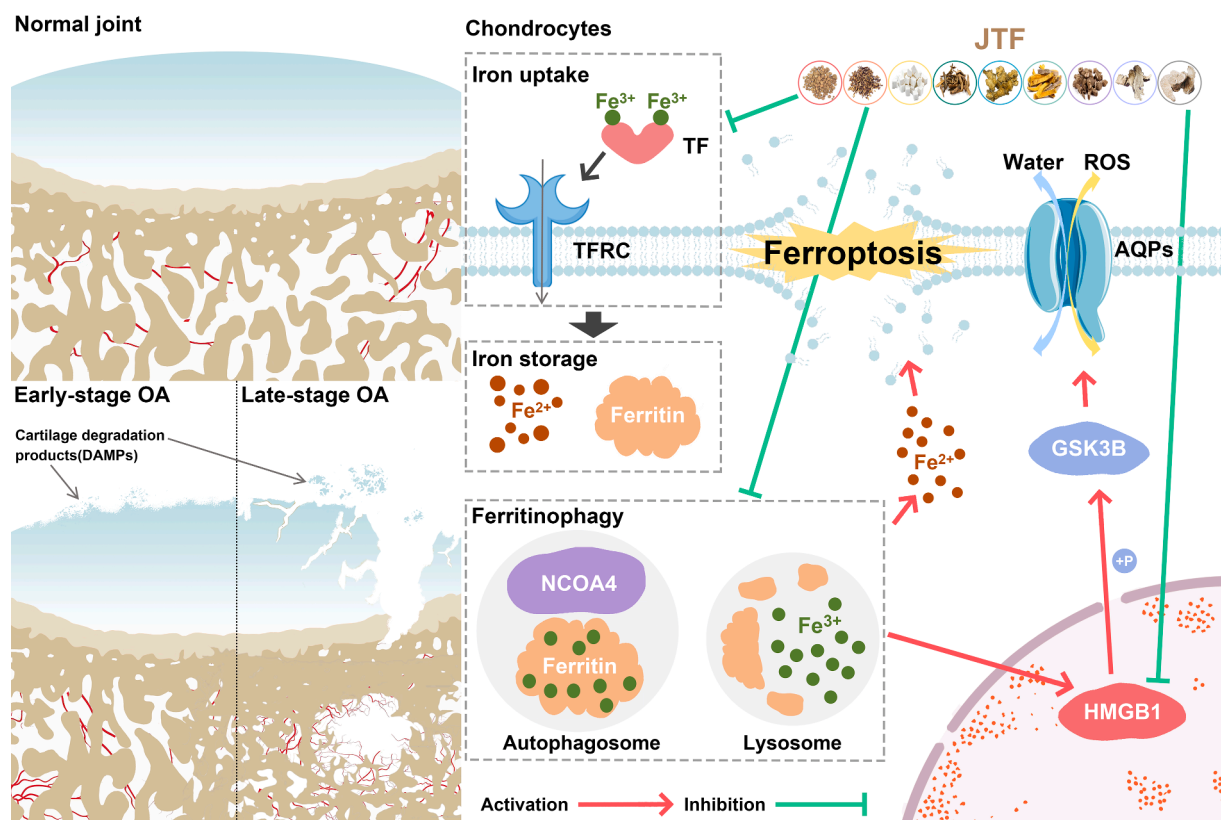


Fig. 5. JTF administration effectively relieves OA progression via inhibiting NCOA4-HMGB1-driven ferroptosis and aquaporin dysregulation.

Fig. 6A–M, O).

Moreover, we observed a substantial elevation in HMGB1 protein expression levels in the cartilage tissues of OA rats (Model vs. Sham, $p < 0.001$, Fig. 6N,P), consistent with the role of damage-associated molecular patterns (DAMPs) in inducing pathological inflammation in OA and promoting HMGB1 synthesis (Murao et al., 2021; Palumbo et al., 2023). Furthermore, we noted a marked reduction in the ratio of p-GSK3B-S9/GSK3B in OA rats (Model vs. Sham, $p < 0.05$, Fig. 6Q,R), indicating GSK3B activation. Notably, the administration of both JTF and CXB effectively reversed these situations (all $p < 0.05$, Fig. 6N, P–R).

Discussion

Emerging clinical evidence reveal the urgent need for novel and effective therapeutics to treat OA. Our study demonstrated that JTF effectively impede the progression of OA by reversing histopathological indicators such as elevated Mankins scores, increased cartilage destruction, and subchondral bone injury. Additionally, JTF normalized abnormal changes in matrix degradation markers, inflammatory factors, edema inducers, and iron metabolism parameters. Through data mining of clinical transcriptomic data and network analysis, we identified NCOA4-HMGB1-driven ferroptosis as a candidate target of JTF against OA, which was further validated through a series of in vivo experiments. Our study represents the first attempt to suggest that JTF may confer protective effects on cartilage and alleviate edema in OA therapy by inhibiting NCOA4-HMGB1-driven ferroptosis and aquaporin dysregulation.

The onset of early-stage OA often manifests with severe pain and edema, potentially attributed to increased ferroptosis activity in chondrocytes, synovium, and subchondral bone (Zhang et al., 2023a). Ferroptosis represents a distinctive form of cell death characterized by the accumulation of iron-dependent lipid peroxides (Stockwell, 2022). Its

significance in OA pathogenesis has become increasingly evident. Epidemiological evidence indicated that iron overload may be a significant etiological factor in the development of joint pathology. Conditions such as hereditary hemochromatosis, hemophilia, and thalassemia, characterized by iron overload, frequently coincide with chronic arthropathy. Notably, individuals with hereditary hemochromatosis have a high risk of developing OA (Cai et al., 2021). TF functions as the primary iron transporter, with TFRC facilitating cellular iron uptake through TF interaction (Shen et al., 2018). Ferritin acts as an iron storage protein within cells. NCOA4 mediates ferritin degradation through lysosomal autophagy, releasing Fe^{2+} under conditions of intracellular Fe^{2+} depletion (Santana-Codina et al., 2021). Our findings align with previous research, indicating that augmented ferritinophagy within articular cartilage could exacerbate iron overload by upregulating NCOA4 expression, a process potentially mitigated by JTF administration.

DAMP-induced inflammation is a pivotal factor in OA pathogenesis (Palumbo et al., 2023). HMGB1, a prototypical DAMP, is released passively during lytic cell death, exacerbating pain, activating osteoclasts, and promoting inflammation (Aulin et al., 2020). Increased HMGB1 levels have been observed in cartilage and synovial membrane of OA patients, contributing to chondrocyte death through MMPs and $\text{IL-1}\beta$ overexpression (Wang et al., 2020). Inhibition of HMGB1 has shown promise in mitigating cartilage degradation in early-stage OA (Aulin et al., 2020). Remarkably, HMGB1 may also be released via ferroptosis-mediated autophagy (Wen et al., 2019), and reducing NCOA4 levels may inhibit HMGB1 release induced by ferritinophagy-mediated ferroptosis (Jin et al., 2022). In contrast, the reduction of ferroptosis and oxidative stress may be accomplished by the inhibition of HMGB1. Inactivation of GSK3B, a serine-threonine kinase implicated in mitochondrial dysfunction, apoptosis, and inflammation, has been associated with enhanced chondrocyte survival in OA. (Guidotti et al., 2015). Our experiments validate these findings,

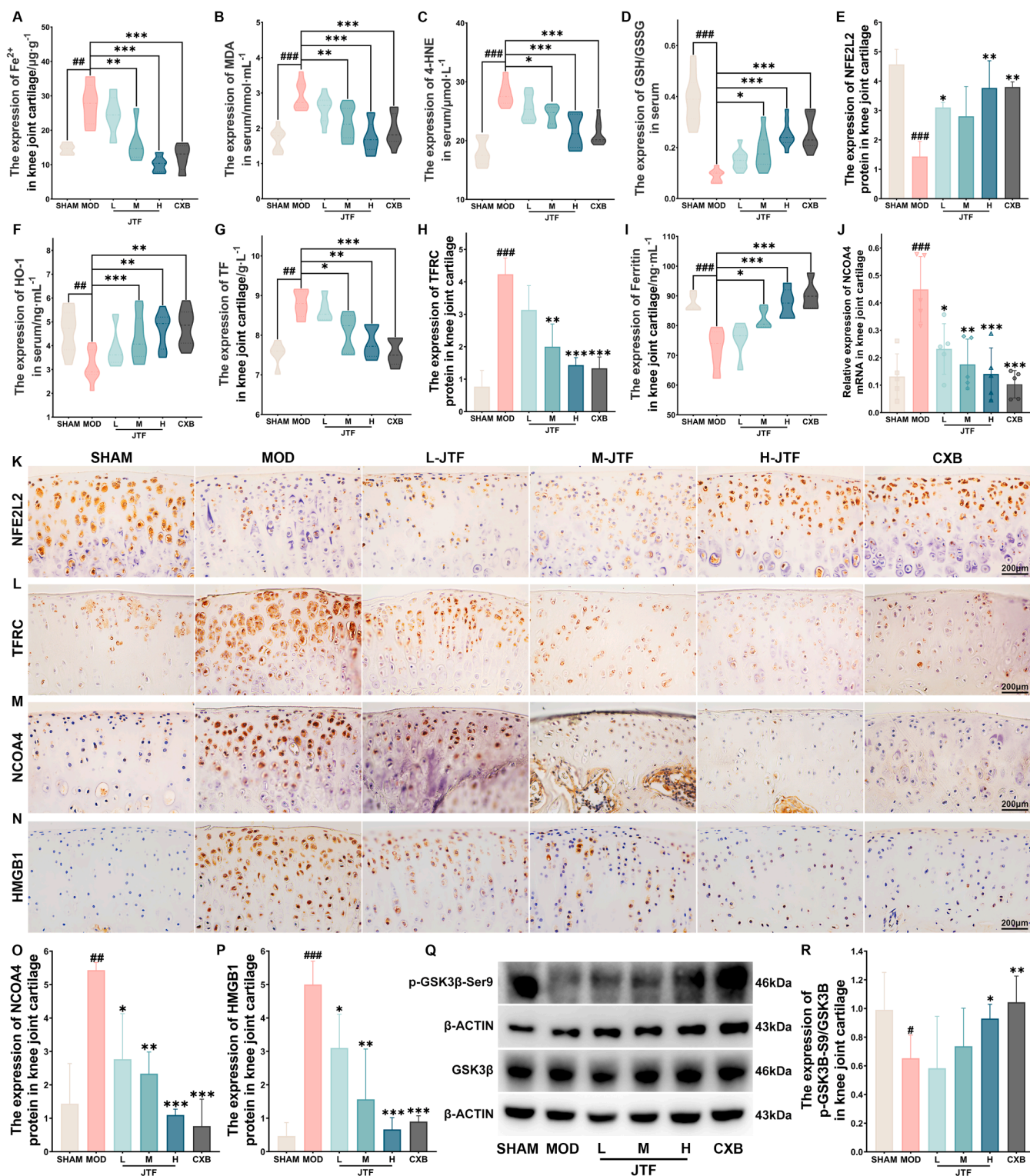


Fig. 6. JTF administration effectively inhibits ferroptosis of chondrocytes by reducing iron accumulation. **A.** Relative expression of Fe^{2+} in the knee joint cartilage tissues of different groups ($n = 5$ per group). **B–D.** Serum levels of MDA, 4-HNE, and GSH/GSSG in different groups ($n = 10$ per group). **E.** The expression of NFE2L2 protein in the knee joint cartilage tissues of different groups ($n = 3$ per group). **F.** Serum levels of HO-1 in different groups ($n = 10$ per group). **G–I.** The expression of TF, TFRC and Ferritin protein in the knee joint cartilage tissues of different groups ($n = 3$ per group). **J.** The expression of NCOA4 mRNA in the knee joint cartilage tissues of different groups ($n = 3$ per group). **K–N.** Representative IHC Images of NFE2L2, TFRC, NCOA4 and HMGB1 proteins in the knee joint cartilage tissues of different groups. **O–P.** Immunoreactive expression of NCOA4 and HMGB1 proteins in the knee joint cartilage tissues of different groups ($n = 3$ per group). **Q–R.** The expression of p-GSK3β-S9/GSK3β proteins in the knee joint cartilage tissues of different groups ($n = 3$ per group). Data are expressed as mean $\pm$ SD (post-hoc Tukey test and Dunn's test). # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ comparison with the sham group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, comparison with the model group.

demonstrating that JTF administration significantly mitigated the abnormal upregulation of HMGB1 and GSK3B proteins, alongside reduced levels of inflammatory cytokines and matrix degradation markers.

Transportation of water within the cartilage matrix and chondrocyte membranes is a crucial aspect of cartilage pathophysiology, particularly concerning the swelling observed in early-stage OA (Mahmoudian et al., 2021). Aquaporins, transmembrane proteins facilitating water movement, also play a pivotal role in regulating hydrogen peroxide (H_2O_2) transportation and reactive oxygen species (ROS) elimination within cells (Bienert and Chaumont, 2014). Dysregulation of aquaporins has been linked to OA development, with AQP1, AQP3, AQP4, and AQP9 showing elevated expression in OA cartilage, contributing to matrix degradation and chondrocyte apoptosis (Gao et al., 2016; Kyung et al., 2021). Conversely, down-regulating AQPs reduces the expression of matrix degradation proteins in OA chondrocytes (Takeuchi et al., 2018). Moreover, accumulating studies revealed that ferroptosis was enhanced by an osmotic mechanism associated with edema (Riegman et al., 2020). Additionally, ferroptosis, influenced by osmotic mechanisms associated with edema, can be modulated by aquaporins, with AQPs affecting extracellular H_2O_2 permeability (Takashi et al., 2020). Intriguingly, H_2O_2 can induce the release of HMGB1 (Tang et al., 2007). Notably, H_2O_2 -induced HMGB1 release can activate AQP1 and AQP4, increasing endothelial cell permeability and causing spinal cord edema (Chen et al., 2019; Sun et al., 2019). Inhibition of GSK3B has also been linked to reduced expression of AQP1 (Liu et al., 2021). Interestingly, our data suggest that JTF administration effectively reduces joint swelling and abnormal upregulation of AQPs proteins.

In conclusion, our findings suggest that JTF administration may effectively suppress the release of DAMPs. and ferroptosis in chondrocytes, restore the cartilage structure and functions, and reduce the knee edema during OA development and progression. Importantly, NCOA4-HMGB1-mediated ferroptosis and aquaporin dysregulation could be one of the targets of JTF against OA. Given the multifaceted nature of OA pathology, which involves edema changes not only in cartilage but also in bone marrow and synovium, further investigation is warranted to comprehensively elucidate the pharmacological mechanisms of JTF against OA.

Data availability

All data were generated in-house, and no paper mill was used. All authors agreed to be accountable for all aspects of work ensuring integrity and accuracy.

CRediT authorship contribution statement

Yudong Liu: Investigation, Writing – original draft, Formal analysis. **Tengteng Xu:** Investigation, Writing – original draft, Formal analysis. **Zhaochen Ma:** Investigation, Writing – original draft, Formal analysis. **Chu Zhang:** Investigation, Formal analysis. **Mingzhu Xu:** Investigation, Formal analysis. **Qun Li:** Investigation, Formal analysis. **Weiheng Chen:** Visualization. **Yanqiong Zhang:** Visualization, Funding acquisition, Supervision. **Chunfang Liu:** Visualization, Funding acquisition, Supervision. **Na Lin:** Visualization, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2024.155593.

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