Research Square

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Protective effect of yellow pond turtle (Mauremys mutica) peptides on cartilage in papain-induced knee osteoarthritis rats via mediating the NF-κB/iNOS-COX-2 pathway

Jia-xing Yan
Huazhong Agricultural University
Zuo-an Li
Fujian Provincial Hospital
Lan-jie Feng
Huazhong Agricultural University
Lu-hong Shen
Huazhong Agricultural University
Jiu-liang Zhang (zjl_ljz@mail.hzau.edu.cn)
Huazhong Agricultural University

Research Article

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Abstract

Knee osteoarthritis (KOA) is a common chronic joint disorder worldwide. The water extracts of yellow pond turtle (*Mauremys mutica*) plastron can be used to repair bones and tendons, but the effect and mechanism of yellow pond turtle peptides (YPTP) on KOA is not clarified. This study aimed to explore the corresponding functional effect and anti-inflammatory activity of YPTP on cartilage in rats with papain-induced KOA by network pharmacology and animal experiments. The network pharmacology results showed that the top ten key target genes of YPTP on KOA included *TNF*, *FPR2*, *APP*, *AGTR2*, *AGTR1*, *STAT3*, *NFKBIA*, *BDKRB2*, *F2*, and *BIRC2*, which were involved in multiple pathways associated with NF-κB signaling. Protein-protein interaction analysis demonstrated that the key targets of YPTP on KOA included COX-2 and iNOS. In addition, YPTP administration (75 and 150 mg/kg) on rats with papain-induced KOA was performed, and knee joint swelling, biomarkers, inflammatory factors, joint microstructure and joint components were assessed. Animal experiment results indicated that YPTP could decrease the expression of COX-2, iNOS and other inflammatory factors in the NF-κB signaling pathway. YPTP could also regulate the expression of MMP-3 and COLLII induced by inflammatory factors, thereby relieving joint pannus, cartilage damage and bone resorption. Our findings indicated that YPTP could inhibit the NF-κB/iNOS-COX-2 pathway and regulate the expression of MMP-3 and COLLII, thereby restricting cartilage degradation and synovial inflammation. This study may provide a theoretical guidance for the selective use of YPTP supplementation on health regulation in the future.

1. Introduction

Yellow pond turtle (*Mauremys mutica*) is a kind of freshwater turtle distributed in Vietnam and south-central China. It is rich in amino acid, minerals, and other essential nutrients. The water extracts of yellow pond turtle plastron are first used as a kind of Chinese traditional medicine, and has been developed as a functional food for centuries[1]. The *Compendium of Materia Medica* records that the plastron of yellow pond turtle has detoxification and anti-inflammation effects, and can help strengthen the bone. A previous study showed that the yellow pond turtle peptides (YPTP) have certain antioxidant activity and immune system enhancing effect by animal experiments[2]. If YPTP with therapeutic effect is applied to the food in daily life, it can be easily absorbed to achieve the purpose of improving human health[3]. Dietary peptides from food proteins have been found to possess strong antioxidant and antibacterial activities, and can relieve pain associated with moderate knee osteoarthritis (KOA)[4]. The ultrafine powder of *Carapax Trionycis* can alleviate the bone damage and recover bone mineral density and calcium metabolism [5]. Peptides extracted from the antler of Formosan sambar deer and red deer have significant anti-inflammatory effect *in vitro*[6]. A designed TLR4 antagonistic peptide could relieve the rheumatoid arthritis symptoms and synovial tissue destruction by impeding inflammatory biomarkers[7]. Some other studies have shown that low molecular weight collagen peptides can increase the organic substance content of bone in osteoporosis rats[8]. Overall, many peptides exhibit important functions in response to diseases through the anti-inflammatory pathway. However, there has been no report on the cartilage protective effect of YPTP in KOA and the related mechanism.

KOA is regarded as a highly prevalent disabling joint disease characterized by cartilage defects of the knee joint. KOA seriously affects the action capability and life healthy of patients[9]. It has been noted that KOA has a high incidence among the aged and sedentary young adults. Conventional drugs are not particularly effective for KOA and can cause serious gastrointestinal reactions. When developing to a certain stage, KOA causes irreversible damage to the hyaline cartilage, and joint replacement may be the only option of treatment[10, 11].

Alleviation of progressive cartilage destruction and inflammation is an important therapeutic approach for inflammatory arthritis. It has been reported that persistent synovial inflammation plays an important role in the cartilage degradation of rat knee[12]. Previously, KOA was found to be related to the cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in the NF-κB signaling pathway, and tumor necrosis factor alpha (TNF-α), prostaglandin E2 (PGE2), nitric oxide (NO), and interleukin-1 (IL-1) were important in the pathological state[13]. The above inflammatory factors can induce the up-regulated expression of metalloproteinase-3 (MMP-3), which may alter the balance between anabolism and catabolism. MMP-3 can lead to the degradation of type II collagen (COLLII). Blocking the over-expression of COX-2 or iNOS can restore the normal expression of MMP-3 and COLLII, thereby reducing the extracellular matrix depletion of cartilage and the extent of cartilage damage and joint metamorphosis[4].

This study aimed to investigate the corresponding functional effects and anti-inflammatory activities of YTPP on papain-induced KOA by network pharmacology and animal experiments. First, a network pharmacology approach was used to theoretically explore the targets of the protective effect of YTPP on KOA. Second, rats with papain-induced KOA were injected intragastrically with YTPP, and related indicators were measured. Knee joint swelling was assessed by vernier caliper and joint microstructure was observed by hematoxylin and eosin (H&E) staining. The proteoglycan was detected by toluidine blue (TB) staining. The biomarkers (CTX-II and COMP) and inflammatory factors of KOA in the serum were measured by ELISA. The expression levels of COX-2, iNOS, and other inflammatory factors in the synovium, and those of MMP-3 and COLLII in the cartilage and meniscus were determined by immunohistochemistry. This study might reveal how YTPP protects cartilage by mediating inflammatory pathways and promotes potential applications of YTPP in KOA prevention and management.

2. Materials And Methods

2.1 Materials and reagents

Yellow pond turtle peptides (YPTP) were prepared by previous research in our laboratory[2]. L-cysteine and papain were purchased from Guangzhou Saiguo Biotech Co., Ltd. (Guangdong, China). The ELISA assay kits of collagen type II C-telopeptide (CTX-II), cartilage oligomeric matrix protein (COMP), prostaglandin E2 (PGE2), interleukin-1beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) were bought from Shanghai Huding Biological Technology Co., Ltd. (Shanghai, China). The assay kit for nitric oxide (NO) was obtained from Nanjing Jiancheng Biotechnology Co., Ltd. (Nanjing, China). All other chemicals and reagents were analytical purity.

2.2 Network pharmacology analysis of YPTP

The composition of YPTP was analyzed with a high performance liquid chromatography electrospray quadrupole time of flight mass spectrometry (HPLC-ESI-Q-TOF-MS/MS) system (AB SCIEX Co., Ltd., Shanghai, China) according to a previous report of our laboratory (Table S1)[2], and the information was imported into the Swiss Target Prediction database (http://www.swisstargetprediction.ch/)[14]. Targets were predicted after the species was set as “Homo sapiens”. The data were exported in Excel for later use. The “knee osteoarthritis” was used as query and the “score > 20” was used as the condition to obtain clusters of disease-related targets in the GeneCards database (https://www.genecards.org/). Afterward, the data were exported in Excel. Jvenn (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to screen the potential targets for the effect of YPTP on KOA, and generate a Venn diagram and substance-protein interaction network relationship. Finally, the data were exported in Excel for later use.

2.3 Gene enrichment and protein-protein interaction (PPI) network construction

The potential targets identified from intersection of the Venn diagram were input in DAVID (https://david.ncifcrf.gov/) with the parameters of ease < 0.05, count ≥ 4, FDR < 0.01, and $P < 0.05$ to obtain their GO biological functions and KEGG pathway enrichment, and the bubble charts were generated by using R. Then, the potential targets of pathways enrichment (top 5) were imported into the String database (https://string-db.org/) based on the species of “Homo sapiens” and the comprehensive score of protein interaction > 0.4. Network construction was performed based on protein-protein interactions (PPI). The network data were exported in CSV format and then input into the software of Cytoscape3.9.0 for analysis and visualization.

2.4 Animals

A total of 42 male SD (SPF, 190–210 g) rats were purchased from the Animal Center for Disease Prevention and Control in Hubei province (Certificate No. SCXK (Hubei) 2020-0019, Wuhan, China). Animal care including room, cage, equipment sanitation was in accordance with the standards for laboratory animals established by the People’s Republic of China (GB14925-2010) and the Guiding Principles in the care and use of animals throughout the experimental period at Center for Laboratory Animals, Huazhong Agricultural University (Permission No. HZAURA-2020-0010). The animals were raised in a quiet environment with proper temperature (22–25°C) and humidity (55 ± 10%) with a free access to water and food (Permission No. SYXK (Hubei) 2020–0084). All animals were kept under a 12 h light/12 h dark cycle.

2.5 Experimental design

The experimental design referred to the following schematic process diagram. After one week of acclimation and exclusion of rats with poor physiological conditions, 32 rats were randomly divided into four groups: normal control group, which were subjected to intra-articular injection of 0.15 mL saline and received an equivalent amount of saline; the KOA model group, which were subjected to intra-articular

injection of 0.15 mL modeling fluid (mixture solution of 4% papain and 0.3 mol/L L-cysteine) prepared 0.5 h in advance and received an equivalent amount of saline; LYPTP group, which were subjected to an intra-articular injection of 0.15 mL modeling fluid and then received a low dose of YPTP (75 mg/kg/day); and HYPTP group, which were subjected to intra-articular injection of 0.15 mL modeling fluid and received a high dose of YPTP (150 mg/kg/day). All the injections were conducted on the left knee joint.

Body weight was recorded during the whole experiment and the left knee joint diameter was measured with a vernier caliper. All rats were intraperitoneally anesthetized with 10% chloral hydrate before the intra-articular injection.

The rats were anesthetized, and blood samples were collected from the eyeball. The blood was centrifuged and the obtained supernatant was stored at − 80°C until detection. After cutting of both sides of the patella, the femoral condyle was exposed. The joint fluid was obtained by injecting 0.5 mL of saline into the left knee joint and then stored at − 80°C. Then, the synovium combined with the patella was rapidly sheared off and placed in 4% paraformaldehyde at an EP tube. Knee joints were transected at the 1/3 position of the distal femur and 1/3 position of the tibia and then fixed in 4% paraformaldehyde. The livers and spleens were collected and weighed. Organ index reflecting the health status of animal organs was calculated as the ratio of organ mass to animal mass as follows:

$$\mathrm{Organindex(mg/g)} = \frac{\mathrm{weightoforgan(mg)}}{\mathrm{bodymass(g)}} \times 100\%$$

2.6 Serum and synovial fluid analysis

Serum and synovial fluid were processed according to the instructions of the kit as mentioned before. CTX-II, COMP, PGE2, IL-1β, IL-6, TNF-α and NO in the serum and IL-1β, IL-6, and TNF-α in the synovial fluid were quantified. Protein was measured using the Bio-Rad assay kit.

2.7 Histology

Intact knee joints were first carefully observed with naked eyes and then decalcified in 10% EDTA at room temperature for approximately two months. The knee joints were longitudinally cut and embedded in paraffin after decalcification. The synovium was cut and embedded in paraffin. Then, sagittal sections (4 μm) were prepared for hematoxylin and eosin (H&E) staining and toluidine blue (TB) staining to observe the structures of meniscus and articular cartilage with an optical microscope.

2.8 Modified Mankin score

After H&E and TB staining, modified Mankin scoring (Table S2) was conducted to evaluate the cartilage damage, cartilage cell number, proteoglycan staining status, and tide line integrity[15]. According to the Mankin scoring principle with a total score of 14 points, 1–5 points were identified as early osteoarthritis; 6–9 points were identified as middle osteoarthritis, and 10–14 points were identified as late osteoarthritis.

2.9 Determination of inflammatory factors in synovium and COLLII and MMP-3 in cartilage and meniscus by immunohistochemistry

After conventional dewaxing and rehydration of the paraffin sections, the tissue sections were placed in a repair box filled with citric acid (pH 6.0) antigen retrieval buffer for antigen retrieval in a microwave oven. Then, the slides were washed with PBS (pH 7.4) and sealed with serum, followed by incubation with the primary antibody and secondary antibody, respectively. 4 μm resin semi-thin sections were placed in PBS (pH 7.4) and shaken on the decoloring shaker for DAB color development. Next, the sections were counterstained with hematoxylin stain solution, examined by microscopy after dehydration and sealing. According to the immunohistochemistry results, the expression levels of iNOS, COX-2, IL-1β and TNF-α in the synovium and COLLII and MMP-3 in the cartilage and meniscus were assessed by image analysis system (Image J).

2.10 Statistical analysis

The knee joint diameter was analyzed by determining the area-under-the-curve (AUC). All data were presented as means ± standard deviation (SD). Differences between groups were determined by one-way analysis of variance (ANOVA). Statistical differences were considered as significant at $P < 0.05$. SPSS software was used for all analysis.

3. Results

3.1 Effects of YPTP on serum CTX-II and COMP

CTX-II and COMP are two biomarkers of KOA, and their levels can indicate the severity of KOA [16]. As shown in Fig. 2A and B, the levels of CTX-II and COMP in the KOA group were significantly higher than those in the normal group, indicating that the KOA rat model was successfully established. Compared with the KOA group, the serum CTX-II levels in LYPTP group and HYPTP group were decreased by nearly 26.26% and 50.40%, respectively ($P < 0.05$). In addition, compared with the model group, the serum COMP levels in the LYPTP and HYPTP groups decreased by 51.44% and 90.12%, respectively ($P < 0.05$). According to the content of two biomarkers (CTX-II and COMP), the severity of KOA followed the order of the KOA group > LYPTP group > HYPTP group. These results indicated that cartilage was degraded and destroyed and synovitis was formed in the KOA group and the YPTP treatment (LYPTP and HYPTP) could reduce the severity of KOA. Therefore, YPTP can be used as a dietary supplement to prevent KOA.

3.2 Key genes of the YPTP targets related to KOA

Based on the sequences of the nine peptides identified in YPTP, 230 targets related to YPTP were screened using the Swiss database, 1918 targets related to KOA were screened using the GeneCards database, and 122 overlapping targets were screened using the Jvenn database (Fig. 1A). The overlapping targets were then imported into the String database to analyze the protein interaction

network, and the results were visualized using Cytoscape 3.9.0. As shown in Fig. 1B, the targets of YPTP mainly included COX-2, iNOS, MMP, and cytokines. The top 30 genes with the highest degree of connectivity with other genes were screened by analyzing the network topology properties (Fig. 1C). The top ten genes were *TNF, FPR2, APP, AGTR2, AGTR1, STAT3, NFKBIA, BDKRB2, F2* and *BIRC2*. *TNF* showed the maximum degree (26) of connectivity, indicating that this gene had important functions in the network. *TNF* (also called TNF-α) signaling can induce the activation of many genes, which is primarily controlled by two distinct pathways, the NF-κB pathway and the MAPK pathway, or apoptosis and necroptosis[17]. *FPR2* belong to the family of seven-transmembrane G protein-coupled receptors. It has the ability to modulate both pro- and anti-inflammatory response[18]. *APP* are a class of proteins whose plasma concentration increase or decrease in response to inflammation[19]. Two important genes *AGTR2* and *AGTR1* are associated with chondrocyte hyperplasia and hypertrophy in the RAS signaling pathway[20]. The *STAT3* and *NFKBIA* genes are typical and widely studied disease targets[21, 22]. The proinflammatory cytokine level in KOA is influenced by the antagonist agent of *BDKRB2* implicated in TLR-2 silencing[23]. The thrombin-cleaved(*F2*) osteopontin level is correlated with the severity of osteoarthritis[24]. *BIRC2* is related to various pathways such as the NF-κB signaling pathway, the TNF signaling pathway, and pathways in cancer according to the KEGG database (https://www.kegg.jp/). Therefore, KOA in rats may be suppressed by regulation of the above signaling pathways.

3.3 GO enrichment and KEGG pathway enrichment analysis

The GO and KEGG enrichment analysis using the DAVID database identified 485 GO items and 87 KEGG pathways (Table S3 & S4). Among the results of the GO enrichment analysis, 348, 49 and 88 were associated with BP, CC and MF, respectively. The top 20 significant GO terms of BP, CC, MF were shown as bubble plots in Fig. 1D-F. The items associated with inflammation had proteolysis, plasma membrane, integral component of the plasma membrane and membrane raft, serine-type endopeptidase activity, endopeptidase activity. The items related with articular cartilage metabolism had extracellular matrix disassembly and collagen catabolic process, metalloendopeptidase activity. 87 pathways were obtained by KEGG enrichment analysis. The top 20 significant KEGG pathways were shown as bubble plots in Fig. 1G. Among the KEGG signaling pathways, 5 pathways with high enrichment of gene targets were screened, which were TNF signaling pathway, HIF-1 signaling pathway, PI3K-AKT signaling pathway, pathways in cancer, NF-κB signaling pathway. As shown in Fig. 1H, the five signaling pathways were integrated together. Some potential targets of YPTP acting on KOA were showed, such as NOS2, PTGS2, IL-6, IL-1β and so on (Table S5 for details). The results showed that YPTP could relieve KOA through inhibition of NF-κB pathways and their target.

3.4 Construction of protein-protein interaction network for the targets of YPTP

The potential targets of YPTY in top 5 pathways were imported into the String database (https://string-db.org/) based on the species “Homo sapiens” as the condition. The protein-protein interaction network (PPI) (Fig. 1I) was visualized using Cytoscape, and the hub proteins with high degree were identified by Analyze Network of Cytoscape. The higher the degree was, the more its color would tend to be blue.

PTGS2(COX-2) showed the maximum degree (12) of connectivity, showing that the potential target for the effect of YPTP on KOA was PTGS2(COX-2). The expression levels of COX-2 and iNOS are closely associated with NF-κB pathway[25]. Therefore, our results suggested that YPTP might prevent the KOA by mediating the NF-κB/iNOS-COX-2 pathway.

3.5 Effects of YPTP on the organ index, weight, and knee joint swelling of KOA rats

As shown in Fig. S1 and Table S6, there was no statistical difference in body weight and organ index between the KOA group and other groups, indicating that YPTP had little impact on the health of rats after about one month of administration. As shown in Fig. 2C, there was no significant difference in knee joint diameter between the different groups on days 7 and 13. On day 31, the knee joint diameter decreased significantly in the YPTP group compared to the KOA group ($P < 0.05$). In addition, compared to the KOA group, the AUC decreased by 57.8% and 97.50% in the LYPTP and HYPTP groups in Fig. 2D respectively ($P < 0.05$), indicating that YPTP might have an inhibitory effect on knee joint swelling in a dose-dependent manner.

3.6 Assessment of articular cartilage structure and joint microstructure with modified Mankin score and visual observation

As shown in Fig. S2, the cartilage surface of the normal group was smooth and glossy, while the KOA group showed severe injury on the cartilage surface and slight joint swelling. Compared to the KOA group, the LYPTP and HYPTP groups showed slight injury in cartilage surface. As shown in Fig. 2E, significant focal cartilage defects and decreased proteoglycans level were observed in the KOA group after H&E and TB staining compared to the normal group. Histomorphometric analysis showed that the articular cartilage surface in the KOA group was rough with local stenosis and adhesions. After YPTP treatment, in LYPTP and HYPTP groups, the cartilage surface damage and the area of joint adhesions were reduced, and the proteoglycans content was increased. The HYPTP group also showed smoother cartilage surface, better arranged cells, and more integrated tide line than the LYPTP group. The modified Mankin score also was performed to assess the severity of KOA. As shown in Fig. 2F and Table S7, the modified Mankin score was significantly lower in the normal (0.08 ± 0.07) and HYPTP (3.27 ± 1.40) groups than in the KOA (11.00 ± 1.50) and LYPTP (9.26 ± 1.39) groups. According to the score, the model group, LYPTP group, and HYPTP group were assessed as late, middle and early osteoarthritis, respectively [15]. These results were consistent with the results of KOA biomarkers and knee joint swelling, suggesting that YPTP could reduce articular cartilage damage.

3.7 Effects of YPTP on serum IL-1β, IL-6, TNF-α, PGE2 and NO

Analysis of serum inflammatory factors revealed that the KOA group had significantly higher levels of IL-6, NO, TNF-α, PGE2 and IL-1β than the normal group (Fig. 3A-E). Compared to KOA group, LYPTP and

HYPTP groups noticeably reduced the levels of these inflammatory factors ($P < 0.05$), indicating that YPTP might improve the structure of cartilage and alleviate synovial inflammation by affecting inflammatory factors.

3.8 Effects of YPTP on synovial fluid IL-1β, IL-6 and TNF-α

The levels of IL-1β, IL-6, and TNF-α were detected in rat synovial fluid. As shown in Fig. 3F-H, the levels of IL-1β, IL-6 and TNF-α were significantly different among groups, and the values followed the order of KOA group > LYPTP group > HYPTP group > normal group ($P < 0.05$). Apparently, YPTP has the potential to relieve inflammatory reaction in KOA rats.

3.9 Effects of YPTP on the expression of IL-1β, TNF-α, COX-2 and iNOS in synovium

Further immunohistochemical experiments were performed to investigate the effect of YPTP on the expression of proteins related to cartilage inflammation in rat synovium. The expression of IL-1β, TNF-α, COX-2, and iNOS in the synovium was semi-quantitatively assessed by immunohistochemistry (Fig. 4A-H; Table S8). The results showed that the KOA group had higher expression of related inflammatory factors than the normal group. Furthermore, the YPTP treatment (LYPTP and HYPTP groups) could significantly decrease the expression of IL-1β, TNF-α, COX-2, and iNOS in a dose-dependent manner relative to the normal group ($P < 0.05$). These results indicated that YTPP might reduce synovial inflammation by reducing the expression of COX-2 or iNOS and other inflammatory factors, thereby protecting the knee joint.

3.10 Effects of YPTP on the expression of COLLII and MMP-3 in articular cartilage

The effects of YPTP on the expression of COLLII and MMP-3 *in vivo* were evaluated by immunohistochemical experiments (Fig. 4I-L and Table S8). Compared to the KOA group, LYPTP and HYPTP groups increased the expression of COLLII by 43.75% and 77.88%, and decreased that of MMP-3 by 24.63% and 80.91%, respectively. These results indicated that YPTP could reduce the structural damage of articular cartilage by regulating the expression of COLLII and MMP-3 in papain-induced knee osteoarthritis rats.

4. Discussion

The production of yellow pond turtle plastron refers to a type of polypeptide with the anti-inflammation effects, antioxidant activity, and immune system enhancing effect and may help strengthen the bone [1–3]. Many peptides also exhibit important functions in response to diseases through the anti-inflammatory pathway[6–8]. However, there has been no report on the cartilage protective effect of YPTP in KOA and the related mechanism. KOA caused by chronic synovial inflammation is a common chronic disease characterized by joint destruction, which is an irreversible pathological change. Currently, there is no curative therapeutic strategy for KOA, and anti-inflammatory agents and physical therapy are the most

common treatments. When KOA develops to a certain stage, it may be inevitable to conduct an arthroplasty or osteotomy[26, 27]. Therefore, it is of great significance to dissect the mechanism for the positive effect of nutraceutical and medicinal food on KOA.

Network pharmacology analysis results implied that YPTP could alleviate KOA through various pathways, such as pathways in cancer, TNF signaling pathway, and PI3K-AKT signaling pathway[28]. The results indicated that the anti-inflammatory targets of YPTP were distributed in multiple signaling pathways, among which COX-2 and iNOS are the most important targets. IL-1β and TNF-α could activate each other, resulting in the activation of the NF-κB pathway. Meanwhile, the biosynthesis of COX-2 and iNOS was activated by IL-1β, IL-6, and TNF-α, which will then promote the expression of PGE2 and NO[29].

To confirm the effect of YPTP on collagen degradation and synthesis, CTX-II and COMP were measured in the serum samples from rats. With the degradation of cartilage, CTX-II and COMP fragments would be increased in the serum and synovial fluid of patients with rheumatoid arthritis. The levels of CTX-II and COMP have been proposed as effective biomarkers of joint injury progression, and their levels in serum can be used to evaluate the severity and progression of KOA[30]. In this study, papain could induce KOA in rats, and the KOA group showed the highest severity of KOA, followed by the LYPTP group and then HYPTP group. In this regard, YPTP might have certain suppressive effect on joint injury progression of KOA.

TNF-α, IL-1β, IL-6, NO, and PGE2 are inflammatory factors closely related to KOA, which play vital roles in the NF-κB/iNOS-COX-2 signaling pathway. TNF-α directly promotes the degradation of cartilage matrix and the destruction of chondrocytes, and induces the production of COX-2, iNOS, IL-6 and IL-1β, aggravating the inflammatory reaction of joint synovium. The production of PGE2 and NO is mainly regulated by inducible COX-2 and iNOS, respectively[31]. NF-κB is a group of protein dimers involved in immune and inflammatory responses[32]. In this study, YPTP showed dose-dependent effects on serum TNF-α, IL-1β, IL-6, NO, and PGE2 levels, which was consistent with the results of knee joint swelling and Mankin scoring. These results revealed that YPTP might regulate IL-1β, IL-6, TNF-α, PGE2, NO, COX-2, and iNOS in the NF-κB/iNOS-COX-2 signaling pathway, and the inhibition of these inflammatory factors could stimulate the repair of cartilage tissue in osteoarthritis patients.

MMP-3 can activate other MMPs to inhibit the degradation of COLLII. Therefore, MMP-3 is a major enzyme for cartilage degradation[33]. COLLII can help maintain the normal structure of cartilage, relieve osteoarthritis, and inhibit the degeneration of bone and joint[34]. These facts are consistent with our research results that YPTP can not only inhibit the degradation of extracellular matrix and reduce the structural damage of articular cartilage by suppressing the expression of MMP-3, but also increase the expression of COLLII to promote cartilage self-repair and control the degeneration of bone.

The results of network pharmacology and *in vivo* pharmacological evaluation suggested that inhibition of COX-2 or iNOS expression might regulate the normal expression of MMP-3 and COLLII. In addition, our findings revealed the inhibition efficacy of YPTP on cartilage damage and bone resorption. Therefore, COX-2, iNOS, MMPs, and COLLII might be potential targets for the prevention of KOA. Further *in vitro*

experiments are needed to verify these targets and how COX-2 and iNOS mediate the activation of MMPs. The reults of this study provide a good foundation for understanding the protective effect of nutraceutical and medicinal food on KOA.

5. Conclusion

This study verified that YPTP has certain protective functions on KOA through network pharmacology analysis and animal experiments. YPTP might affect the expression of KOA-related inflammatory factors in the NF-κB/iNOS-COX-2 signaling pathway, of which COX-2 and iNOS were key targets. In the LYPTP and HYPTP groups of the *in vivo* experiments, IL-1β, TNF-α, COX-2, and iNOS are down-regulated, resulting in down-regulation of MMP-3 and proteoglycans, and up-regulation of COLLII synthesis. Therefore, YPTP may reduce the degradation of cartilage, alleviate synovial inflammation, and maintain normal cartilage structure. The findings indicated that COX-2, iNOS, MMP-3, and COLLII might be the key targets for treating KOA. Meanwhile, this study provides a good basis for further application of YTPP as a nutritional and medicinal food in health regulation in the future.

Abbreviations

KOA, knee osteoarthritis,

COX-2, cyclooxygenase-2,

iNOS, inducible nitric oxide synthase,

NF-κB, nuclear factor kappa B,

TNF-α, Tumor necrosis factor alpha,

PGE2, prostaglandin E2,

NO, nitric oxide

IL-1β, interleukin-1β,

MMP-3, metalloproteinase-3,

COLLII, type II collagen,

YPTP, yellow pond turtle peptides

H&E, hematoxylin and eosin staining,

TB, toluidine blue staining,

CTX-II, collagen type II C-telopeptide,

COMP, cartilage oligomeric matrix protein,

PPI, Protein-Protein Interaction,

AUC, area under the curve,

BP, biological processes,

CC, cellular components,

MF, molecular functions

Declarations

Animal Ethics: The protocol was reviewed and accepted by the Tab of Animal Experimental Ethical Inspection of Laboratory Animal Centre, Huazhong Agriculture University, ID number HZAURA-2020-0010

Consent for publication:

All authors have reviewed the manuscript and approved to submit to your journal. The authors declare that there are no conflicts of interest.

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Not Applicable.

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Authors' contributions:

Jia-xing Yan: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Jiu-liang Zhang:** Writing - review & editing, Project administration, Funding acquisition. **Lan-jie Feng:** Validation, Formal analysis. **Lu-hong Shen:** Validation, Resources. **Zuo-an Li:** Writing - review & editing, Funding acquisition.

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Scheme

Scheme 1 is available in Supplementary Files section.

Figures

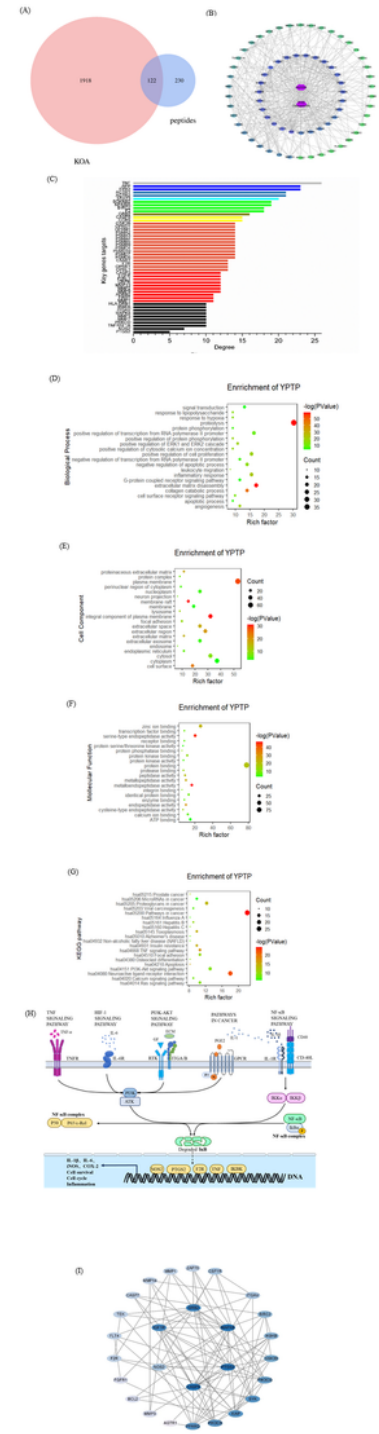

Figure 1

(A) Venn diagrams of YPTP targets (230 in total), KOA targets (1918 in total), and overlapping targets (122) generated by Jvenn. (B) Protein interaction network relationship of the YPTP targets related to KOA of YPTP for KOA (degree > 5) by using the software of Cytoscape3.9.0. (C) Analysis results of key gene targets for the effect of YPTP on KOA (the top 30 of the degree). (D) Bubble plots of biological processes in GO enrichment analysis of YPTP. (E) Bubble plots of cell components in GO enrichment

analysis of YPTP. (F) Bubble plots of molecular functions in GO enrichment analysis of YPTP. (G) Bubble plots of KEGG pathway enrichment of YPTP. The value of -log(PValue) represents the significance of enrichment. The higher the corresponding -log(PValue) was, the more its color would tend to be red, otherwise, the color tends to be blue. Bubble size indicates the number of items. (H) Pathway map of potential targets related to immune inflammation was integrated in the treatment of KOA by YPTP, some potential targets of YPTP acting on KOA were showed, such as NOS2, PTGS2, IL-6, IL-1β and so on (Table S5 for details). (I) PPI network pharmacology diagram of the potential targets of top 5 pathways by using the software of Cytoscape3.9.0. The higher the degree was, the more its color would tend to be deep.

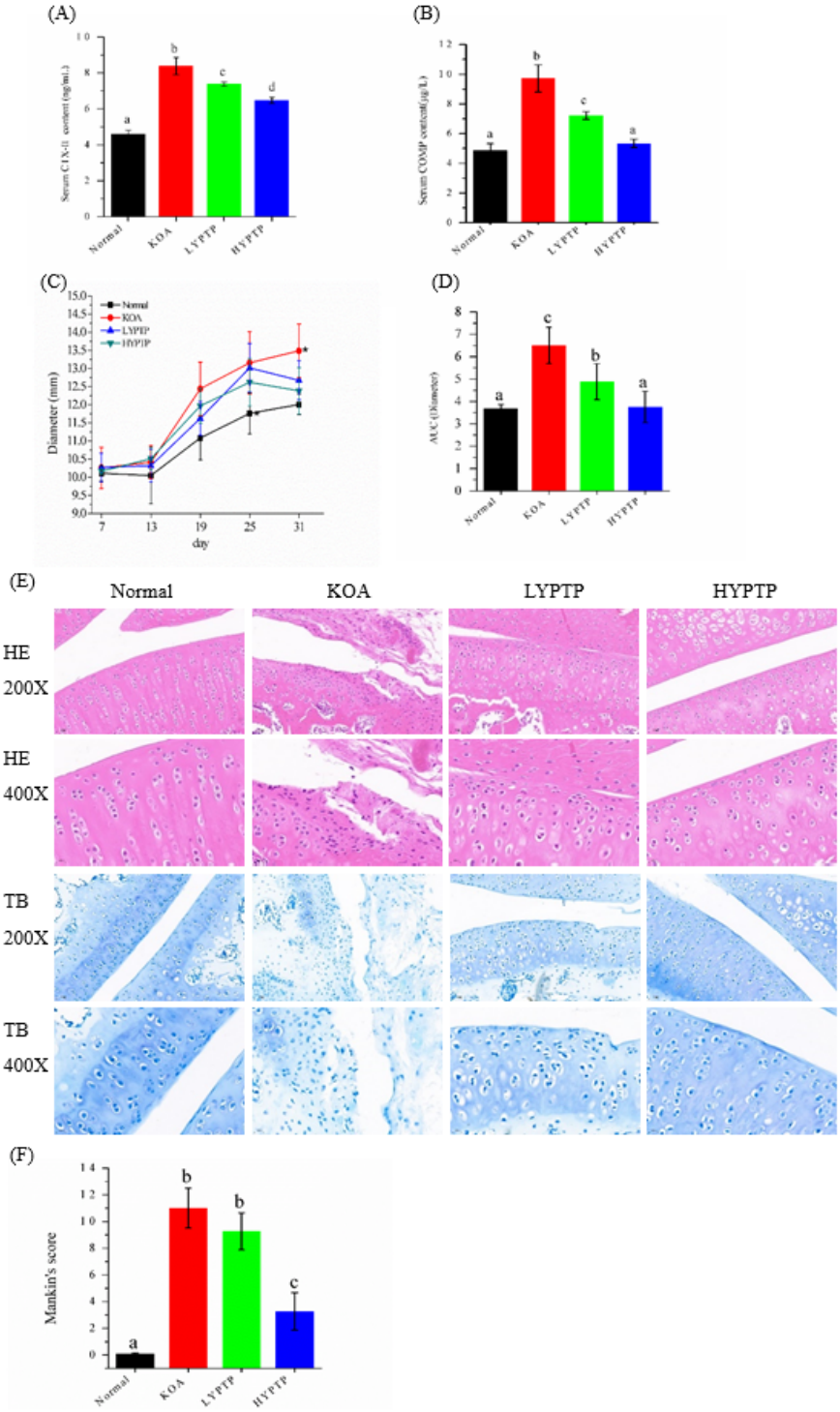

Figure 2

Effects of YPTP on serum CTX-II (A) and COMP (B) in papain-induced KOA rats. Data are expressed as mean ± standard deviation (SD) (n = 8). Different letters indicate significant differences ($P < 0.05$). (C) Effects of YPTP on joint diameter. (D) The area under the curve (AUC) is the quantification of joint diameter of Fig. C. Data are expressed as the means ± SD (n = 8), * indicates $P < 0.05$. (E) Effects of YPTP on the articular cartilage. Histopathological alterations were evaluated by H&E and TB staining.

Images were acquired at a magnification of 200x and 400x. (F) The modified Mankin scores. Different letters above the bar indicate significant differences defined as $P < 0.05$.

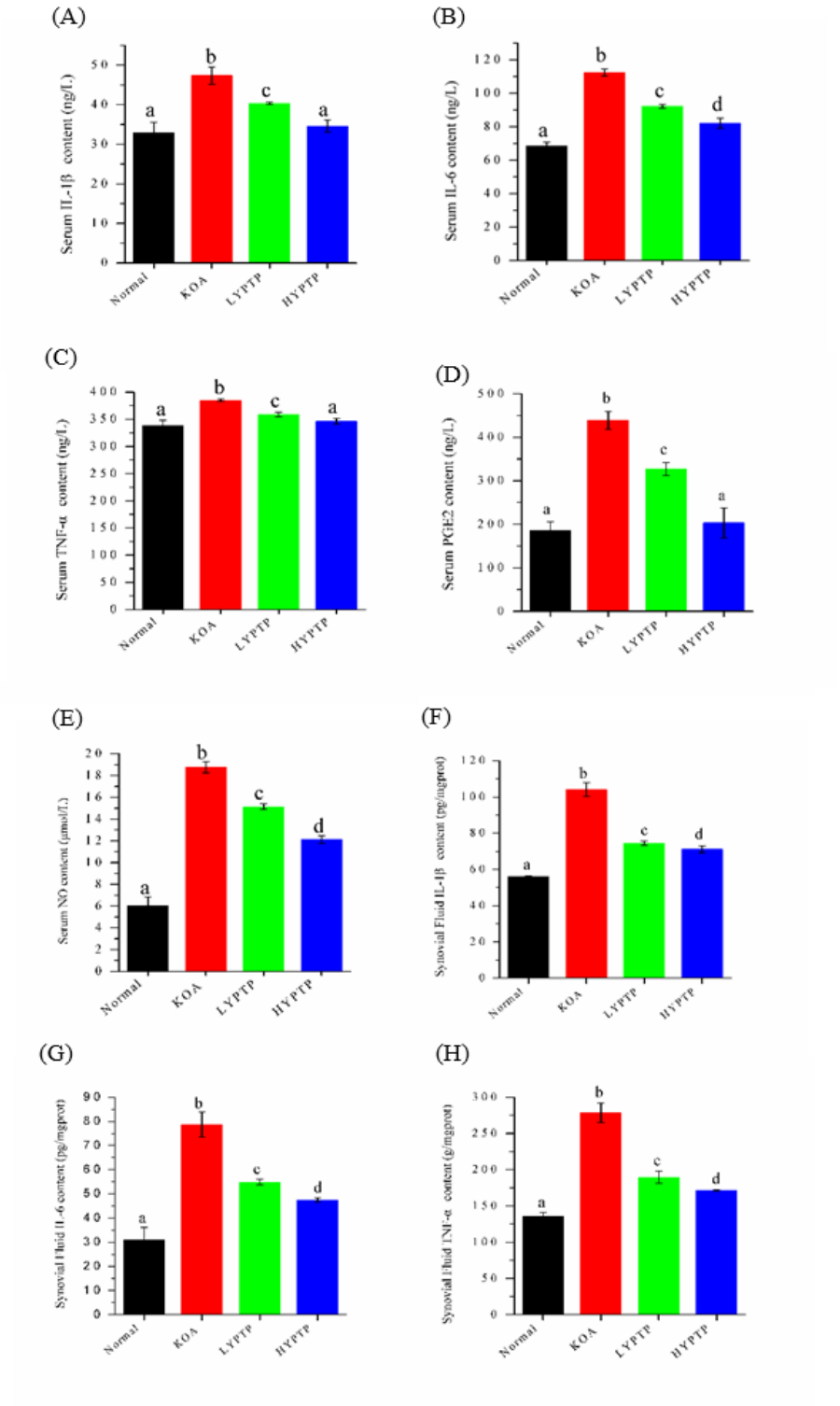

Figure 3

Effects of YPTP on serum IL-1β (A), IL-6(B), TNF-α (C), PGE2 (D),NO (E) and synovial fluid IL-6 (D), S IL-1β (E) and TNF-α (F) in papain-induced KOA rats. Data are expressed as means ± SD (n = 8). Different letters

indicate statistical significance defined as $P < 0.05$.

Figure 4

A-D: Representative images of immunohistochemical (IHC) staining of COX-2, iNOS, IL-1β and TNF-α. E-H: The semi-quantitative results of COX-2, iNOS, IL-1β and TNF-α are shown as means ± SD (n = 8). I-J: Representative images of IHC staining of COLLII and MMP-3. K-L: The semi-quantitative results of COLLII and MMP-3. Data are shown as means ± SD (n = 8). Different letters indicate statistical significance defined as $P < 0.05$.

Supplementary Files

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- PD1.png