

The Protective Effect of Selenium Nanoparticles in Osteoarthritis: In vitro and in vivo Studies

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Introduction: Osteoarthritis (OA) is a common chronic joint disease characterized by articular cartilage degeneration. OA usually manifests as joint pain, limited mobility, and joint effusion. Currently, the primary OA treatment is non-steroidal anti-inflammatory drugs (NSAIDs). Although they can alleviate the disease's clinical symptoms and signs, the drugs have some side effects. Selenium nanoparticles (SeNPs) may be an alternative to relieve OA symptoms.

Materials and Results: We confirmed the anti-inflammatory effect of selenium nanoparticles (SeNPs) in vitro and in vivo experiments for OA disease in this study. In vitro experiments, we found that SeNPs could significantly reduce the expression of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), the major inflammatory factors, and had significant anti-inflammatory and anti-arthritis effects. SeNPs can inhibit reactive oxygen species (ROS) production and increased glutathione peroxidase (GPx) activity in interleukin-1beta (IL-1 β)-stimulated cells. Additionally, SeNPs down-regulated matrix metalloproteinase-13 (MMP-13) and thrombospondin motifs 5 (ADAMTS-5) expressions, while up-regulated type II collagen (COL-2) and aggrecan (ACAN) expressions stimulated by IL-1 β . The findings also indicated that SeNPs may exert their effects through suppressing the NF- κ B p65 and p38/MAPK pathways. In vivo experiments, the prevention of OA development brought on by SeNPs was demonstrated using a DMM model.

Discussion: Our results suggest that SeNPs may be a potential anti-inflammatory agent for treating OA.

Keywords: osteoarthritis, NF- κ B p65 signaling pathway, p38/MAPK signaling pathway, selenium nanoparticles

Introduction

Osteoarthritis (OA) is a prevalent chronic degenerative joint disease that is more common in middle-aged and elderly people.¹ Its primary characteristics are subchondral sclerosis, joint swelling, synovitis, and gradual loss of articular cartilage.² The clinical manifestations of OA are slow joint pain, tenderness, stiffness, joint swelling, limited mobility, and joint deformity, which seriously hinders work and affects human life quality.^{3,4} Inflammation and catabolism are closely related to the occurrence and development of OA.⁵ The potential pathogenesis of OA is still elusive.⁶ Articular cartilage degradation is primarily associated with the protein overproduction that breaks down cartilage, including matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and other catabolic enzymes.⁷ Inflammatory factors, such as IL-1 β , play a vital role in bone and joint damage and inflammation.⁸ The production of ADAMTS and MMPs can be increased by IL-1 β , stimulating extracellular matrix (ECM) degradation, and decreasing aggrecan (ACAN) and type II collagen (COL-2) synthesis.^{9,10}

Meanwhile, IL-1 β can induce the release of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which can create nitric oxide (NO) and prostaglandin E2 (PGE2).¹¹ IL-1 β can induce inflammatory cytokines interleukin (IL)-6 and

NO expressions with excessive reactive oxygen species (ROS) production.¹² ROS or free radicals cause oxidative stress, the primary pathophysiology of OA.¹³ Increasing evidence suggests that neutralizing IL-1 β -induced inflammatory mediators may help to treat OA.¹⁴ Patients with advanced OA are treated with total knee replacement, whereas patients with mild disease are mostly treated with nonsteroidal anti-inflammatory drugs (NSAIDs).¹⁵ Although these treatments can improve a patient's range of motion and clinical symptoms, they do not slow or reverse the degenerative process, and long-term side effects are significant.^{16,17} This has prompted researchers to investigate alternate solutions.

Selenium is vital to human health due to its anti-inflammatory, potent antioxidant, and antiviral properties.^{18,19} Selenium provides several health advantages and is crucial for thyroid hormone metabolism, male reproduction, the immune system, anti-inflammatory processes, and removing free radicals.^{20–22} According to studies, a selenium deficiency can cause growth retardation, bone and cartilage metabolism dysfunction, and joint anomalies.²³ Selenium can lessen oxidative stress-induced cartilage damage by enhancing chondrocytes' antioxidant activity, removing ROS, and reducing the negative effects of ROS on cartilage homeostasis.²⁴ Selenium in the form of selenomethionine inhibits the pro-inflammatory genes activated by IL-1 β , thereby lowering the inflammatory response.²⁵ Numerous research has demonstrated the anti-inflammatory properties of selenium.^{26,27} Selenium bioavailability and toxicity are the primary factors restricting its application.²⁸ Selenium nanoparticles (SeNPs) have attracted attention due to their unique physical and chemical properties.²⁹ Nanotechnology has shown its potential to treat OA in drug delivery, biological scaffolds, genetic engineering, and lubricants.³⁰ Nanoscale materials exhibit new properties different from isolated atoms and blocky materials.³¹ The smaller the nanoparticle, the greater its activity.³² With the decrease of size, the ratio of surface to volume increases sharply, which enhances the interaction between nanomaterials and biological systems and improves their functions.³³ In addition to pain relief, nanoparticle therapy is also expected to alleviate cartilage deterioration and even promote regeneration.³⁴ Furthermore, the nanoparticles have excellent bone conductivity, biocompatibility, and biological functions that promote cell adhesion, proliferation, and osteogenic differentiation.³⁵ Previous studies have demonstrated that highly dispersed selenium (called selenium nanoparticles) has similar biological activities and effects to sodium selenite and selenomethionine, yet with lower toxicity and acceptable bioavailability for inducing selenoproteins.³⁶

This study utilizes chondrocytes and a mouse OA model to assess the anti-inflammatory effects of SeNPs generated by the microbial reduction approach. We assessed the anti-inflammatory effect of SeNPs on OA development using a mouse experiment model *in vivo*. We verified the protective effect of SeNPs on chondrocyte damage caused by IL-1 β in this study. We also discuss the mechanism of inhibiting related pathways to delay OA development.

Materials and Methods

Materials and Reagents

Recombinant mouse IL-1 β was purchased from Novoprotein Scientific Inc. (Shanghai, China). Collagenase II, 0.25% trypsin, Penicillin–streptomycin solution (100 x), SYBR Green master mix, and cDNA Synthesis reagent were purchased from Yi Sheng Biotechnology (Shanghai) Co., LTD. Medium (DMEM)/F12 was purchased from Shanghai Xiaopeng Biological Technology Co., LTD. Australia Origin Fetal Bovine Serum was purchased from Hangzhou Ouyuan Biotechnology Co., LTD. Phosphate-buffered saline (PBS) was purchased from Sangon Biotech (Shanghai) Co., Ltd. RNA-Quick Purification kit was purchased from Shanghai Yi Shan Biological Technology Co., LTD. Reactive Oxygen Species (ROS) Assay Kit, Phosphatase inhibitor mixture A, Hoechst 33342 were purchased from Beyotime Institute of Biotechnology. Reduced glutathione (GSH) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute. PageRuler™ Prestained Protein Ladder and Pierce™ BCA were purchased from Thermo Fisher Scientific. EDTA Decalcification solution was purchased from SenBeiJia Biological Technology. A 4% universal tissue fixative was purchased from Wuhan Xavier Biotechnology. A 20 \times TBST Buffer was purchased from Solarbio (Beijing, China). ELISA kits were purchased from Wuhan yunclone Technology. RIPA Lysis Buffer and PMSF were purchased from Hangzhou Verde Biotechnology.

Preparation and Characterization of SeNPs

SeNPs were synthesized by microbial reduction using Na₂SeO₃ as the starting precursor. *Saccharomyces cerevisiae* was inoculated into YPD medium (yeast extract 1%, peptone 2%, glucose 2%) for activation, and then incubated in a shaker

at 30 °C for 12 h, from which 10% was inoculated into a shaker containing 30 mL medium with 100 g/L mother liquor. The shaker was incubated for 12 h, and a certain amount of sodium selenite was added until the final concentration of the medium was 2 g/L. Distilled water was added and the resulting precipitate was mixed evenly. The medium was incubated at 30 °C for 48 h, and then autolysis at 40 °C for 24 h. After ultrasonic crushing, SeNPs were separated by centrifugation. Particle size and zeta potential were measured by dynamic light scattering (Malvern, United States). The morphology of SeNPs particles was observed by transmission electron microscopy (Hitachi, Japan). The particle composition was determined by Energy Dispersion X-ray spectroscopy (Shimadzu, Japan).

Isolation and Culture of Primary Chondrocytes

This study was approved by the Animal Ethical and Welfare Committee of Zhejiang Provincial People's Hospital (Hangzhou, China). All procedures associated with the care of animals were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Wild-type embryonic young mice born 1–2 days ago had their cartilage tissue removed from the knee joint, cut into small pieces, thoroughly washed with sterilized PBS, centrifuged at 1000 rpm for 3 minutes three times, discarded the supernatant, added to 12-well culture plates, and digested with type II collagenase at a concentration of 2 mg/mL for four hours before single cells were collected by short-term centrifugation. In DMEM/F12 with 10% fetal bovine serum and 1% antibiotic (penicillin/streptomycin) at 37 °C and 5% CO₂, chondrocytes were cultivated in a 100 mm petri dish. To prevent phenotypic alterations, F1 cells were subcultured when the cell density reached 80% and employed in the experiment.

Cell Viability Assay

CCK-8 reagent was used to test the impact of SeNPs on primary chondrocyte viability following the manufacturer's recommendations. Chondrocytes were inoculated in 96-well plates at a cell density of 5×10^3 for each well. After 12 h, chondrocytes were treated with different concentrations of SeNPs (10 nM, 20 nM, 50 nM, 100 nM, 200 nM, 400 nM, 800 nM, 1200 nM, 1600 nM, 2000 nM) for 24 h or 48 h. Change to medium containing 10% CCK-8 reagent and incubate away from light, then measure the absorbance at 450 nm with a microplate reader (Synergy LX, BioTek, United States).

Glutathione Peroxidase Activity

Glutathione peroxidase (GPx) activity in cell lysates was measured according to the instructions of the kit. The principle of determination is that dithiodinitrobenzoic acid reacts with sulfhydryl compounds to produce a yellow compound, which can be quantified by colorimetric measurement.

Measurement of IL-6, NO, Reactive Oxygen Species

Intracellular ROS production was determined using 2, 7-dichlorodi-hydrofluorescein diacetate (DCFH-DA) fluorescence intensity changes in chondrocytes treated with IL-1 β and different concentrations of SeNPs. The cells were incubated in a serum-free medium containing 10 μ m DCFH-DA at 37 °C for 30 min. The maximum excitation wavelength was 480 nm, and the maximum emission wavelength was 525 nm. The ROS generation was measured by flow cytometry (BD Biosciences, New Jersey, United States) according to the fluorescence signal intensity. The concentration of IL-6 in the cell culture supernatant was determined using an ELISA kit and the level of nitric oxide (NO) in the cell culture supernatant was determined using the Griess Reaction kit according to the instructions.

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from mouse chondrocytes using the RNA-Quick Purification kit according to the manufacturer's instructions and then reverse transcribed into cDNA using cDNA Synthesis reagent, which was diluted 1:10 with nuclease-free water. Real-time quantitative Polymerase Chain Reaction (Rt-qPCR) using the Roche LightCycler 480 Detection System. The real-time PCR program was as follows: initial denaturation at 95 °C for 2 min, denaturation at 95 °C for 15s, annealing at 60 °C for 30s, and extension at 72 °C for 30s, a total of 40 cycles. Target mRNA levels were normalized to GAPDH levels, and the relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method. The primers of COL-2, ACAN, MMP-13, ADAMTS-5, iNOS,

COX-2, and GAPDH were designed by NCBI Primer-Blast tool, which was as follows: COL-2: forward: CAGGATG CCGGAAAATTAGGG, reverse: ACCACGATCACCTCTGGGT; ACAN: forward: GTGGAGCCGTGTTTCCAAG, reverse: AGATGCTGTTGACTCGAACCT; MMP-13: forward: TGTTTGCAGAGCACTACTTGAA, reverse: CAGTCACCTCTAAG CCAAAGAAA; ADAMTS-5: forward: CCACGACCTCAAGAACTTTT, reverse: CTGCATCGTAGTG CTCTTCG; iNOS: forward: GGAGTGACGGCAAACATGACT, reverse: TCGATGCACAACCTGGGTGAAC; COX-2: forward: TTCCAATCCATGTCAAACCGT, reverse: AGTCCGGGTACAGTCACACTT; GAPDH: forward: TGACCTCA ACTACATGGTCTACA, reverse: CTTCCATTCTCGGCCTTG.

Western Blotting Analysis

The mouse chondrocytes were washed with ice-cold PBS twice, and then the protein was extracted from the mixture of RIPA buffer and PMSF (100:1). The protein content was determined by high-speed centrifugation at 4 °C (14,400 rpm, 15 min) after 30 min of ice cracking. The protein concentration of the samples was determined by the BCA protein assay kit. The same amount of protein was separated by prefabricated gel with denatured protein and transferred to the PVDF membrane (Millipore, United States). After sealing the membrane with 5% non-fat milk at room temperature for 2 h, the membrane was washed with TBST three times for 10 min and incubated with primary antibody: COL-2 (diluted 1:3000, #28459-1-AP, Proteintech Group), ACAN (diluted 1:1000, #DF7561, Affinity, Cincinnati, OH, USA), MMP-13 (diluted 1:2500, #18165-1-AP, Proteintech Group), anti-ADAMTS-5 (diluted 1:1000, ab41037, Abcam Corporation, USA), anti-iNOS (diluted 1:1000, ab178945, Abcam Corporation, USA), COX-2 (diluted 1:2000, #12375-1-AP, Proteintech Group), GAPDH (diluted 1:50,000, #60004-1-IG, Proteintech Group), p38 MAPK (diluted 1:1000, #14064-1-AP, Proteintech Group), Phospho-p38 MAPK (diluted 1:2000, #28796-1-AP, Proteintech Group), IκBα (diluted 1:2000, #10268-1-AP, Proteintech Group), anti-p-IκBα (diluted 1:1000, ab133462, Abcam Corporation, USA), NF-κB p65 (diluted 1:2000, #10745-1-AP, Proteintech Group), anti-NF-κB p-p65 (diluted 1:1000, ab76302, Abcam Corporation, USA), Lamin B1 (diluted 1:3000, #12987-1-AP, Proteintech Group), at 4 °C overnight. The membrane was washed with TBST three times for 10 min each and incubated with the secondary antibody (goat Anti-Rabbit IgG, diluted 1:3000, #ZB-2301, Zhongshan Golden Bridge Biotechnology; goat Anti-Mouse IgG, diluted 1:50,000, #HA1006, HuaAn Biotechnology) at room temperature for 2 h. Then, the membranes were washed with TBST three times, reactive protein bands were found using enhanced chemiluminescence (ECL), and the intensity of these membranes was determined using ChemiDoc™ MP Imaging System (Bio-Rad, United States).

Immunofluorescence Assay

F1 generation chondrocytes were cultured in confocal dishes for 12 h, treated with IL-1β and SeNPs or without SeNPs for 24 h, washed once with PBS, then fixed with 4% paraformaldehyde for 15 min, washed three times with PBS, and washed for 3 min each time. The samples were incubated with 0.2% Triton X-100 (prepared with PBS) for 30 min, then added with 5% normal goat serum, and blocked for 30 min at room temperature. Chondrocytes were mixed with COL-2 (1:100), and MMP-13 (1:100) antibody was incubated at 4 °C overnight. On the next day, the cells were washed with PBS, incubated with fluorescein-conjugated goat anti-rabbit IgG antibody (diluted 1:200, #ZF-0311, Zhongshan Golden Bridge) for 1 h, then stained with Hoechst for 15 min, washed with PBS for 3 times, and observed under the laser confocal microscopy (Leika TCS SP8 Confocal, LEIKA Inc, California, United States).

Mice OA Models

Thirty 8-week-old C57BL/6 male wild type (WT) mice were purchased from Slack Laboratory Animal Co., LTD., Shanghai. All experiments were carried out by the requirements of the animal Ethics and Use Committee of Zhejiang Provincial People's Hospital. All procedures associated with the care of animals were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. The medial meniscus (DMM) was surgically destroyed, and the tibial ligament of the medial meniscus was severed to establish a mouse model of osteoarthritis. Then, they were randomly divided into the sham control group (n=10), the OA group (n=10), and the SeNPs treatment group (n=10). Four weeks after the DMM operation, 5μL solution containing 50 nM SeNPs was injected into the knee joint of the mouse in the SeNPs treatment

group, and the administration lasted for 5 weeks every week. All mice were sacrificed 8 weeks after DMM surgery to preserve joint tissue for further analysis.

Experimental Design

For measuring the cartilage protection of various concentrations of SeNPs in vitro, chondrocytes were treated with IL-1 β and different concentrations of SeNPs (20 nM, and 50 nM). The negative control group was treated with 5 ng/mL IL-1 β , while the blank control group received no treatment. The cells were taken 24 h after being incubated.

For in vivo experiments, the medial collateral ligament and medial meniscus of the mouse knee joint were removed as previously mentioned, destabilizing the joint stability of experimental animals. Each mouse in the OA and SeNPs therapy groups had the medial meniscus of their knee joint surgically destabilized. In the sham control group, the knee joint was cut without removing the medial collateral ligament and the medial meniscus. Every five days, SeNPs were injected into the joint cavity in the therapy group. Sterile normal saline was administered identically to both the OA group and the sham control group. For specimen preparation and histological examination, joint tissue was saved.

Histopathologic Analysis and Immunohistochemistry

Samples obtained from mouse knee joints were immobilized in 4% paraformaldehyde for 48 h and then recalcified in 10% EDTA solution for 4 weeks. Then, the tissues were embedded in paraffin wax and cut into slices with a thickness of 6 μ m. The sections were stained with hematoxylin and Safranin-O/Fast Green and the histological changes of stained sections were evaluated by an optical microscope and the International Society for the Study of Osteoarthritis (OARSI) scoring system. COL-2 and MMP-13 were incubated at 4 °C for the duration of the night in immunohistochemistry. After that, tissue sections were treated with secondary antibodies for two hours at room temperature. The sections were examined and photographed under an optical microscope (Nikon Ti, Japan). Calculate the percentage of positive cells by counting the number of stained cells that are positive across the entire joint surface of each specimen.

Statistical Analysis

All data are presented as mean \pm standard deviation. Multiple groups of data were compared using the one-way analysis of variance (ANOVA). Comparisons between the two sets of data were made using the Student's *t*-test. A *P*-value < 0.05 is considered to be a significant difference.

Results

Particle Characterization of SeNPs

We measured the size of nanoparticles using dynamic light scattering to investigate the particle characteristics of SeNPs. According to findings, SeNPs have an average particle size of 222.9 nm (Figure 1A). The Zeta potential of SeNPs was measured with an average value of -18.7 mV (Figure 1B). Transmission electron microscopy indicated that nanoparticles were spherical (Figure 1C), and the particles were monodispersed in the solution without agglomeration. Energy Dispersion X-ray spectroscopy exhibits that the particles primarily comprise selenium (Figure 1D), indicating that the synthesized nanoparticles had high purity. These results lay a foundation for the follow-up experiments.

Effect of SeNPs on the Viability of Mouse Chondrocytes

Chondrocytes were exposed to a range of concentrations (10 nM, 20 nM, 50 nM, 100 nM, 200 nM, 400 nM, 800 nM, 1200 nM, 1600 nM, and 2000 nM) of SeNPs for 24 or 48 h to assess the cytotoxic effects of SeNPs on mouse chondrocytes. The CCK-8 assay was used to measure cell viability. SeNPs have no cytotoxicity to chondrocytes after 24 h of treatment when the concentration is less than 50 nM. In contrast, cell viability was significantly inhibited at concentrations of 100 nM or higher (Figure 2A and Figure 2B). Similar results were observed when chondrocytes were treated with SeNPs for 48 h compared to untreated cells (*P* < 0.05). These results indicated that SeNPs lower concentrations do not affect the cell vitality of chondrocytes. Therefore, 20 nM and 50 nM of SeNPs were selected for subsequent experiments.

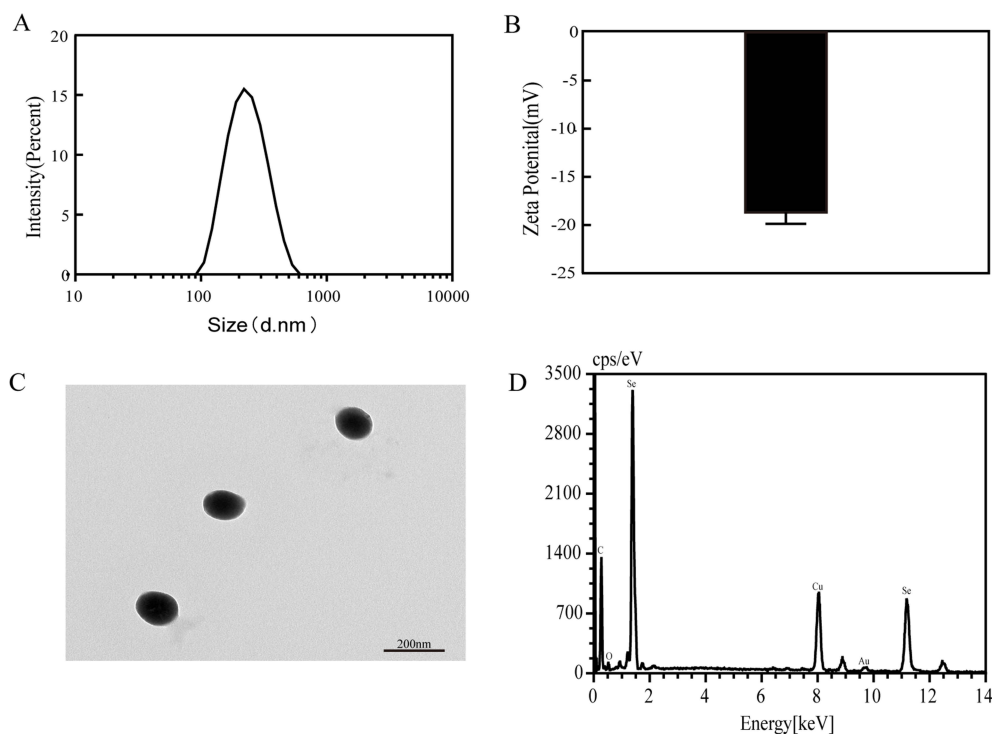


Figure 1 Particle characterization of SeNPs. **(A)** The corresponding size distribution of SeNPs. **(B)** The average zeta potential value of SeNPs. **(C)** A transmission electron microscopy image of SeNPs. **(D)** Energy Dispersion X-ray spectroscopy (EDX) of SeNPs. **Abbreviation:** SeNPs, selenium nanoparticles.

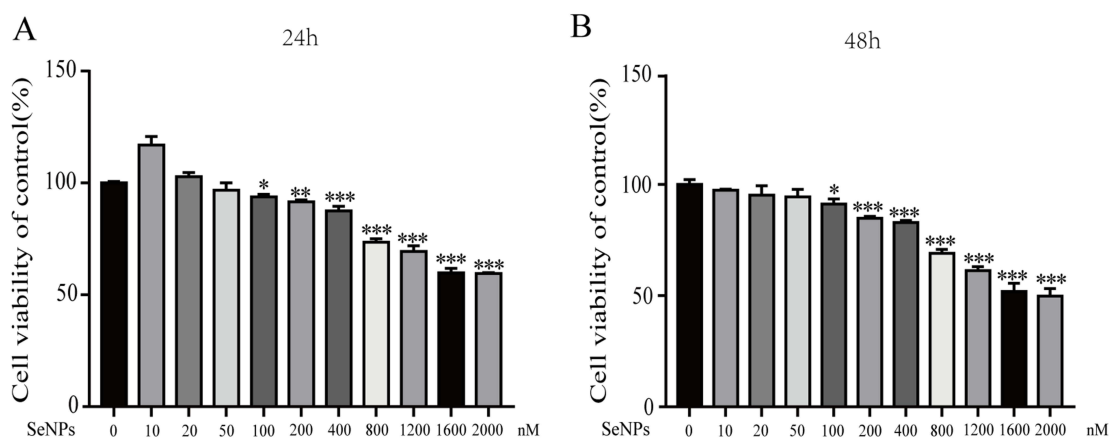


Figure 2 Effect of SeNPs on the viability of mouse chondrocytes. Cells were treated with different concentrations of SeNPs (10 nM, 20 nM, 50 nM, 100 nM, 200 nM, 400 nM, 800 nM, 1200 nM, 1600 nM, 2000 nM) for 24 h **(A)** or 48 h **(B)** and analyzed using a CCK-8 assay. All data are presented as mean \pm standard deviation ($n=3$). * $P < 0.05$ vs the control group; ** $P < 0.01$ vs the control group; *** $P < 0.001$ vs the control group. **Abbreviation:** SeNPs, selenium nanoparticles.

Effect of SeNPs on ROS Generation and Glutathione Peroxidase Activity

Oxidative stress is an imbalance between the production of ROS and scavenging by the antioxidant defense system and substantially impacts OA.³⁷ IL-1 β was used for stimulating the generation of ROS and oxidative stress in chondrocytes to explore whether SeNPs could alleviate oxidative stress. Chondrocytes treated with IL-1 β exhibited a significantly higher ROS production than untreated controls (**Figure 3A**). However, SeNPs subdued the ROS production induced by IL-1 β . Glutathione peroxidase (GPx), a crucial part of the body's antioxidant defense mechanism, is strongly regulated by selenium in its expression and activity.³⁸ Then, we detected GPx activity to confirm the effect of SeNPs on GPx in chondrocytes after treatment with or without IL-1 β . The results revealed that GPx activity was dramatically suppressed after IL-1 β administration compared to the

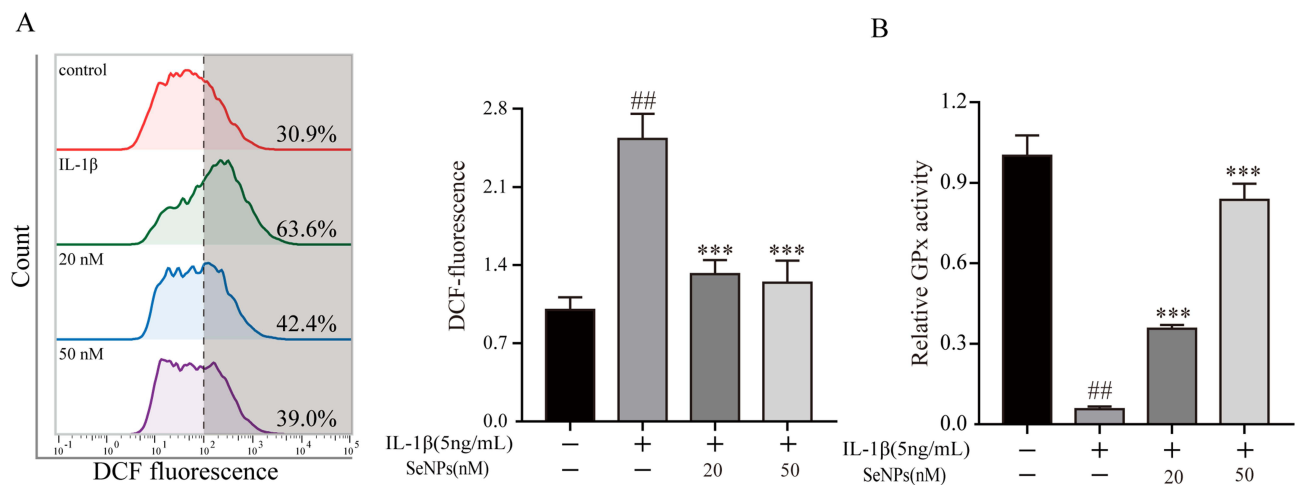


Figure 3 Effects of SeNPs on the level of reactive oxygen species (ROS) and the activity of glutathione peroxidase (GPx) in mouse chondrocytes induced by IL-1 β . **(A)** Intracellular ROS levels were detected by DCFH-DA staining and flow cytometry. **(B)** The activity of GPx was determined by the glutathione (GSH) assay kit. All data are presented as mean \pm standard deviation ($n=3$). ## $p < 0.0001$ vs compared with the control group; *** $p < 0.001$ vs compared with IL-1 β group.

Abbreviations: SeNPs, selenium nanoparticles; IL, interleukin; ROS, reactive oxygen species; GPx, glutathione peroxidase; DCFH-DA, 2, 7-dichlorodihydrofluorescein diacetate.

control group. However, SeNPs can significantly reduce the inhibitory effect of IL-1 β on GPx activity (Figure 3B). These results suggest that SeNPs can counteract excessive ROS generation and increase GPx activity in chondrocytes.

Effects of SeNPs on IL-1 β -Induced ECM Synthesis and Degradation in Mouse Chondrocytes

MMPs and ADAMTS overexpression leads to ECM degradation, resulting in reduced collagen and aggrecan synthesis in the pathogenesis of OA.³⁹ Western blot and Rt-qPCR analysis were conducted to evaluate whether SeNPs have an impact on the expression of COL-2, ACAN, MMP-13, and ADAMTS-5 with or without IL-1 β treatment to confirm whether SeNPs can protect against IL-1 β -induced ECM degradation in chondrocytes. After IL-1 β treatment, mRNA and protein expression of ADAMTS-5 and MMP-13 increased significantly, and SeNPs can decrease their up-regulation caused by IL-1 β (Figure 4A–E). Meanwhile, SeNPs dramatically decreased the mRNA and protein downregulation of COL-2 and ACAN caused by IL-1 β stimulation. Immunofluorescence results consistently presented that SeNPs markedly inhibited COL-2 protein degradation and MMP-13 expression (Figure 4F and Figure 4G). Thus, these results reveal that SeNPs prevent ECM degradation in chondrocytes.

Effect of SeNPs on IL-6, NO, iNOS, and COX-2 Expression of Mouse Chondrocytes

Next, we investigated the effects of SeNPs on IL-1 β -induced production of inflammatory mediators, including COX-2, iNOS, IL-6, and NO, in chondrocytes. The cells were treated with different concentrations of SeNPs combined with IL-1 β for 24 h. Griess reaction and ELISA kit were used to determine the concentrations of endogenous NO and IL-6 in the cell suspension. The results revealed that NO and IL-6 levels decreased significantly dose-dependently when combined with SeNPs compared to chondrocytes treated with IL-1 β alone (Figure 5A). Then, we evaluated the effect of SeNPs on iNOS and COX-2 expressions in IL-1 β -stimulated mouse chondrocytes using Western blot and Rt-qPCR. The findings demonstrated that IL-1 β dramatically boosted COX-2 and iNOS expressions at the mRNA and protein levels. However, iNOS and COX-2 expressions produced by IL-1 β were reduced by SeNPs in a dose-dependent manner (Figure 5B and Figure 5C). These results indicated that SeNPs could inhibit the production of IL-1 β -induced inflammatory mediators and cytokines in chondrocytes in a concentration-dependent manner.

Effects of SeNPs on IL-1 β -Induced NF- κ B and P38/MAPK Pathways

We examined the phosphorylation status of signaling proteins in primary IL-1 β -stimulated chondrocytes to determine whether the inhibitory effect of SeNPs was associated with IL-1 β -induced changes in signaling pathways. Western blot demonstrated that IL-1 β stimulates translocation of NF- κ B p65 from the cytoplasm to the nucleus and I κ B α degradation in chondrocytes. SeNPs significantly inhibited the IL-1 β regulation on mouse chondrocytes dose-dependently (Figure 6A). The p38/MAPK

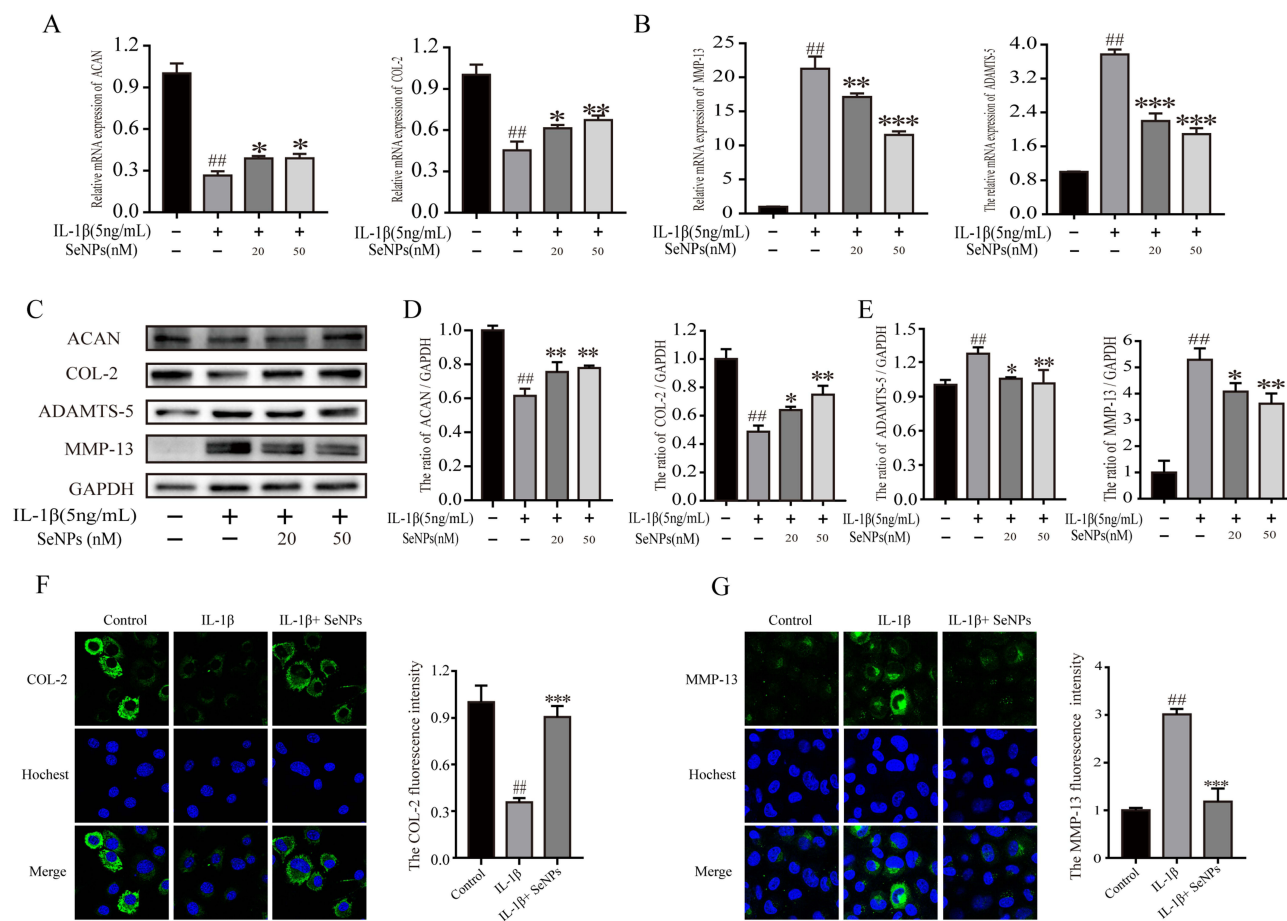


Figure 4 Effect of SeNPs on extracellular matrix degradation of mouse chondrocytes induced by IL-1 β . **(A and B)** The mRNA expression levels of ACAN, COL-2, ADAMTS-5, and MMP-13 in mouse chondrocytes were measured by Rt-qPCR. **(C–E)** The protein expression of ACAN, COL-2, ADAMTS-5, and MMP-13 was examined by Western blot analysis. **(F and G)** Immunofluorescence was used to assess the amounts of COL-2 and MMP-13 protein expression. All data are presented as mean \pm standard deviation (n=3). ^{###}*P* < 0.0001 vs compared with the control group; ^{*}*P* < 0.05 vs compared with IL-1 β group; ^{**}*P* < 0.01 compared with IL-1 β group; ^{***}*P* < 0.001 vs compared with IL-1 β group. **Abbreviations:** SeNPs, selenium nanoparticles; IL, interleukin; Rt-qPCR, Real-time quantitative Polymerase Chain Reaction; ACAN, aggrecan; COL-2, type II collagen; ADAMTS, a disintegrin and metalloproteinase thrombospondin motifs; MMP, matrix metalloproteinase.

pathway is also activated when IL-1 β stimulates OA chondrocytes, resulting in MMPs production and inflammatory mediator release. IL-1 β greatly increased p38 phosphorylation, while SeNPs treatment suppressed it (Figure 6B). Consequently, SeNPs may regulate NF- κ B and p38/MAPK signaling pathways to limit MMPs expression and act as an anti-inflammatory.

Histopathological Analysis and Morphological Evaluation

DMM OA model was established in mice to study whether SeNPs have a protective effect on the cartilage developed by OA in vivo. The sham surgery control group's cartilage surface was stained with Safranin-O and hematoxylin-eosin, exhibiting a smooth and complete cartilage surface (Figure 7A), while the OA group's cartilage surface was severely damaged. Aggrecan decreased significantly, and cell density decreased. Comparatively, the SeNPs treatment group alleviated the destruction of articular cartilage, maintained aggrecan in cartilage, and reduced the damaging effects. The Safranin O staining results revealed that the OARSI score of the OA group was significantly higher than that of the sham operation control group, whereas the OARSI score of the SeNPs group was significantly lower than that of the OA group (Figure 7B). Immunohistochemistry was also used to assess COL-2 and MMP-13 expressions in the cartilage matrix. COL-2 positive cells were reduced, MMP-13 positive cells increased in the OA group compared to the sham surgery control group. After receiving a SeNPs treatment, COL-2 positive cells rose, while MMP-13 positive cells reduced (Figure 7C and Figure 7D). The results demonstrated that SeNPs could effectively shield chondrocytes of arthritis mice, reduce the loss of ECM, promote ECM synthesis protein (COL-2) expression, and inhibit the ECM degrading enzyme (MMP-13) expression.

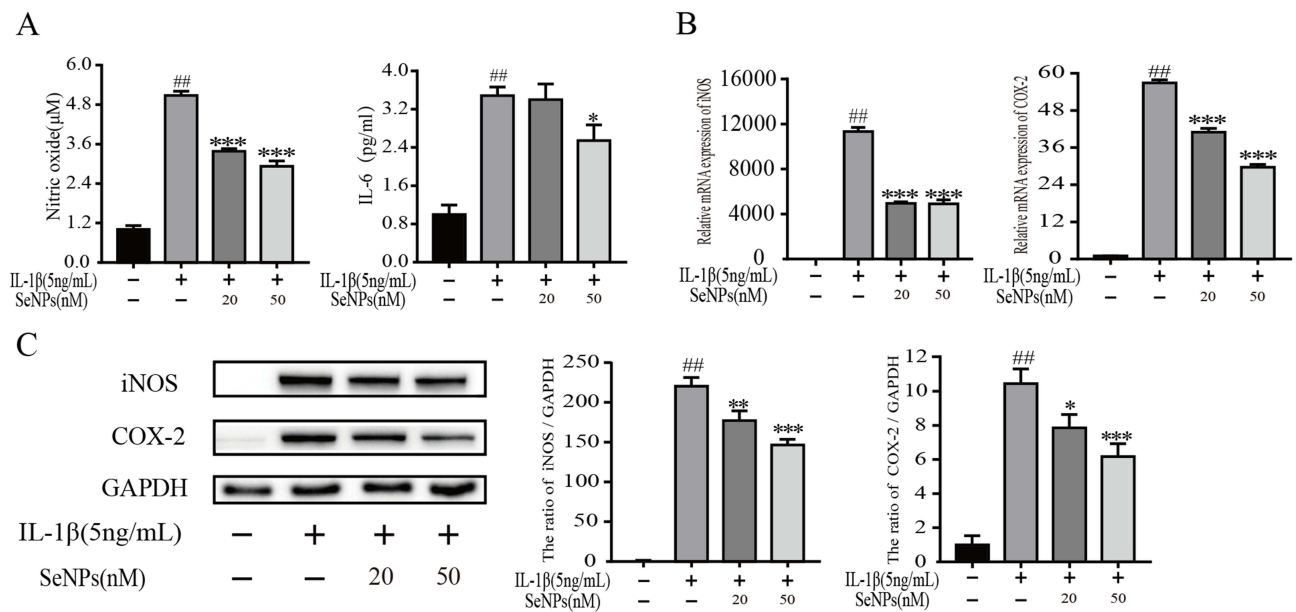


Figure 5 Effects of SeNPs on the expression of NO, iNOS, COX2, and IL-6 in mouse chondrocytes induced by IL-1β. **(A)** The production of IL-6 was measured by ELISA and NO was detected by Griess. **(B)** The mRNA expression levels of iNOS and COX-2 were measured by Rt-qPCR. **(C)** The protein expression of iNOS and COX-2 assessed by Western blot and quantification analysis. All data are presented as mean ± standard deviation (n=3). ^{##}P < 0.0001 vs compared with the control group; ^{*}P < 0.05 vs compared with IL-1β group; ^{**}P < 0.01 compared with IL-1β group; ^{***}P < 0.001 vs compared with IL-1β group.

Abbreviations: SeNPs, selenium nanoparticles; IL, interleukin; Rt-qPCR, real-time quantitative Polymerase Chain Reaction; NO, nitric oxide; iNOS, nitric oxide synthase; COX-2, cyclooxygenase-2.

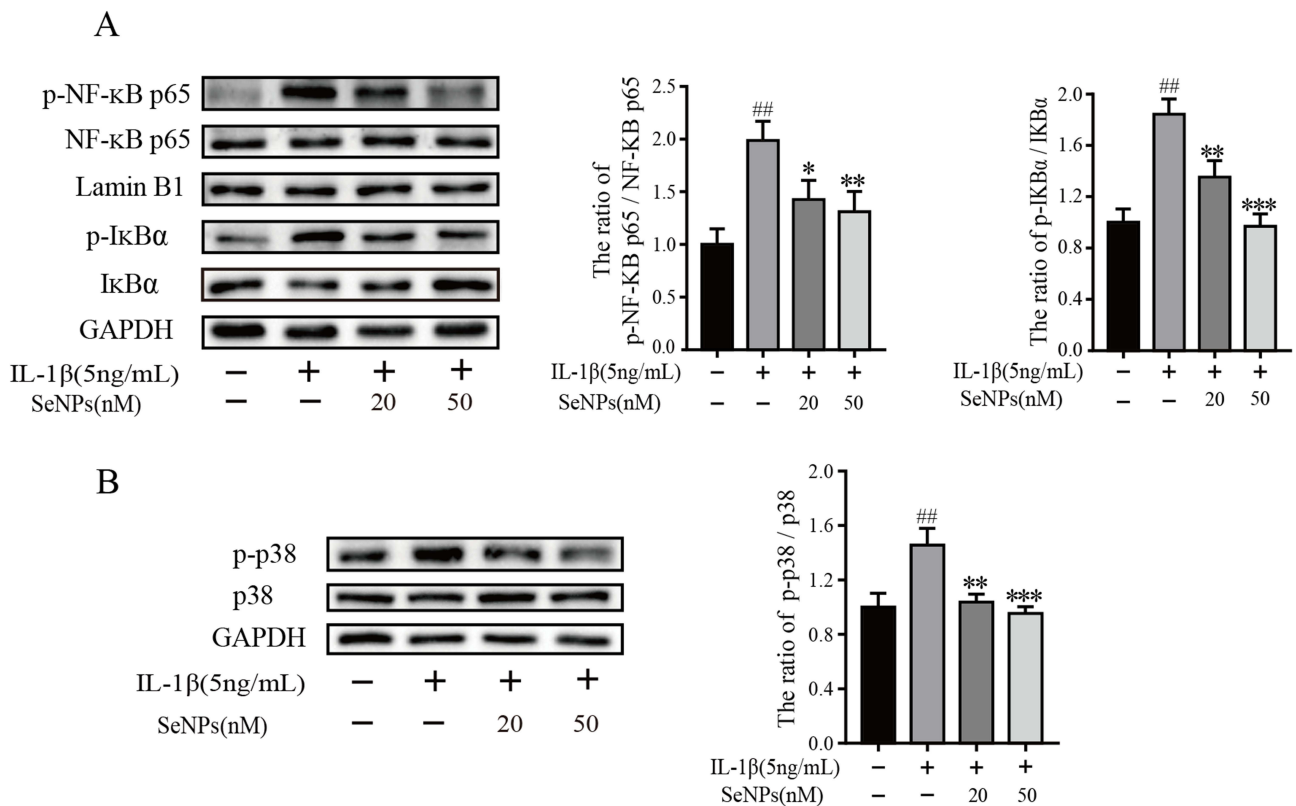


Figure 6 Effect of SeNPs on IL-1β-induced NF-κB and p38 signaling pathways. Chondrocytes were pretreated with different concentrations of SeNPs for 2 h and then stimulated with IL-1β (5 ng/mL) for 30 min. The expression of phosphorylated NF-κB p65 (p-NF-κB p65), total NF-κB p65, phosphorylated 38 (p-p38), total p38, phosphorylated IκBα (p-IκBα), total IκBα were determined by Western blot and quantification analysis **(A and B)**. All data are presented as mean ± standard deviation (n=3). ^{##}P < 0.0001 vs compared with the control group; ^{*}P < 0.05 vs compared with IL-1β group; ^{**}P < 0.01 compared with IL-1β group; ^{***}P < 0.001 vs compared with IL-1β group.

Abbreviations: SeNPs, selenium nanoparticles; IL, interleukin; IκB, inhibitor of kappa B; NF-κB, nuclear factor κB.

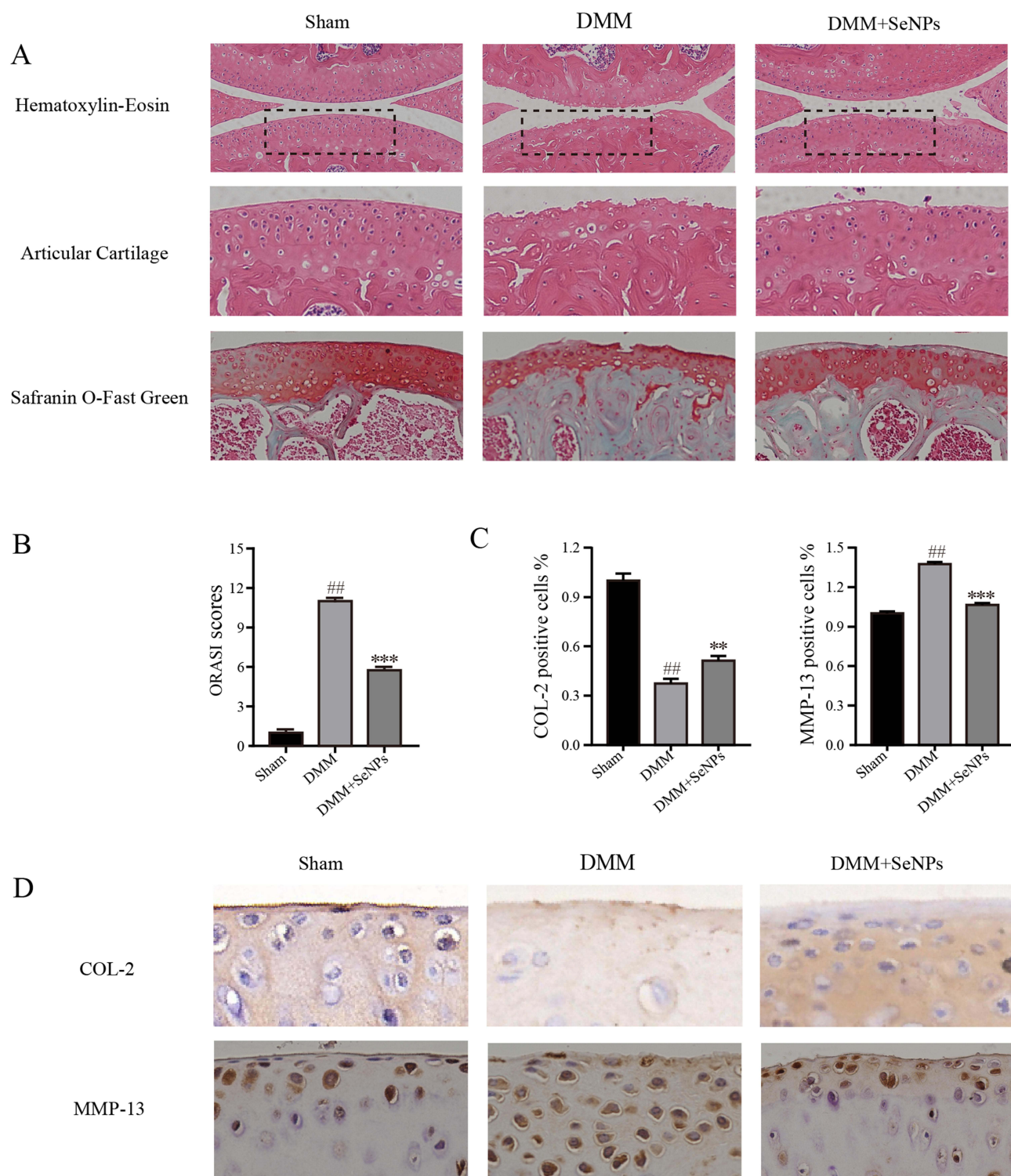


Figure 7 SeNPs alleviated the development of OA in a surgically induced mouse DMM model. **(A)** Histological analysis was performed by safranin O staining, Haematoxylin-Eosin, and evaluation of OA. Stain, H-E, safranin O, magnification, 10 \times ; scale bar, 200 μ m. **(B)** Sham surgery, DMM, and DMM + SeNPs were scored by the International Society for Osteoarthritis Research (OARSI). **(C and D)** The effects of SeNPs on cartilage matrix degradation in OA model mice were evaluated by immunohistochemistry of COL-2 and MMP-13. Stain, immunohistochemistry, magnification, 40 \times ; scale bar, 50 μ m. All data are presented as mean \pm standard deviation (n=10). ^{##}*P* < 0.0001 vs Sham group; ^{**}*P* < 0.01 vs DMM group; ^{***}*P* < 0.001 vs DMM group.

Abbreviations: SeNPs, selenium nanoparticles; OA, osteoarthritis; DMM, destabilization of the medial meniscus; COL-2, type II collagen; MMP, matrix metalloproteinase; DMM, the destabilization of medial meniscus.

Discussion

OA, a common chronic degenerative joint ailment, is primarily caused by an imbalance between catabolic and anabolic processes.⁴⁰ Currently, the primary goal of OA treatment is to reduce OA patients' complaints of joint pain, swelling, and dysfunction.^{41,42} Other OA medications and NSAIDs are only momentarily beneficial, and excessive use might have negative side effects.⁴³ Therefore, it is necessary to discover safe and effective strategies to alleviate the clinical symptoms of OA. Nanoparticle technology, widely used in the healthcare industry, can reduce the negative effects on the body.⁴⁴ Micronutrient selenium, needed for chondrogenesis, has anti-inflammatory properties.⁴⁵ Selenium is a dual-purpose element since it has slightly greater toxic concentrations than those needed for basic physiological processes.⁴⁶ The toxicity of high concentrations limits the long-term use of selenium products to treat Kashin-Beck disease, osteoarthropathy, or other illnesses.⁴⁷ Meanwhile, nano-sized elemental selenium exhibits potential biological effects and reduces the risk of selenium toxicity.⁴⁸ Therefore, this study created a type of SeNPs utilizing less harmful and more bioavailable microorganisms. We confirmed that the effect of SeNPs (0–50 nM) on mouse OA chondrocytes could not be attributed to cytotoxicity. We discovered that SeNPs significantly inhibited iNOS, COX-2, MMP-13, and ADAMTS-5 expressions in IL-1 β -induced inflammatory responses, promoting COL-2 and ACAN synthesis in chondrocytes. Furthermore, our findings demonstrated that SeNPs could effectively inhibit NF- κ B p65 activation and p38/MAPK phosphorylation caused by IL-1 β and had a protective effect on cartilage degradation in mice with DMM-induced OA.

ROS are oxygen-containing free radicals, including superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH).⁴⁹ Previous research has demonstrated that OA progression is significantly related to oxidative stress.⁵⁰ High ROS concentrations can cause oxidative stress, resulting in mitochondrial dysfunction and a breakdown of the epigenetic controls over cell signaling and gene expression.^{51,52} Oxidative stress causes inflammation, chondrocyte apoptosis, cartilage degradation, subchondral bone dysfunction, and unbalanced bone resorption and remodeling.^{53,54} When ROS production increases, the cellular defense systems against oxidative stress are activated, effectively removing ROS molecules from the cell.⁵⁵ GPxs are a family of selenium-dependent and independent antioxidant enzymes that catalyzes the conversion of harmful H₂O₂ and numerous hydroperoxides, including lipid peroxide and DNA peroxide, into water and alcohol, respectively.⁵⁶ This process frequently employs GSH as a reducing agent, shielding biofilm and cellular components from oxidative stress.⁵⁷ We stimulated chondrocytes with IL-1 β to decrease glutathione concentration and increase ROS levels. When OA chondrocytes were treated with SeNPs, we observed a significant decrease in ROS levels while increasing GPx activity, indicating that SeNPs may protect chondrocytes by reducing ROS production while increasing GPx activity.

ACAN and COL-2 are the primary structural components of the chondrocyte extracellular matrix, and they are crucial to preserve structural integrity.⁵⁸ ACAN and COL-2 degradation accelerate OA development.⁵⁹ Therefore, OA might be treated by preventing ACAN and COL-2 degradation. MMPs are crucial regulators in OA development, preventing ACAN and COL-2 synthesis.⁶⁰ Among all MMPs, MMP-13 plays a major role in OA progression due to its ability to break down COL-2, constituting the primary structure of the cartilage ECM.⁶¹ ADAMTS family plays a key role in cartilage degradation in OA.⁶² ADAMTS-5 is a key enzyme associated with arthritis and participates in the cleavage of ACAN in OA.⁶³ IL-1 β is a proinflammatory cytokine widely used to stimulate the OA microenvironment in vitro.⁶⁴ We evaluated the protective impact of SeNPs on IL-1 β -induced chondrocyte damage using IL-1 β to produce an oxidative stress microenvironment. Our findings demonstrated that chondrocytes treated with IL-1 β dramatically raised MMP-13 and ADAMTS-5 mRNA and protein expression levels, while ACAN and COL-2 mRNA and protein levels dropped. SeNPs can increase ACAN and COL-2 synthesis while inhibiting MMP-13 and ADAMTS-5 synthesis in cells.

According to a report, IL-1 β promotes iNOS and COX-2 expressions in OA chondrocytes, which are responsible for NO and PGE2 up-regulation, respectively.⁶⁵ The inflammatory mediators, NO and PGE2, are crucial in OA development.⁶⁶ Previous research has demonstrated that decreasing inflammatory cytokines, including NO and PGE2, can weaken OA progression because NO and PGE2 can inhibit ECM synthesis and induce ECM degradation.^{67,68} Our results revealed that SeNPs inhibited IL-1 β -induced NO and IL-6 production, as well as iNOS and COX-2 expression in chondrocytes. These findings suggested that SeNPs had anti-inflammatory effects on IL-1 β stimulated chondrocytes.

NF- κ B exists in the cytoplasm and binds to NF- κ B (I κ B) repressor.⁶⁹ IL-1 β stimulation can trigger phosphorylation of I κ B α and p65, I κ B protein is degraded by phosphorylation, and NF- κ B p65 is transferred from cytoplasm to the nucleus to regulate inflammatory mediator production.⁷⁰ The MAPKs family is divided into at least three components: extracellular signal-regulated kinase 1/2 (ERK 1/2), c-Jun interterminal kinase (JNK), and p38/MAPK, activated by phosphorylation of

specific tyrosine and threonine residues by upstream kinases in response to inflammation and other extracellular signals.^{71–73} According to previous studies, NF- κ B and MAPK are the most important transcription factors in regulating MMPs, COX-2, and iNOS expressions in OA.^{74,75} IL-1 β can easily activate the MAPK pathway (ERK, P38, and JNK) and NF- κ B p65 phosphorylation, inducing inflammatory mediator expressions and leading to ECM degradation.⁷⁶ In this study, we treated chondrocytes with IL-1 β enhanced p38/MAPK and I κ B α phosphorylation, promoting the translocation of NF- κ B p65 from the cytoplasm to the nucleus. However, SeNPs treatment suppressed I κ B α and p38/MAPK phosphorylation and attenuated the nuclear translocation of NF- κ B p65. The results presented that SeNPs could inhibit the inflammatory reaction induced by IL-1 β via NF- κ B and p38/MAPK signals, thus playing a protective role in chondrocytes.

Besides in vitro study, we employed the DMM model to evaluate the protective effect of SeNPs on cartilage degradation in vivo. The DMM model is widely used to evaluate the efficacy of drug treatment for OA due to its mechanical instability.⁷⁷ A weekly intra-articular injection of SeNPs reduces cartilage degeneration during OA progression. The morphological and histological evaluation confirmed knee cartilage degeneration and structural changes were less severe in the SeNPs treatment group than in the control group. These in vivo results are consistent with the in vitro effects of SeNPs and support the consistency and reproducibility of this OA model.

Conclusion

Our results suggest that SeNPs inhibit IL-1 β -induced inflammatory mediator expression by inhibiting NF- κ B and p38/MAPK activation in chondrocytes (Figure 8). Meanwhile, SeNPs can increase the synthesis of COL-2 and ACAN. In vivo experiments, SeNPs can suppress ECM degradation and chondrocyte loss, and reduce the degree of articular cartilage

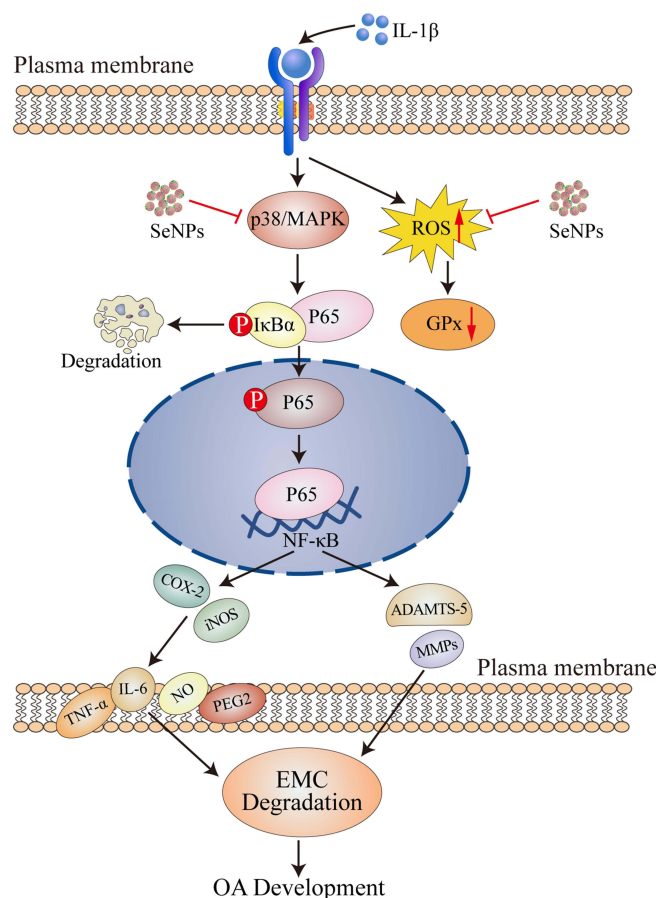


Figure 8 Molecular mechanism of SeNPs on IL-1 β -induced mouse chondrocytes.

Abbreviations: OA, osteoarthritis; SeNPs, selenium nanoparticles; IL, interleukin; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; GPx, glutathione peroxidase; I κ B, inhibitor of kappa B; NF- κ B, nuclear factor κ B; ACAN, aggrecan; COL-2, type II collagen; ADAMTS, a disintegrin and metalloproteinase thrombospondin motifs; MMP, matrix metalloproteinase; NO, nitric oxide; iNOS, nitric oxide synthase; COX-2, cyclooxygenase-2.

irregularity and knee joint stenosis in mice model of OA. These findings suggest that SeNPs may be a potential anti-inflammatory agent for treating OA.

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Disclosure

The authors report no conflicts of interest in this work.

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