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Ameliorating Osteoarthritis in Mice Using Silver Nanoparticles

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Abstract

Knee osteoarthritis (KOA) is one of the most commonly encountered degenerative diseases of the joints in people over 45 years of age. Currently, there are not any effective therapeutics for KOA,and the only end-point strategy is total knee arthroplasty (TKA); therefore, KOA is associated with economic burdens and societal costs. The immune inflammatory response is involved in the occurrence and development of KOA. We previously established a mouse model of KOA using type II collagen. Hyperplasia of the synovial tissue was present in the model, alongside a large number of infiltrated inflammatory cells. Silver nanoparticles have substantial anti-inflammatory effects and have been widely used in tumor therapy and surgical drug delivery. Therefore, we evaluated the therapeutic effects of silver nanoparticles in a collagenase II-induced KOA model. The experimental results showed that silver nanoparticles significantly reduced synovial hyperplasia and the infiltration of neutrophils in the synovial tissue. Hence, this work demonstrates the identification of a novel strategy for OA and provides a theoretical basis for preventing the progress of KOA.

Introduction

Knee osteoarthritis (KOA) is one of the most frequent forms of osteoarthritis and involves a complex disease process in the entire synovial joint[1]. As the world population is gradually aging, the incidence of KOA is increasing substantially. Consistent pain in the knee joint commonly prompts patients with KOA to seek medical treatment. The etiology of the pain in KOA may be related to the inflammatory response, synovial hyperplasia, and cartilage degeneration[2].

The synovium tissues are composed of two types of cells: synovial fibroblasts and macrophages[3,4,5]. Synovial fibroblasts produce synovial fluid. Synovial macrophages are normally dormant and are activated by the inflammatory

response. Initial inflammation of the synovium causes knee joint pain[6].

The synovium tissue inflammatory immune response plays a crucial part in the pathogenesis of KOA. Previous studies have confirmed that there are inflammatory responses in the synovium tissues in KOA, known as synovitis, and the synovitis degree of KOA is closely related to the inflammatory cell infiltration of the synovium tissues[7,8,9]. Synovitis is an inflammatory reaction of the synovium, and its pathological characteristics are the proliferation of synovial cells, new vessel formation, and the infiltration of inflammatory cells[5,10,11].

The goal of KOA treatment is to relieve the inflammatory reaction of the synovium and delay the progression of the disease. Currently, the major clinical drugs for treating KOA are nonsteroidal anti-inflammatory drugs (NSAIDs); however, they exhibit significant side effects, such as nephrotoxicity[12,13]. Intra-articular glucocorticoid injections are another option for treating KOA; however, the glucocorticoid spreads quickly and could be rapidly metabolized by the joint effusion. Meanwhile, diabetic patients with underlying hyperglycemia should be cautious about ongoing steroid injections[14]. In summary, there is no available drug therapeutic strategy for KOA. Therefore, the exploration of new drugs for treating KOA is extremely urgent.

The size of silver nanoparticles is less than 100 nm. Due to their prominent anti-inflammatory, antibacterial, and antioxidant effects, they have been widely utilized invarious aspects of healthcare and medicine, such as wound healing and burn injury[15,16]. They are also used in targeted drug delivery, medical imaging, and molecular diagnosis[17]. Silver (Ag) has greater anti-inflammatory and anti-bacterial action than other metal nanoparticles, such as copper (Cu), zinc (Zn), and iron (Fe)[15]. Silver nanoparticles, a new type of nanomaterial, have broad-spectrum and potent antimicrobial properties. A previous study found that in burn injury and peritonitismouse models[18,19], silver nanoparticles could effectively inhibit the production of inflammatory factors and promote wound healing. A previous study also demonstrated that silver nanoparticles improved the healing of diabetic wounds by promoting the synthesis of growth factors and collagen deposition[20].

Based on the anti-inflammatory effects of silver nanoparticles, we aimed to use silver nanoparticles to treat type II collagen-induced KOA in mice. The results suggested that the number of inflammatory infiltration cells of the synovial joint in mice was significantly reduced with this treatment. The results also suggested that silver nanoparticles could significantly relieve the symptoms of KOA in mice. Therefore, the application of silver nanoparticles may support the development of new treatment options for clinical KOA.

Protocol

All animal work was approved by the Animal Ethical and Welfare Committee (AEWC) of Guangzhou Forevergen Medical Laboratory Animal Center (2018-0186).

1. Establishment of the KOA mouse model

1. Maintain BALB/c mice (18-24 g; 12-14 weeks old) in an environment with 70% humidity and at 26 °C with a light/dark cycle of 12 h. For this experiment, the animals were maintained in the Guangzhou Forevergen Medical Laboratory Animal Center.
2. Use type II collagen to establish a KOA mouse model as described previously[21]. Perform the intra-articular injection as described below.

1. Apply 2% sodium pentobarbital (40 mg/kg) for anesthesia and buprenorphine (0.05 mg/kg, subcutaneous injection) for analgesia. Then, fix the mouse limbs with the tape, remove the hair with the razor, and disinfect with an alternating scrub of 0.1% iodophor and alcohol three times.
2. Wear sterile gloves and use sterile scissors to sequentially expose the skin, subcutaneous tissue, and infrapatellar ligament. Keep the incision area below 0.5 cm.
   NOTE: A heating blanket was used to maintain the body temperature of the mice during the operation.
3. Use a 1 mL insulin syringe to inject 10 U of 30 mg/kg (0.4 mg/mL) type II collagenase into the joint cavity (beneath the infrapatellar ligament)[22].
   NOTE: The angle between the needle and the skin should be approximately 15°; then, the direction of the needle should be changed, and the needle should be withdrawn altogether.
4. After the injection, suture the subcutaneous tissue first, and then the skin. Sterilize the suture area with 0.1% iodophor. Place the mice into the individually ventilated cages (IVCs) separately after they wake up from the anesthesia.

2. Synthesis of silver nanoparticles

NOTE: The preparation of silver nanoparticles has been described previously in detail[19]. The whole formulation process is carried out on ice. After preparation, the mixture is stored at 4 °C; otherwise, the mixture easily solidifies at room temperature.

1. Add a total of 400 µL of collagen type I (4 mg/mL) to a 1.5 mL microcentrifuge tube, and place on ice.
2. Add a total of 200 µL of phosphate-buffered saline (PBS) to the above collagen, mix the solution well, and place it on ice.
3. Finally, add 400 µL of silver nanoparticles to the above solution, and then mix sufficiently. The final concentration of the nanoparticle solution is 1 mM.
   NOTE: The mean diameter of the silver nanoparticles ranges from 5 nm to 15 nm[23]. This was confirmed by electron microscopy.

3. Silver nanoparticle treatment of type II collagenase-induced KOA mice

1. Remove the type II collagenase-induced KOA mice from their cages 1 week later, and inject them with silver nanoparticles. Inject the silver nanoparticles once per week, and collect the specimens 30 days later.
2. Inject a total of 2% sodium pentobarbital (dose: 2 mL/kg) for anesthesia *via* an intraperitoneal injection, and then fix, prepare the skin, and sterilize as described in steps 1.2.
3. Wear sterile gloves and use sterile scissors to sequentially expose the skin, subcutaneous tissue, and knee ligaments.
4. Use a 1 mL insulin syringe, and enter the joint cavity at a 15° angle with the needle. Slowly inject approximately 20 µL of the silver nanoparticle collagen mixture, and slowly withdraw the needle[24].
5. Suture the subcutaneous tissue and skin in turn, and sterilize. Place the mice into individually ventilated cages (IVC) separately after they wake up from the anesthesia.
6. Perform this injection of the silver nanoparticle collagen mixture (20 µL) four times at a frequency of once per week.

NOTE: The mice treated with the silver nanoparticle collagen mixture should be kept in individual cages. Mice fighting may occur when they are maintained together, and this would affect the experimental results. During the injection, there will be a feeling of tension when the needle reaches the joint cavity, and swelling occurs in the knee joint after the injection. The combination of these two methods allows the researcher to ensure that the drug has been successfully injected into the knee joint.

4. Collection of the knee joint and synovial tissue

1. Sacrifice the mice with carbon dioxide asphyxiation or any other protocol approved by the relevant animal ethics committee.
2. Sterilize and dissect the skin and subcutaneous tissue sequentially, and fully expose the knee joint.
3. Harvest the knee joints, including the femur and tibia, and remove the musculature tissues.
4. Collect the knee joint tissues, including the femur, tibia, and surrounding soft tissues (ligament and capsula), in 10% formalin for preservation and fixation.

5. Hematoxylin-eosin staining

1. After overnight fixation, paraffin-embed the sections, and use a microtome to cut the paraffin-embedded tissue into 0.4 µm thickness. Use the prepared sections for further staining (hematoxylin-eosin staining, Safranin O/ Fast Green, and immunohistochemical (IHC) staining).
2. Deparaffinize the sections twice with xylene, soak in 100%, 95%, 80%, and 70% ethanol in sequence for 5 min each, and rehydrate.
3. Stain the sections with hematoxylin (0.1 g/100 mL) for 5 min, and then directly place in 1% HCl for 10 s and eosin (0.5 g/100 mL) for 1 min.
4. Observe the histopathological changes of the synovium under a microscope.

6. Safranin O/Fast Green

1. Embed the tissue in paraffin, and prepare the histologic sections as described in step 5.1.
2. Deparaffinize the sections twice with xylene, and rehydrate them with an ethanol series (such as 100%, 95%, 80%, and 70% ethanol in distilled water, each for 5 min).
3. Stain the prepared sections with the hematoxylin, and wash them with PBS three times for 2 min each.
4. Differentiate the sections with hydrochloric acid alcohol, and wash them with PBS three times for 2 min each.
5. Immerse the sections in 0.02% Fast Green staining solution for 5-10 min, followed by 0.1% Safranin O staining for 1-2 min.
6. Differentiate the sections with 1% acetic acid, followed by PBS washing.
7. Detect and analyze fibrocartilage formation in the sections.

7. Immunohistochemical (IHC) staining

1. Embedded the tissue in paraffin, and prepare the histologic sections as described in step 5.1.
2. Deparaffinize the sections twice with xylene, and rehydrate them with an ethanol series (such as 100%, 95%, 80%, and 70% ethanol in distilled water, each for 5 min). Immerse the sections in Tris-EDTA buffer (10 mM

Tris base, 1 mM EDTA solution; pH 9.0), and heat in a microwave oven at 95 °C for 10 min to perform antigen retrieval.

3. Expose the sections to 3% hydrogen peroxide solution for 10 min to remove endogenous peroxidase.
4. Treat the sections with 5% goat serum to block nonspecific binding.
5. Add the diluted primary antibodies (1:1,000 dilution) against CD177, and incubate overnight at 4°C. Then, wash the sections with PBS three times.
6. Soak the sections with PBS, and incubate the sections with the appropriate secondary antibody (HRP-conjugated polymer anti-rabbit system) for 30 min at room temperature.
7. Perform the visualization of IHC staining by using 3,3'-diaminobenzidine (DAB) as a chromogen.
8. View the sections under a microscope, and analyze the acquired images.

Representative Results

The KOA mouse model was induced using type II collagenase. Starting 1 week after model induction, the prepared silver nanoparticle collagen mixture was injected into the joint cavity once per week for 4 weeks (**Figure 1**). The weights of the mice in each group were observed and recorded daily. The results showed that the average body weight of the KOA mice was significantly lower than that of the mice in the normal control group. However, the average body weight of the mice in the type II collagenase + AgNPs group was higher compared to the KOA mice, although this difference was not statistically significant (**Figure 2**). After 30 days, the synovial tissues of the knee joints were collected from the mice and subjected to pathological examination. The hyperplasia, vascular proliferation, inflammatory infiltration of the synovium, and cartilage damage were analyzed[5,10,11]. The results showed that the synovial thickness of the mice in the KOA group was significantly higher compared with the normal control group. In the group treated with the silver nanoparticle collagen mixture, the synovial membrane thickness was reduced compared with the KOA group (**Figure 3**). There was vascular hyperplasia in the synovium of the KOA mice compared to the normal control group, and vascular hyperplasia was significantly reduced in the synovium of mice treated with the silver nanoparticle collagen mixture (**Figure 4**). The results of the Safranin-O staining showed that the cartilage matrix of KOA mice was destroyed, while the mice treated with the silver nanoparticle collagen mixture showed a significantly better cartilage matrix (**Figure 5**). The morphological feature scores in each group were assessed as described previously[22]. The results were as follows: 0 ± 0 for the saline group, 7 ± 0.63 for the type II collagen group, and 4.2 ± 1.17 for the type II collagenase + AgNPs group (**Figure 6**). CD177 is a major neutrophil marker[25]. CD177 is expressed in 40%-60% of neutrophils under normal conditions. However, the expression of CD177 in neutrophils increases significantly during acute inflammation. The results of the IHC staining demonstrated that the infiltrated neutrophils in the synovial region were significantly reduced in the group treated with AgNPs compared with the KOA group (**Figure 7**), which suggests that treatment with AgNPs could improve the symptoms of KOA.

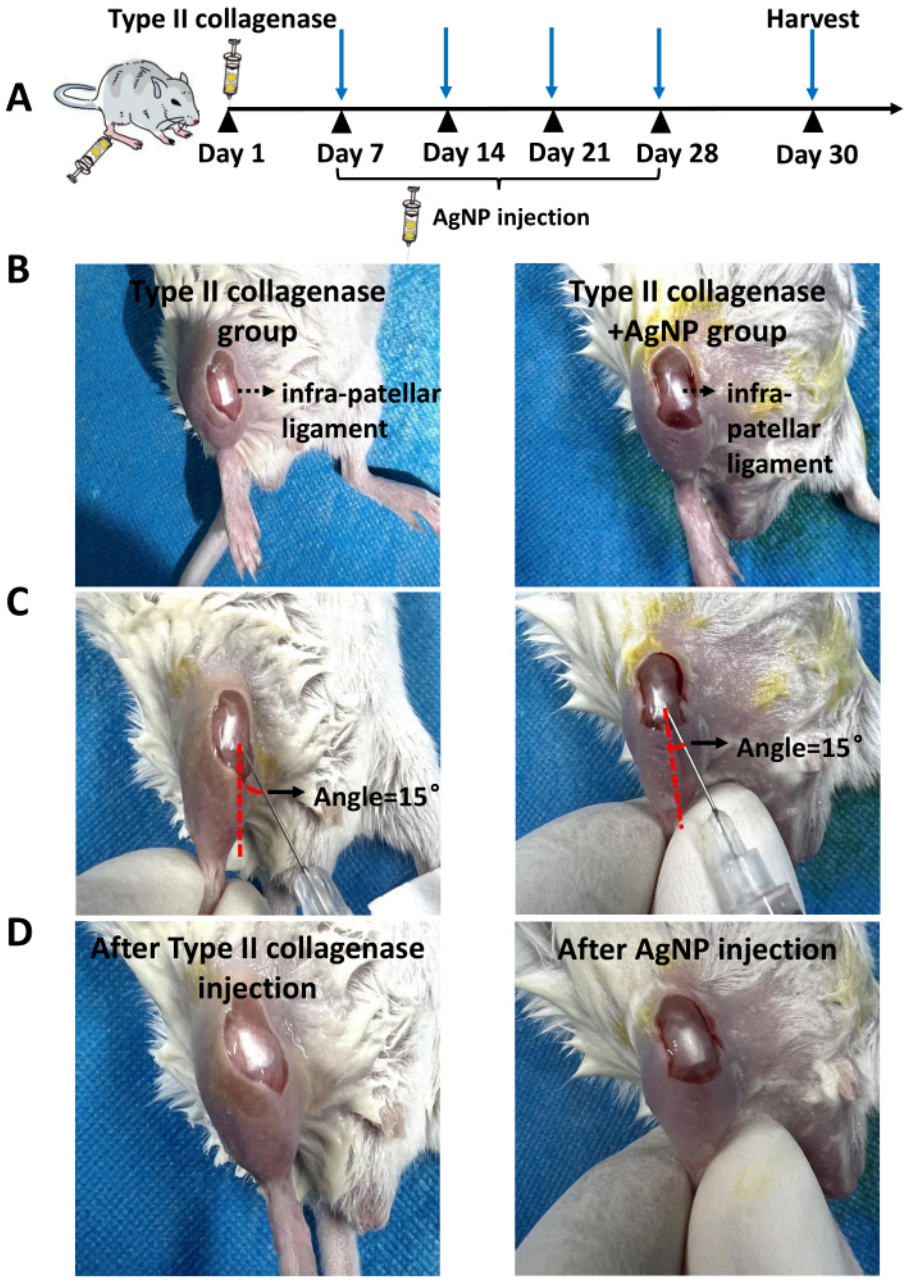

Figure 1: Injection location. (**A**) Representative images of the type II collagenase injection. (**B**) Representative images after the type II collagenase injection. (**C**) Representative images of the silver nanoparticle collagen mixture injection in the KOA mouse model. (**D**) Representative images after the silver nanoparticle collagen mixture injection in the KOA model mice. The red dotted line represents the line parallel to the mouse knee ligaments. The black arrow represents the angle between the insulin syringe needle and the skin. Please click here to view a larger version of this figure.

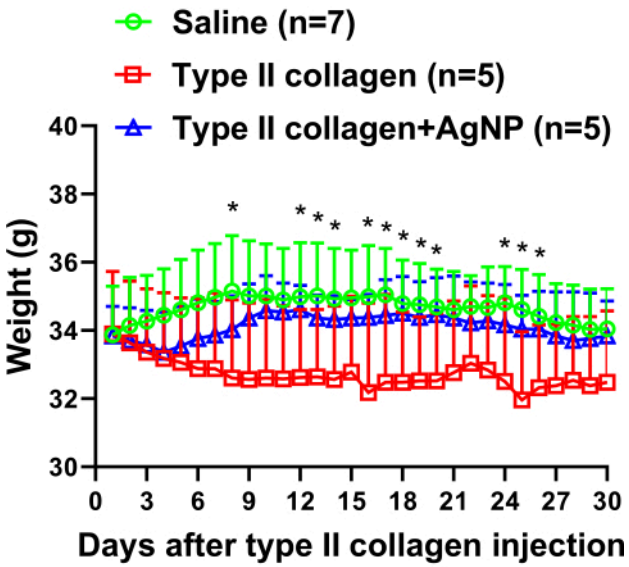

Figure 2: Body weight changes of the mice in each group. This panel shows the average weight of the mice in each group at different time points; the x-axis indicates the number of days after the injection of type II collagenase, and the y-axis indicates the fold-change in body weight. Saline group (n = 7), type II collagenase group (n = 5), type II collagenase + AgNPs group (n = 5). *p < 0.05. Please click here to view a larger version of this figure.

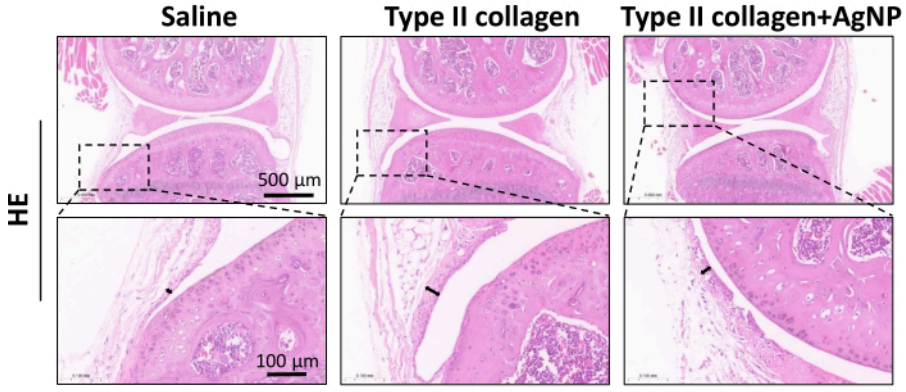

Figure 3: Hematoxylin-eosin (H&E) staining representing synovial hyperplasia. The synovial tissues in each group of mice were collected, fixed, sectioned, and stained with H&E at 30 days after surgery. The double arrows represent the detected synovial thickness. Scale bar = 0.1 mm. Please click here to view a larger version of this figure.

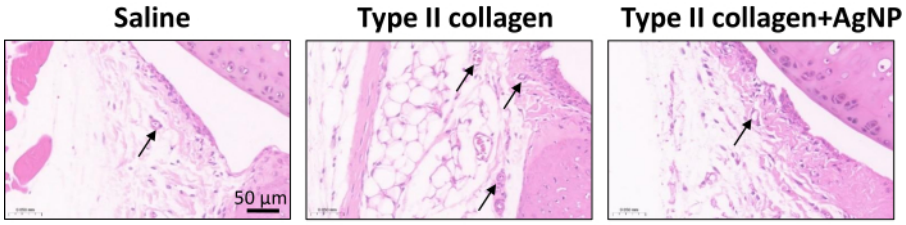

Figure 4: Representative image of perisynovial vascular hyperplasia. The arrows indicate the vessels. Scale bar = 0.05 mm. Please click here to view a larger version of this figure.

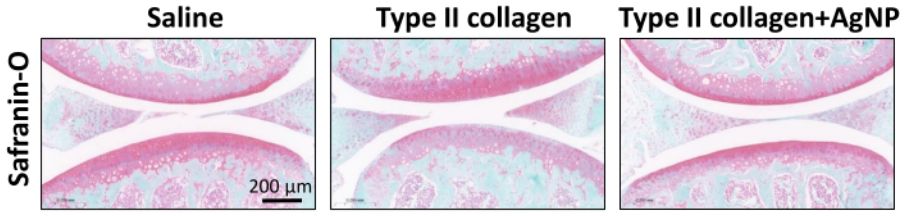

Figure 5: Safranin-O staining of the knee joint in each group of mice. Scale bar = 0.2 mm. Please click here to view a larger version of this figure.

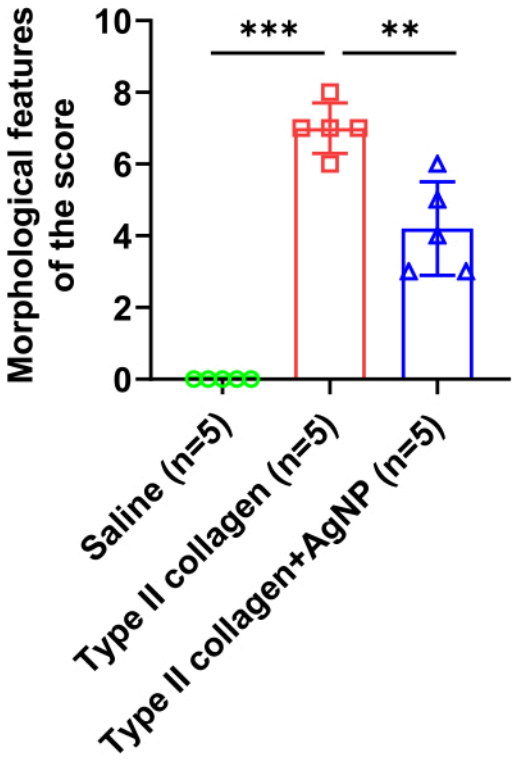

Figure 6: Morphological feature scores in each group. The synovial tissue was used to measure the morphological feature score for the mice in each group. Five tissue sections in each group were selected to analyze the degree of hyperplasia/enlargement of the synovial lining cell layer, the degree of neutrophil infiltration in the synovial tissue, and the degree of activation of the synovial stroma (**Table 1**). The average value was used as the final score. $^{**}p < 0.01$ and $^{***}p < 0.001$ with a Student's t-test for each cohort compared to the untreated KOA group. Please click here to view a larger version of this figure.

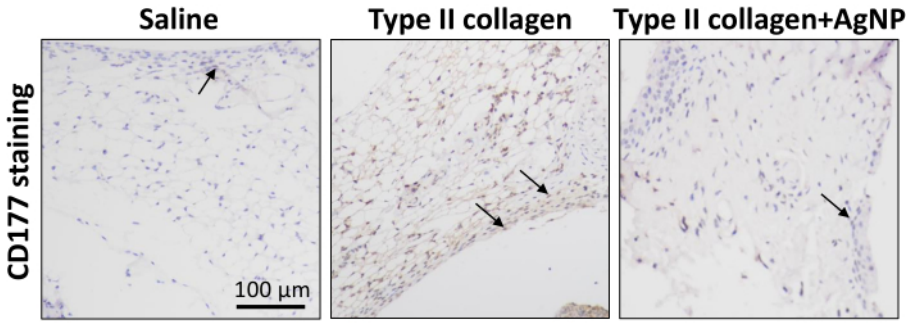

Figure 7: Immunohistochemical staining for a neutrophil marker in the synovial tissue in each group of mice. Immunohistochemical staining was used to detect the expression of the neutrophil marker CD177 in the synovial tissue of the mice in each group. The arrows indicate neutrophils. Scale bar = 100 µm. Please click here to view a larger version of this figure.

Table 1: Scoring morphological features. Please click here to download this Table.

Discussion

Silver nanoparticles exhibit anti-inflammatory, antibacterial, antioxidant, and immunomodulatory effects, meaning they could protect cells and tissues from damage by reducing the production of reactive oxygen species[26]. Some researchers are concerned about the toxicity of silver nanoparticles[27]. The toxicity of silver nanoparticles is directly related to the presence of free silver ions. Due to the nanoscale size of silver nanoparticles, they could easily interfere with biomolecules, cells, and human organs[15,28,29]. Several studies have reported that silver nanoparticles could induce oxidative stress and impair mitochondrial function in human cells[30]. In addition, Ag can be detected in human organs, especially in the liver and spleen, after the use of large quantities of silver nanoparticles. Researchers have also reported that silver nanoparticles have the ability to cross the blood-brain barrier *via* transsynaptic transport and accumulate in the brain[31]. A systematic report of the biotoxicity of silver nanoparticles has not beenconducted, although some researchers acknowledge the safety of silver nanoparticles[32].

In this study, we prepared a silver nanoparticle collagen mixture. Indeed, the duration period of silver nanoparticles in human tissues is brief, but the duration period of silver nanoparticles can be prolonged when applied with a collagen mixture; this not only reduces the trauma but also the dose of the drugs. Considering the toxicity of silver nanoparticles, the dose of silver nanoparticles applied in this study was 30 mg/kg, in line with previous research[33].

A few vital considerations of the experimental operation are as follows. Type II collagenase should be stored at −20 °C after preparation to prevent degradation due to enzymatic cleavage. The preparation of the silver nanoparticle collagen mixture must be carried out on the ice continuously at room temperature because the silver nanoparticle collagen mixture becomes a semisolid gel rapidly and then cannot be used for injection. The solution should be stored at 4 °C after preparation. A 1 mL insulin syringe with a smaller needle

should be chosen for intra-articular administration, and this could effectively prevent the leakage of the injected drugs. The needle should be inserted at an angle of 15° to inject the silver nanoparticle collagen mixture. When the needle isnonresistant, this indicates that the needle has reached the knee joint cavity. After injecting, the angle of the injection should be changed, and the needle should be withdrawn slowly to avoid leakage of the injected drug.

In this study, silver nanoparticles effectively improved the symptoms of type II collagenase-induced KOA in mice, demonstrating the anti-inflammatory effect of silver nanoparticles. Several studies have reported the presence of apoptosis in cells incubated *in vitro* with silver nanoparticles[34,35,36]. The reduction in synovial hyperplasia could have been caused by the silver nanoparticles due to their involvement in the impairment of mitochondrial function, or these outcomes may have been mediated by reactive oxygen species. Vascular hyperplasia was observed in the synovium of mice in the KOA model group. It was possible that chemokines drove neutrophils from the blood vessels to the synovial tissue during this process and that the burst of inflammation caused the cells to consume more oxygen, thus leading to vascular hyperplasia. Hence, further experiments are required to prove the reliability of this hypothesis. This study provides theoretical benefits for research into the treatment of clinical KOA. In future studies, we aim to combine the anterior cruciate ligament (ACL) method along with the chemically induced KOA model method to observe the effect of silver nanoparticles. The experimental results show that silver nanoparticles can significantly decrease the infiltration of inflammatory cells in the synovium in KOA mice, but the mechanisms of this effect still need further study, which might unravel the pathogenesis of KOA.

Disclosures

The authors have no conflicts of interest to disclose.

Acknowledgments

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