

Original Article

Panax notoginseng saponins alleviates knee osteoarthritis by suppressing intramuscular inflammation

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Abstract: Reducing proinflammatory markers in the quadriceps muscle of knee osteoarthritis (KOA) patients is necessary for improvement in performance of daily life activities. The present study explored the effects of panax notoginseng saponins (PNS) on levels of vastus lateralis inflammatory factors in KOA rabbit models. Compared with the sham group, total muscle mass was decreased in the KOA group. In contrast, treatment with PNS increased total muscle weight, indicating the enhancement of muscle force following PNS therapy. Additionally, treatment with PNS significantly decreased knee joint diameter more than that of KOA group. Furthermore, enzyme-linked immunosorbent assay (ELISA) indicated that treatment with PNS reduced KOA-induced upregulation of interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor α (TNF α) in serum and vastus lateralis muscle tissues. These data suggest that local and circulating inflammatory responses could be relieved by PNS treatment. This study also evaluated the effects of PNS on NF- κ B activation. Compared with the KOA group, treatment of PNS significantly suppressed activation of NF- κ B p65 subunit. Expression of I κ B α was increased by PNS treatment after 24 weeks. These data suggest an anti-inflammatory role of PNS in the vastus lateralis muscle tissues of KOA rabbits. These findings, for the first time, show the protective role of PNS through suppressing proinflammatory mediators, within the muscle, thereby improving muscle function and gait in knee OA.

Keywords: Panax notoginseng saponins, inflammatory response, vastus lateralis muscle, knee osteoarthritis, NF- κ B signaling

Introduction

For older adults, knee osteoarthritis (KOA) is a major contributor to pain and functional disability [1, 2]. According to statistics, one-half of people over 80 years old may suffer from arthritis to different degrees [3]. In progression of KOA, patients are characterized by overall affection in the entire joint structure, with enhanced inflammation in the synovial membrane and synovial tissues [4, 5]. Undoubtedly, increased inflammation response plays a key role in KOA patients. Recently, enhancement of inflammatory responses in the skeletal muscle surrounding the knee has been shown to be necessary for development of KOA [6]. Thus, means of suppressing inflammatory response in the skeletal muscle deserves further study.

The presence of proinflammatory markers, including interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor α (TNF α), may be attributed to muscle atrophy and impaired muscle regeneration [7, 8]. In addition, inflammatory factors within the vastus lateralis muscle decrease the capacity of skeletal muscle mass maintenance, thereby leading to further deterioration of muscle mass and strength [9, 10]. Studies have shown that the vastus lateralis, the largest component muscle of the quadriceps femoris, exerts a key role in the extension of knees and maintenance of normal walking [11, 12]. Decrease of muscle strength and function further leads to decreased physical function and disability in KOA patients [13, 14]. Reducing proinflammatory markers in the quadriceps muscle of KOA patients is necessary for

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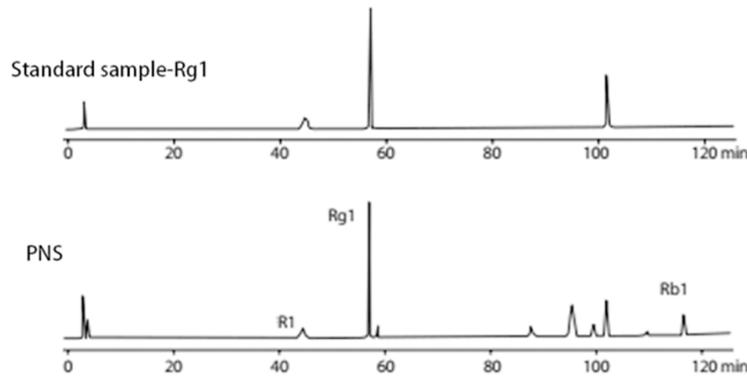


Figure 1. Chemical standardization of freeze-dried PNS powder for injection by HPLC fingerprint analysis.

improvement in the performance of daily life activities.

Panax notoginseng saponins (PNS) are a class of effective free radical-scavengers, characterized by antioxidant properties and anti-inflammation functions [15]. Previous studies have shown that PNS inhibits cancer progression and improves atherosclerosis [16, 17]. PNS has also been shown to inhibit radiation-induced osteoporosis via modulating bone formation and resorption [18]. However, whether PNS could improve inflammatory response in the vastus lateralis of KOA patients has not been explored.

The present study explored the effects of PNS on levels of inflammatory factors of vastus lateralis in KOA rabbit models. Further study demonstrated that PNS could suppress inflammatory response in muscle tissues of KOA rabbits.

Materials and methods

Animal surgery

One hundred and twenty 1-year-old skeletally mature female New Zealand White rabbits (weight: average 5.7 kg, range 4.8±6.6 kg; Sibeifu, Beijing, China) were used, in accordance with an experimental protocol approved by Zhongshan Hospital Affiliated to Dalian University Animal Care Committee. Animals were housed in pairs in floor pens to permit normal activity. Rabbits received a standard diet and water ad libitum. Briefly, after achieving anesthesia with an intravenous injection of 250 mg/kg of 10% chloral hydrate, a 2 cm lat-

eral para-patellar skin incision was made. Next, the patella was dislocated medially to expose the knee joint and the anterior cruciate ligament was transected visually with a #15 blade. The joint was then repositioned, irrigated with sterile saline, and closed with 4-0 nylon. After surgery, all rabbits were housed in separated cages and had ad libitum access to food and water. All animals were euthanized 8 weeks after the operation.

Rabbits were randomly divided into three groups of 40 rabbits each at 1, 2, 4, 8, 12, and 24 weeks postoperative. The KOA group received four weekly intra-articular injections of 300 μ L saline.

Isolation of the panax notoginseng saponins

A freeze-drying powder of panax notoginseng saponins for injection (without excipients; PNS content is 100%) was purchased from Kunming Pharmaceutical Corporation (Kunming, Yunnan province, China). Standard reagents, including notoginsenoside R1, ginsenoside Rg1, and ginsenoside Rb1 (purity > 99%), were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). HPLC analyses of compounds in PNS were carried out as follows: In brief, the separation was performed on an Elite LaChrom system (Hitachi, Tokyo, Japan), equipped with a Hypersil C18 column (5 μ m; length, 200 mm; inside diameter, 4.6 mm, Interchim, Montlucon, France), and the column temperature was maintained at room temperature. A 20 μ L sample was injected into the column and eluted with a constant flow rate of 1.0 mL/min. The mobile phase consisted of acetonitrile and H₂O (25:75, v/v). A programmable UV detector (Model 526, ESA Inc, Chelmsford, MA) set at 203 nm was used for analyses. Data acquisition was performed using LaChrom Elite software (VWR, Darm Stadt, Germany) and a standard curve was used to calculate concentrations for samples based on peak areas. As shown in **Figure 1**, the notoginsenoside R1, ginsenoside Rg1, and ginsenoside Rb1 contents in the freeze-dried powder of panax

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notoginseng saponins for injection were 0.12 mg, 0.36 mg, and 0.38 mg, respectively.

Histopathology examination

Rabbit knee joints were dissected after euthanasia. Knee joint tissues were processed with 3% hydrochloric acid (HCl) solution for 5-7 days, with a periodic change of HCL every 24 hours for complete decalcification of joints. Afterward, the joints were fixed in neutral buffered formalin (10%) for 2 days. Decalcified joints of rabbits were again dehydrated in different series of alcohol and joints were cleared and embedded in liquid paraffin. Decalcified joints were sliced into 5 μm pieces, with hematoxylin and eosin used for staining the tissue and preparing slides. Slides were prepared for microscopic evaluation under light microscope (original magnification 40 \times , DXIT 1200, Nikon, Japan). H&E stained joint slides were examined for bone and cartilage destruction.

Measurement of hyaluronic acid

Rabbit hyaluronic acid (HA) ELISA kit (SBJ-T0271-96T, Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing City, China) was used to measure serum hyaluronic acid (HA), according to manufacturer instructions.

Measurement of keratan sulfate

Rabbit keratin sulfate (KS) ELISA kit (SBJ-T0272-96T, Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing City, China) was used to measure serum KS, according to manufacturer instructions.

Enzyme-linked immunosorbent assay (ELISA)

Serum or frozen vastus lateralis muscle tissues (approximately 100 mg) were homogenized in a lysis buffer (50 mmol/L Tris-HCl, 300 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 0.02% sodium azide) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were centrifuged at 16000 \times g for 15 minutes at 4°C and supernatants were used to quantify levels of IL-1 β , IL-6, and TNF α by way of a sandwich enzyme-linked immunosorbent assay, following manufacturer instructions (R&D Systems, Minneapolis, Minnesota, USA). Samples were read at a 450 nm wavelength using a microplate reader (Model 3550; Thermo Fisher Scientific, Waltham, MA, United States).

Western blot analysis

Approximately 10 mg of vastus lateralis muscle was taken from 24 week-euthanized rabbits in the sham, KOA, and PNS groups. After washing twice with phosphate-buffered saline (PBS), samples were grinded and lysed in radio immunoprecipitation assay buffer and collected by centrifugation (14,000 rpm for 20 minutes, 4°C). Protein concentrations of the samples were determined and the desired volume of each sample for equal protein was calculated. About 50 μg protein of each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (NC). The NC membrane was initially blocked with 5% skim milk (TBS-T) and shaken at room temperature for 1 to 2 hours. Next, the blocking solution diluted primary antibodies, p-p65 (#4887, 1:1000, Cell Signaling Technology, Inc., Boston, MA, USA), p65 (#8242, 1:1000, Cell Signaling Technology, Inc., Boston, MA, USA), I κ B α (#4814, 1:1000, Cell Signaling Technology, Inc., Boston, MA, USA), and β -actin (#4970, 1:1000, Cell Signaling Technology, Inc., Boston, MA, USA), were added to the NC membrane and incubated at 4°C overnight, respectively. Subsequently, the NC membrane was washed with TBS-T three times (each time for 15 minutes). In the dark, an enhanced chemiluminescence (ECL) reagent (Millipore Co., Billerica, MA, USA) was used to expose the membrane to film. To quantitatively analyze Western blot detection of protein bands, each group included four rabbits, as shown in **Figure 5A** and **5B**. Data were quantified using ImageJ 1.8.0 (National Institutes of Health, Bethesda, MD, USA). Beta-actin was used as an internal control.

Muscle mass assessment

Heads of the quadriceps muscle group (rectus femoris, vastus medialis, VL, and vastus intermedius muscle) were isolated and dissected, individually. Wet mass was measured using a commercial balance with a measuring accuracy of 0.001 g (Mettler-Toledo, Switzerland).

Joint width assessment

On the day of sacrifice, the width of experimental and contralateral knees was measured in all animals using a commercially available digital caliper. Prior to measurement, hind limbs were shaved, the joint space between femur and

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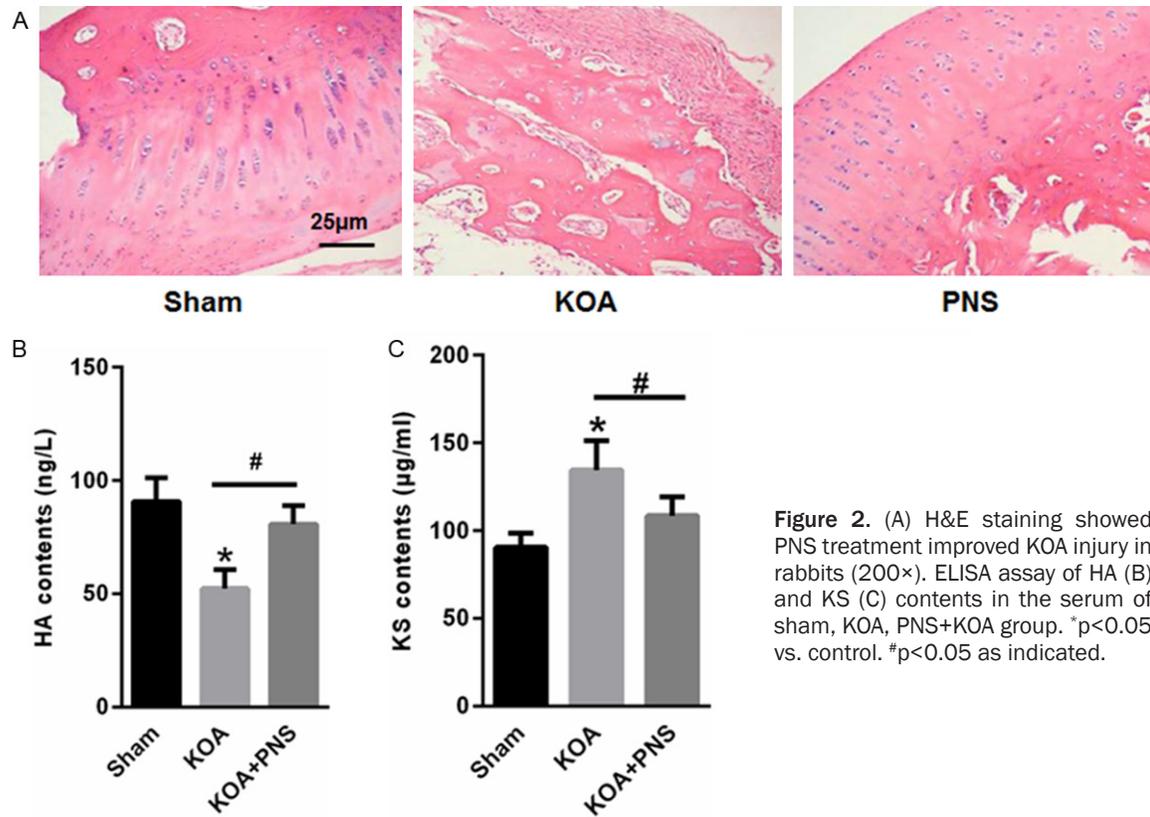


Figure 2. (A) H&E staining showed PNS treatment improved KOA injury in rabbits (200 \times). ELISA assay of HA (B) and KS (C) contents in the serum of sham, KOA, PNS+KOA group. * $p < 0.05$ vs. control. # $p < 0.05$ as indicated.

tibia was identified, and the largest medial-lateral diameter was marked. Measurements were repeated three times and average value was recorded.

Statistical analysis

Statistical analysis was performed and comparison among the groups was computed on SPSS 17.0. Data were analyzed by one-way ANOVA analysis followed by post hoc test and pair wise comparison of samples means examined by least significant difference (LSD) with ($\bar{x} \pm s$). P -values < 0.05 were considered statistically significant.

Results

Animals

Throughout the course of this study, there were no surgical complications or infections and no animals died.

Establishment of KOA models in rabbits

In the sham group, there was no damage of articular cartilage and articular cartilage cells

were arranged orderly. In contrast, in the KOA group, the articular cartilage surface became thinner, hardened, and rough. Furthermore, some articular cartilage almost disappeared with cartilage cell atrophy and a concentrated nucleus. The number of cartilage cells significantly decreased while the arrangement was disorderly. After PNS treatment, most articular cartilage damage was not obvious. The spindle shaped articular cartilage cells were arranged disorderly (**Figure 2A**). This study also analyzed serum HA and KS contents. Compared with the sham group, serum HA contents were decreased in the KOA group. After PNS treatment, serum HA contents were significantly increased (**Figure 2B**). In addition, serum KS contents were increased in the KOA group, but were reduced after PNS treatment (**Figure 2C**), suggesting a protective role of PNS in KOA progression.

PNS increases total muscle mass and decreases the knee joint diameter of KOA rabbits

Next, this study tested total muscle mass in the sham, KOA, and PNS groups. Compared with the sham group (21.8 ± 3.5 g), total muscle

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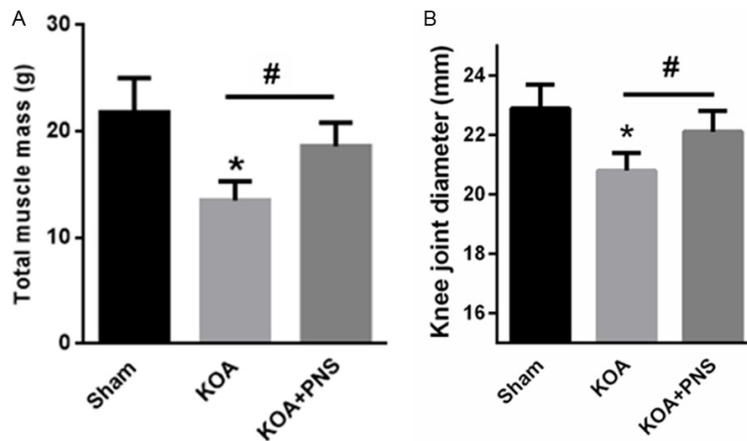


Figure 3. PNS improves muscle injury. A. Treatment with PNS increased total muscle weight than that of KOA group. B. Treatment with PNS significantly decreased the knee joint diameter than that of KOA group. * $p < 0.05$ vs. control. # $p < 0.05$ as indicated.

mass was decreased in the KOA group (13.3 ± 2.8 g). In contrast, treatment with PNS increased total muscle weight to 19.6 ± 3.9 g, indicating enhancement of muscle force following PNS therapy (**Figure 3A**). Compared with the sham group (22.9 ± 1.9 mm), knee joint diameter of the KOA group was significantly increased in the knees (20.8 ± 1.4 mm). However, treatment with PNS significantly decreased knee joint diameter to 22.1 ± 1.1 mm, suggesting improved joint function (**Figure 3B**).

PNS reduces expression of inflammation factors

Next, inflammation factors were tested, including IL-1 β , IL-6, and TNF α in the serum and vastus lateralis muscle of KOA and PNS-treated rabbits. ELISA assay showed that levels of IL-1 β , IL-6, and TNF α were significantly upregulated in the serum of KOA rabbits (**Figure 4A**). In contrast, treatment with PNS decreased IL-1 β , IL-6, and TNF α levels (**Figure 4A**). Furthermore, ELISA assay indicated that treatment with PNS reduced KOA-induced upregulation of IL-1 β , IL-6, and TNF α in vastus lateralis muscle tissues (**Figure 4B**). These data suggest that local and circulating inflammatory responses could be relieved by PNS treatment.

PNS suppresses NF- κ B activation in vastus lateralis muscle of KOA rabbits

This study explored whether PNS could inhibit activation of NF- κ B in vastus lateralis muscle tissues. First, it was found that phosphoryla-

tion levels of p65 were increased in vastus lateralis muscle tissues of KOA rabbits but levels of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha I κ B α , were decreased in muscle tissues of KOA rabbits (**Figure 5A**). Compared with the KOA group, treatment of PNS significantly suppressed activation of NF- κ B p65 subunit. Additionally, expression of I κ B α was increased by PNS treatment after 24 weeks. These data suggest an anti-inflammatory role of PNS in the vastus lateralis muscle tissues of KOA rabbits (**Figure 5B**).

Discussion

It has been widely reported that presence of inflammatory factors in the vastus lateralis muscle reduces skeletal muscle mass, further leading to leg disuse due to pain and disease symptoms [6, 19]. The vastus lateralis is a major component of the quadriceps femoris, having been suggested to exert a key role in knee extension and maintenance of daily function, including walking. Therefore, deterioration in muscle strength and function, especially the quadriceps, will adversely affect physical function and result in disability in people with KOA [20, 21]. Recent studies have suggested that increased proinflammatory markers in the quadriceps muscle directly aggravate the performance of daily life activities. Therefore, suppression of inflammatory responses in the vastus lateralis muscle deserves further study.

The present study mainly explored the role of PNS, a traditional Chinese medicine, in the improvement of muscle inflammatory markers. Under normal conditions, inflammation is a systemic response to insult and/or injury in affected tissues, thereby transmitting information and enhancing recovery from cell injuries. However, sustained inflammation will result in cell and tissue injury [22]. Within the muscle itself, upregulation of many cytokines (TNF α , IL-1 β , IL-6, and IL-8) has been extensively identified in inflamed tissues [22, 23]. Increased pain and joint inflammation may decrease muscle strength and function [24]. In line with previ-

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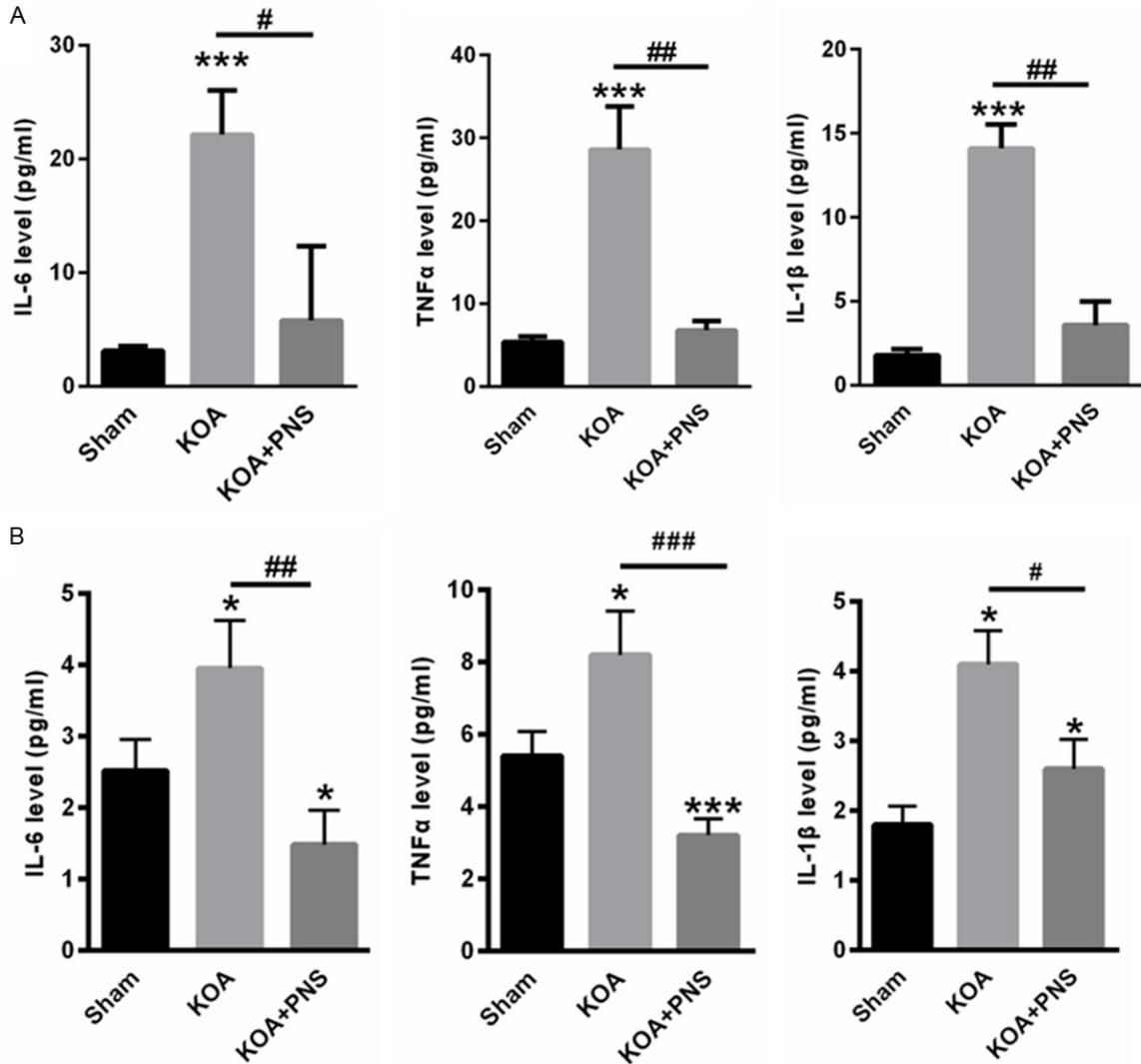


Figure 4. PNS reduced expression of inflammation factors. A. ELISA assay showed that levels of IL-1 β , IL-6, and TNF α were significantly upregulated in the serum of KOA rabbits. B. ELISA assay indicated that treatment with PNS reduced KOA-induced upregulation of IL-1 β , IL-6, and TNF α in the vastus lateralis muscle tissues. * $p < 0.05$, ** $p < 0.01$ vs. control. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ as indicated.

ous studies, this study found that muscle mass was reduced and inflammation factors, including TNF α , IL-1 β , and IL-6, were enhanced in KOA rabbits. In contrast, treatment with PNS significantly enhanced muscle mass and relieved inflammation response in KOA rabbits. It was hypothesized that reduced inflammatory response in vastus lateralis by PNS treatment may contribute to improvement of KOA.

The importance of intramuscular inflammation in knee OA has been widely accepted by clinicians. Thus, means of suppressing intramuscular inflammation may be effective therapy methods to improve the functional capacity of

KOA patients. Regarding inflammatory signaling, *NF- κ B1* should be emphasized, since the activation of NF- κ B signaling pathways may lead to release of pro-inflammatory cytokines, further resulting in cartilage damage and bone metabolism disturbance [25, 26]. Five members are included in the NF- κ B family, including p105 (constitutively processed to p50), p100 (processed to p52 under-regulated conditions), p65 (also known as RelA), RelB, and c-Rel [27]. These subunits exist in the form of homo- and heterodimers. In resting cells, they are normally inactive in the cytoplasm by combination with inhibitors, the I κ B proteins [28]. Inhibition of I κ B kinase targets them for degradation, leading to

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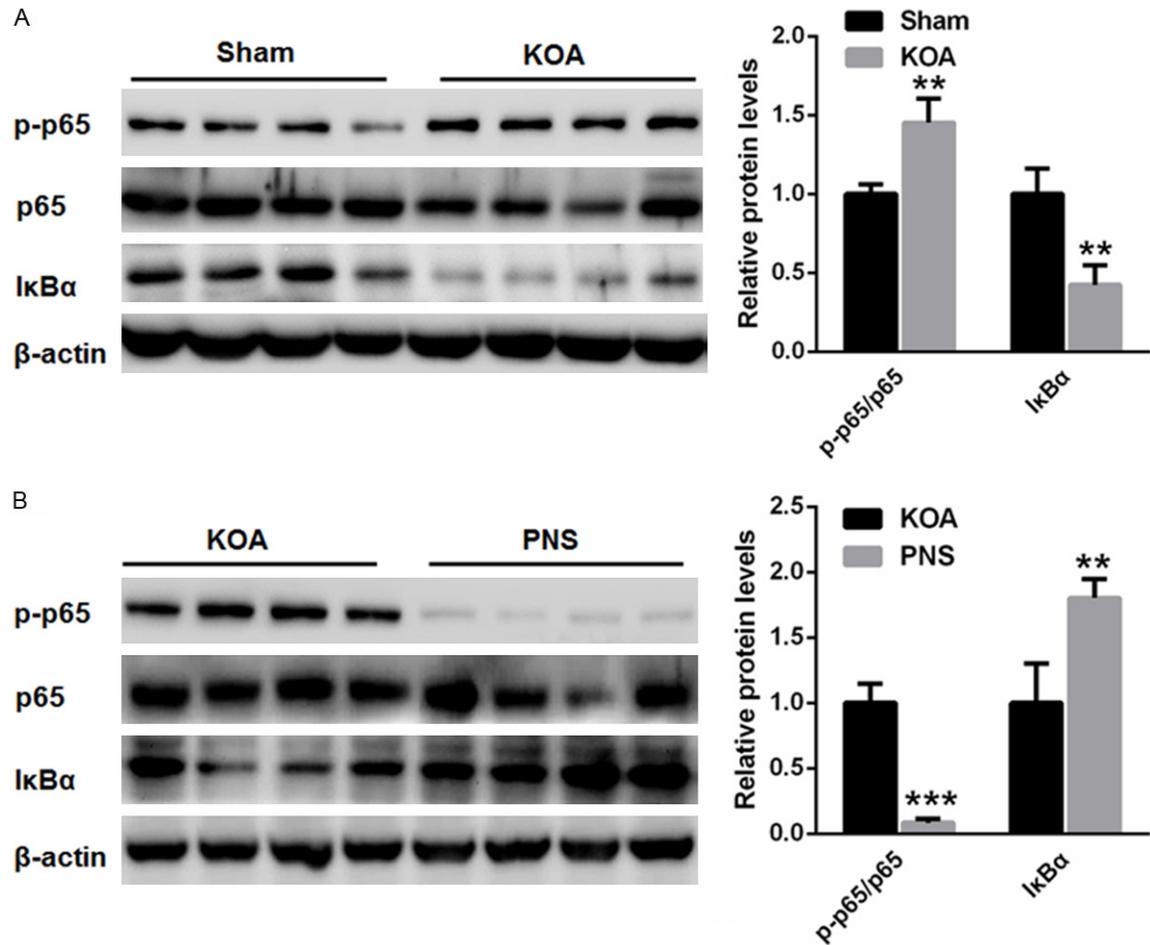


Figure 5. PNS suppressed NF-κB activation in vastus lateralis muscle of KOA rabbits. A. Activation of NF-κB signaling was identified in the vastus lateralis muscle tissues of KOA rabbits than that of sham control. B. Treatment of PNS significantly suppressed activation of NF-κB p65 subunit in the vastus lateralis muscle tissues of KOA rabbits. ** $p < 0.01$, *** $p < 0.001$ vs. control.

the release of NF-κB subunits for nuclear translocation and transactivation of multiple responsive genes. It has been reported that the p65 canonical pathway of NF-κB exerts a key role in modulating early molecular events [28]. Therefore, this study examined the effects of PNS on NF-κB signaling in vastus lateralis. Results showed that PNS treatment suppressed NF-κB activation in muscle tissues, suggesting PNS as an attractive therapeutic reagent in KOA.

These findings, for the first time, show the potential protective role of PNS through suppression of proinflammatory mediators, within the muscle, thereby improving muscle function and gait in knee OA. However, further study is necessary to investigate potential application in the KOA population.

Disclosure of conflict of interest

None.

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