

# Specnvezhenide Decreases Interleukin-1 $\beta$ -Induced Inflammation on Rat Chondrocytes via NF- $\kappa$ B and wnt/ $\beta$ -Catenin Pathways and Reduces Joint Destruction in Osteoarthritic Rats

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**Highlights**

Chiyuan Ma and Jisheng Ran contributed equally to this study.

All authors were involved in the study and preparation of the manuscript.

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All conference has been list at the end of the paper with mark in the manuscript.

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## Abstract

As a chronic disease, osteoarthritis (OA) leads to degradation of both cartilage and subchondral bone, of which the development is related to proinflammatory cytokines like interleukin-1 $\beta$ . In the present study, the anti-inflammatory effect of Specnvezhenide in osteoarthritis and mechanism of it was studied in vitro and in vivo. The results showed that Specnvezhenide decreases interleukin-1 $\beta$ -induced expression of matrix-degrading enzymes and reduces the activation of NF- $\kappa$ B and wnt/ $\beta$ -catenin pathways in vitro. Furthermore, Specnvezhenide treatment prevents the degeneration of both cartilage and subchondral bone in rats OA model. As conclusion, to the best of our knowledge, we report firstly that Specnvezhenide decreases interleukin-1 $\beta$ -induced inflammation on rat chondrocytes by inhibiting activation of NF- $\kappa$ B and wnt/ $\beta$ -catenin pathways, and has therapeutic potential in OA treatment.

Keywords: Specnvezhenide; osteoarthritis; chondrocyte; NF- $\kappa$ B; wnt/ $\beta$ -catenin

## Introduction

Osteoarthritis (OA) is one of the most common degenerative joint disease with cartilage loss and synovial inflammation<sup>1</sup>. Genetic and environmental factors could increase the risk for development of OA including sex, obesity, and injuries<sup>2</sup>. These factors would cause repeated abnormal microenvironment in the joints, leading to its degeneration. Smooth function of joints is provided by cartilage at articular surface because of tensile strength of matrix induced by chondrocytes<sup>3</sup>. Chondrocytes take part in the synthesis and degradation of cartilage matrix and inflammatory microenvironment could damage this function of chondrocytes<sup>4</sup>. The inflammatory cytokines interleukin-1-beta (IL-1 $\beta$ ) and tumor necrosis factor alpha (TNF- $\alpha$ ) are key players in OA. Stimulation of these cytokines would enhance degeneration of cartilage matrix by up-regulating matrix-degrading enzymes and down-regulation of chondrocyte-specific proteins<sup>5</sup>. Disfunction and degradation of cartilage would cause loss of smooth mobility of joints and OA. Recently, agents extracted from plants have received great interest in the treatment of OA. Specnvezhenide (SPN, Chemical Abstracts Service number 39011-92-2, C31H42O17, molecular weight, 686.62) is an extracted agent of the fruits of *Ligustrum lucidum*. Additionally, the affect of SPN on some inflammation-related signal pathways has been reported before<sup>6</sup>. However, as SPN is just newly discovered in the last few decades, its pharmaceutical value is poorly studied<sup>6a</sup>. In the present study, we report firstly that SPN exerts anti-inflammatory properties in rats OA model and inhibits IL-1 $\beta$ -induced inflammation on chondrocytes by reducing NF- $\kappa$ B and wnt/ $\beta$ -catenin signaling in vitro.

## Materials and methods

### Animals

Sprague Dawley rats (200-250g; 6 weeks old, Animal Center of Zhejiang University) were used in this study. Water and food were provided routinely in the facility. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the 2<sup>nd</sup> Affiliated Hospital, School of medicine, Zhejiang University, Hangzhou, China (2015-107).

### Materials

Specnvezhenide was obtained from Shanghai Pureone Biotechnology. Recombinant rat IL-1 $\beta$  was

purchased from R&D Systems, Abingdon, UK. Dulbecco's Modified Eagle's Medium (DMEM), penicillin and streptomycin, fetal bovine serum (FBS), 0.25% trypsin were obtained from Gibco RRL, Grand Island, NY, USA. Collagenase II were purchased from Sigma-Aldrich, St, Louis, MO, USA.

#### CCK-8 assay.

CCK-8 was used to evaluate the cytotoxicity of SPN according to the manufacturer's instruction. Rat chondrocytes were seed in 96-well plates at a density of 5000/wel. After incubation with different concentrations (0, 10, 50, 100, 200  $\mu$ M) of SPN for 24 h, cells were incubated with 10  $\mu$ L CCK-8 for 4 h. The optical density (OD) was read at a wavelength of 450 nm with a reader.

#### Cell culture and treatment.

Rat chondrocytes were prepared as previously described<sup>7</sup>. Briefly, six-weeks-old Sprague Dawley rats were sacrificed, and cartilage from the hip joints was digested with 0.25% pancreatic enzymes for 30 min to remove other tissues and cells. Then, the cartilage fragments were digested with 0.2% collagenase II to isolate the cells. The chondrocytes were grown in DMEM with 10% FBS, 100U/mL penicillin, and 100mg/mL streptomycin at 37°C with 5% CO<sub>2</sub>. Cells were seeded in six-well plates for analysis. Subconfluent cells were pretreated with SPN at different concentration for an hour then incubated with or without IL-1 $\beta$  (5ng/ml) for 24 hours.

#### RNA extraction and Real-Time PCR

We used TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to extract RNA from cells according to the protocol. Total RNA was used to synthesize cDNA by reverse transcription (cDNA synthesis kit, Takara). Power SYBR Green PCR Master Mix (Applied Biosystems) was used for real-time PCR. The expression of MMP3, MMP9, Collagen II, and sox9 was analyzed using primer sequences listed in Table 1. GAPDH worked as endogenous controls. For real-time PCR, 10  $\mu$ L reaction mixture contained SYBR Green and each primer. PCR was set using StepOnePlus system (Applied Biosystems). The program included 1 cycle of denaturation at 95°C for 1 min and 40 cycles of denaturation at 95°C for 15 s, primer annealing, and extension at 63°C for 25 s, followed by melt curve analysis. Data were analyzed for fold difference using formula:  $2^{-\Delta\Delta CT}$ .

Table 1 Primers used for Real-Time PCR

Gene*	Primer Sequences (5'-3')		Amplicon Size (bp)
	Forward	Reverse	
Rat MMP3	CAGGCATTGGCACAAGGTG	GTGGGTCACTTTCCTGCAT	110
Rat MMP9	GCAAACCCTGCGTATTTCAT	GATAACCATCCGAGCGACCTTT	73
Rat Collagen II	GAGTGAAGAGCGGAGACTACTG	GTCTCCATGTTGCAGAAGACTTTCA	83
Rat sox9	CCAGCAAGAACAAGCCACAC	CTTGCCCAGAGTCTTGCTGA	131
Rat GAPDH	GAAGGTCGGTGTGAACGGATTG	CATGTAGACCATGTAGTTGAGGTCA	127

\* Rat means species is rat

### Protein extraction and Western blot analysis

Radioimmunoprecipitation assay (RIPA) containing protease and phosphatase inhibitors was used to prepare whole cell extract after treatment. Nuclear and Cytoplasmic Extraction Reagents (Bosterbio, Wu Han, China) were used to prepare nuclear and cytoplasmic extracts. Equal amounts of cell extract were separated by 10% SDS-PAGE, and electro-transferred to polyvinylidene difluoride membranes. After blocked with 5% Bull Serum Albumin (BSA, Sigma-Aldrich, St, Louis, MO, USA) for 2 hours, the membranes were blotted with primary antibodies at 4 °C overnight, then incubated for 1 hour with secondary antibody. In the present study, antibodies (Ab) against  $\beta$ -catenin (cell signal, #8480p), NF- $\kappa$ B p65 (cell signal, #4764S), phosphor- NF- $\kappa$ B p65 (cell signal, #3031), GAPDH (cell signal, #5174), and TATA-box binding protein (TBP, Boster, BA3586-2) were used. GAPDH worked as endogenous controls in whole cell and cytoplasmic protein assessment. TBP worked as endogenous controls in nuclear protein analysis.

### Induction of OA in rats

Rat model of OA was developed by surgical excision of anterior cruciate ligament (ACL) and medial meniscus (MM) on the knee joints. In brief, SD rats were anesthetized by pentobarbital (40mg/kg). A medial para-patellar approach was used here, and patella was retracted laterally to expose medial meniscus and ACL. ACL was transected and medial meniscus was removed. The operational wound was closed in layers by sutures.

45 rats were used in the present study, and were divided randomly into three groups. Rats in control group received sham surgery as sham control (NC). 200 $\mu$ l SPN solution (200 $\mu$ M) was injected intra-articularly in OA rats every 7 days in SPN group 1 week post surgery, whereas OA group rats received equal volume of PBS. Rats were sacrificed after 8 weeks of treatment, and the knees were preserved in 4% paraformaldehyde solution.

### $\mu$ CT analysis

The  $\mu$ CT analysis was conducted using a  $\mu$ CT system (vivaCT80, Scanco Medical, Zurich, Switzerland) to evaluate the structure of subchondral bone. Reconstruction of the sections was done by MicView software. The region between articulating surface of condyles and growth plate was assessed. Various trabecular bone indices such as trabecular bone volume fraction (BV/TV; %), trabecular number (Tb. N.; 1/mm), and connectivity density (Conn.D) were evaluated using CT Analyzer software.

### Histological analysis

4% paraformaldehyde solution fixed knees were decalcified and embedded in paraffin, sectioned at 5  $\mu$ m thickness. The sections of interior joint were stained with Safranin O-fast green (SO). The OARSIS assessment system with grading method (0-6) was used to evaluate the sections<sup>8</sup>.

### Statistical analysis

The results are presented as mean  $\pm$  standard deviation of three experiments. Statistical differences were performed with SPSS 12.0 version. One-way ANOVA with a subsequent post hoc Tukey's

test was used for multiple comparisons.  $P < 0.05$  is considered to be significant with statistical meaning.

## Results

### Effect of SPN on rat chondrocytes viability and gene expression in vitro

Cytotoxicity of SPN on rat chondrocyte was evaluated by CCK-8 assay here. As illustrated in Fig 1, SPN didn't have significant cytotoxic effects on rat chondrocytes at concentrations of 0-200  $\mu\text{M}$  after 24h incubation. Thus, SPN (10, 50, 100, 200  $\mu\text{M}$ ) was used in the following experiments.

Then, effect of SPN on chondrocyte-specific gene expression of rat chondrocytes was observed. Chondrocytes were treated with SPN at different concentrations (10, 50, 100, 200  $\mu\text{M}$ ) for 24h without IL-1 $\beta$ . As shown in Fig 1, SPN treatment did not influence the expression of Collagen II, and sox9. Also, the expression of cartilage matrix-degrading enzymes such as MMP-3 and MMP-9 did not show any significant change. These results show that SPN treatment does not have any significant effect on rat chondrocytes viability and gene expression.

### Effect of SPN on IL-1 $\beta$ -induced chondrocyte-specific gene degradation and cartilage matrix-degrading enzymes expression on rat chondrocytes in vitro

Next, we investigated the effect of SPN on IL-1 $\beta$ -induced chondrocyte-specific gene degradation and cartilage matrix-degrading enzymes expression in rat chondrocytes. Chondrocytes were pretreated with SPN at different concentrations (10, 50, 100, 200  $\mu\text{M}$ ) for 1h, then incubated with IL-1 $\beta$  (5ng/ml) for 24h. In the present study, the expression of MMP3, MMP9, Collagen II, and sox9 were evaluated by Real-Time PCR. As shown in Fig 2, the expression of MMP3, MMP9 was down-regulated by SPN treatment, but the pretreatment did not provide any significant improvement of chondrocyte-specific gene degradation. According to the results, SPN treatment could protect the cartilage by down-regulation of IL-1 $\beta$ -induced expression of MMPs in a dose-dependent manner.

### Effect of SPN on IL-1 $\beta$ -induced activation of NF- $\kappa\text{B}$ and wnt/ $\beta$ -catenin signaling on rat chondrocytes in vitro

To understand the anti-inflammatory mechanism of SPN, we investigated the effect of SPN on IL-1 $\beta$ -induced activation of NF- $\kappa\text{B}$  and wnt/ $\beta$ -catenin signaling in rat chondrocytes in vitro by Western blot. Here, the phosphorylation level of NF- $\kappa\text{B}$  p65 in cell was evaluated to assess the activation of NF- $\kappa\text{B}$  signaling, and level of  $\beta$ -catenin in the whole cell was investigated to assess the activation of wnt/ $\beta$ -catenin signaling. Fig 3 showed that the increasing phosphorylation level of NF- $\kappa\text{B}$  p65 and level of  $\beta$ -catenin could be inhibited by SPN treatment. The data indicates that SPN exerted an inhibitory property on IL-1 $\beta$ -induced phosphorylation of NF- $\kappa\text{B}$  and increasing of  $\beta$ -catenin in rat chondrocytes. Furthermore, this kind of inhibition is dose-dependent, so we selected 200  $\mu\text{M}$  as the concentration of SPN treatment in the following signaling study and animal experiment.

### Effect of SPN on IL-1 $\beta$ -induced nucleus translocation of NF- $\kappa\text{B}$ p65 and $\beta$ -catenin in vitro

According to the previous studies, activation of NF- $\kappa\text{B}$  and wnt/ $\beta$ -catenin signaling involves nucleus translocation of NF- $\kappa\text{B}$  p65 and  $\beta$ -catenin<sup>9</sup>. Here, to further investigate the effect of SPN IL-1 $\beta$ -induced activation of NF- $\kappa\text{B}$  and wnt/ $\beta$ -catenin signaling, we used Western blot to observe

the nucleus translocation of NF- $\kappa$ B p65 and  $\beta$ -catenin in SPN treatment for IL-1 $\beta$ -induced inflammation. According to Fig 4, SPN treatment (200 $\mu$ M) reduced nuclear protein level of NF- $\kappa$ B p65 and  $\beta$ -catenin when compared with IL-1 $\beta$  stimulated group, meanwhile, the cytoplasmic protein level of those two proteins had no significant difference. Our study confirmed the nucleus translocation of NF- $\kappa$ B p65 and  $\beta$ -catenin in IL-1 $\beta$ -induced inflammation and showed that SPN would reduce IL-1 $\beta$ -induced nucleus translocation of NF- $\kappa$ B p65 and  $\beta$ -catenin.

#### Effect of SPN treatment on cartilage degeneration in rat model of OA

As SPN treatment could protect the cartilage by down-regulation of IL-1 $\beta$ -induced expression of MMPs in vitro, we investigated its cartilage protective role in vivo. We developed an OA model in SD rats by surgical transaction of ACL and MM. Rats were sacrificed after 8 weeks. OASRI-based histological assessment was used to evaluate the OA grade. SO stained sections of knee joints in OA rats showed disrupted and discontinuous cartilage, mean OASRI grade was 4.6 which was significantly higher than the sham control. Meanwhile, the average OASRI grade of SPN group was 2.95, as illustrated in Fig 5, lower than OA group with significant difference. This part demonstrated that SPN treatment plays a cartilage-protective role in rat model of OA.

#### Effect of SPN treatment on degeneration of trabecular microarchitecture of subchondral bone in OA rats

It is well-known that degenerative changes into the subchondral region are usually accompanied with the degeneration in articular cartilage<sup>10</sup>. Transection of ACL and MM changes the biomechanical environment in the joint and results in the development of OA. In the present study,  $\mu$ CT was used to investigate the change of femoral subchondral bone. In the femoral subchondral bone, trabecular bone volume fraction (BV/TV; %), trabecular number (Tb. N.; 1/mm), and connectivity density (Conn.D) were evaluated (Fig. 6). In OA-induced rats, trabecular BV/TV, Tb.N, and Conn D were all lower than the sham control (NC). These data confirmed the degenerative changes of the subchondral region in OA. Furthermore, we also investigated the effect of SPN treatment on this kind of degeneration. The results showed that SPN treatment didn't provide significant improvement of the degenerative change of trabecular BV/TV and Tb.N, but Conn D of SPN treated OA rats was slightly higher than the OA rats with statistical difference. The data suggested that SPN treatment might preserve femoral subchondral bone microarchitecture in OA.

#### Discussion

It has been long that nonsteroidal anti-inflammatory drugs (NSAIDs) are used to treat OA in clinical practice. However, most of NSAIDs could only relieve symptoms such as pain and swelling, but could not prevent the degeneration of cartilage and trabecular microarchitecture of subchondral bone in OA. Moreover, long-time use of NSAIDs could result in a range of serious side effects. Specnvezhenide (SPN) is an extracted agent of the fruits of *Ligustrum lucidum*, which is newly discovered in the past few decades. Its pharmaceutical value was only studied in the neuron system<sup>6a</sup> and treatment of diabetic retinopathy<sup>6b</sup>. To the best of our knowledge, we report for the first time that SPN could prevent the degeneration of OA and regulates the activation



of NF- $\kappa$ B and wnt/ $\beta$ -catenin signaling in this study.

It is well accepted that cartilage degradation in OA is mediated by MMP family<sup>11</sup>. High level of MMPs is regarded as one of the main expressions of OA<sup>5b, 7b, 12</sup>, as well as low level of chondrocyte-specific proteins like sox9 and Collagen II<sup>13</sup>. Thus, downregulating the expression of MMPs is a strategy to prevent OA. Firstly, we observed that SPN treatment at different concentrations (0, 10, 50, 100, 200  $\mu$ M) does not affect rat chondrocytes viability, and SPN does not alter gene expression under nonpathological conditions, which means that SPN does not significantly affect the normal cartilage. On the other hand, SPN down-regulated the IL-1 $\beta$ -induced expression of matrix-degrading enzymes including MMP-3 and MMP-9 with significant difference. Interestingly, SPN treatment didn't provide significant improvement on the downregulation of sox9 and Collagen II caused by IL-1 $\beta$  (5ng/ml). This suggests that SPN exerts its protective action by downregulating the expression of matrix-degrading enzymes induced by IL-1 $\beta$  on rat chondrocytes.

NF- $\kappa$ B and wnt/ $\beta$ -catenin pathways are two of the major pathways involved in OA development<sup>14</sup>. When NF- $\kappa$ B pathway is activated by inflammatory mediators like IL-1 $\beta$ , NF- $\kappa$ B-p65 that stays in the cytoplasm in resting status would translocate into nucleus and up-regulates plenty of inflammation-related genes, such as MMPs, COX-2 and PGE2, so NF- $\kappa$ B signaling is a therapeutic target in OA<sup>15</sup>. We found that SPN treatment markedly reduced nucleus translocation of NF- $\kappa$ B-p65 caused by IL-1 $\beta$  stimulation. Interestingly, we also found that SPN treatment significantly inhibited IL-1 $\beta$ -induced phosphorylation of NF- $\kappa$ B-p65 in whole cell. On the other hand, when wnt/ $\beta$ -catenin pathway is staying off-state,  $\beta$ -catenin is targeted to its proteasomal degradation through the  $\beta$ -TrCP/Skp pathway<sup>16</sup>. Thus,  $\beta$ -catenin level stays low at resting situation because of continuous degradation. When wnt/ $\beta$ -catenin pathway is activated,  $\beta$ -catenin becomes stable, proteasomal degradation was reduced and stabilized  $\beta$ -catenin is translocated into nucleus up-regulating plenty of inflammation-related genes like MMPs<sup>16</sup>. As illustrated in Fig 3&4, SPN not only reduced IL-1 $\beta$ -induced increasing level of  $\beta$ -catenin in whole cell, but also inhibited the nucleus-translocation of  $\beta$ -catenin. Taken together, our results indicated that SPN treatment inhibits IL-1 $\beta$ -induced inflammation via regulating NF- $\kappa$ B and wnt/ $\beta$ -catenin pathways in vitro.

We further examined the in vivo effect of APN in a rat OA model. The present opinion of OA progress is that change of microarchitecture in subchondral bone comes along with the degeneration in cartilage<sup>17</sup>. In the animal experiment, we evaluated the degeneration in both articular cartilage (fig 5) and subchondral bone (fig 6). In OA group, erosion in the cartilage was observed and large mass of cartilage matrix loss was usual. Even deformation appeared in some knees. In SPN group, the structural integrity of cartilage was maintained in most knees. Grading results based on OARSI assessment showed a significant protection of articular cartilage in the presence of SPN. Previous study indicates that change of biomechanical environment like ACL-transaction-caused instability of joint could influence subchondral bone and results in concentration and activity of osteoclasts that would promote subchondral bone resorption<sup>18</sup>. In the present study, resorption of subchondral bone in OA rats was investigated in  $\mu$ CT analysis. The improvement of trabecular microarchitecture of femoral subchondral bone in the presence of SPN was also observed according to the result of connectivity density (Conn.D, fig 6). Thus, our results suggest that SPN has a therapeutic effect for OA in vivo.

## Conclusion

In conclusion, we demonstrate firstly that Specnvezhenide regulates IL-1 $\beta$ -induced expression of MMPs and activation of NF- $\kappa$ B and wnt/ $\beta$ -catenin signaling in chondrocytes, and also plays a therapeutic role in degeneration of articular cartilage and subchondral bone in OA.

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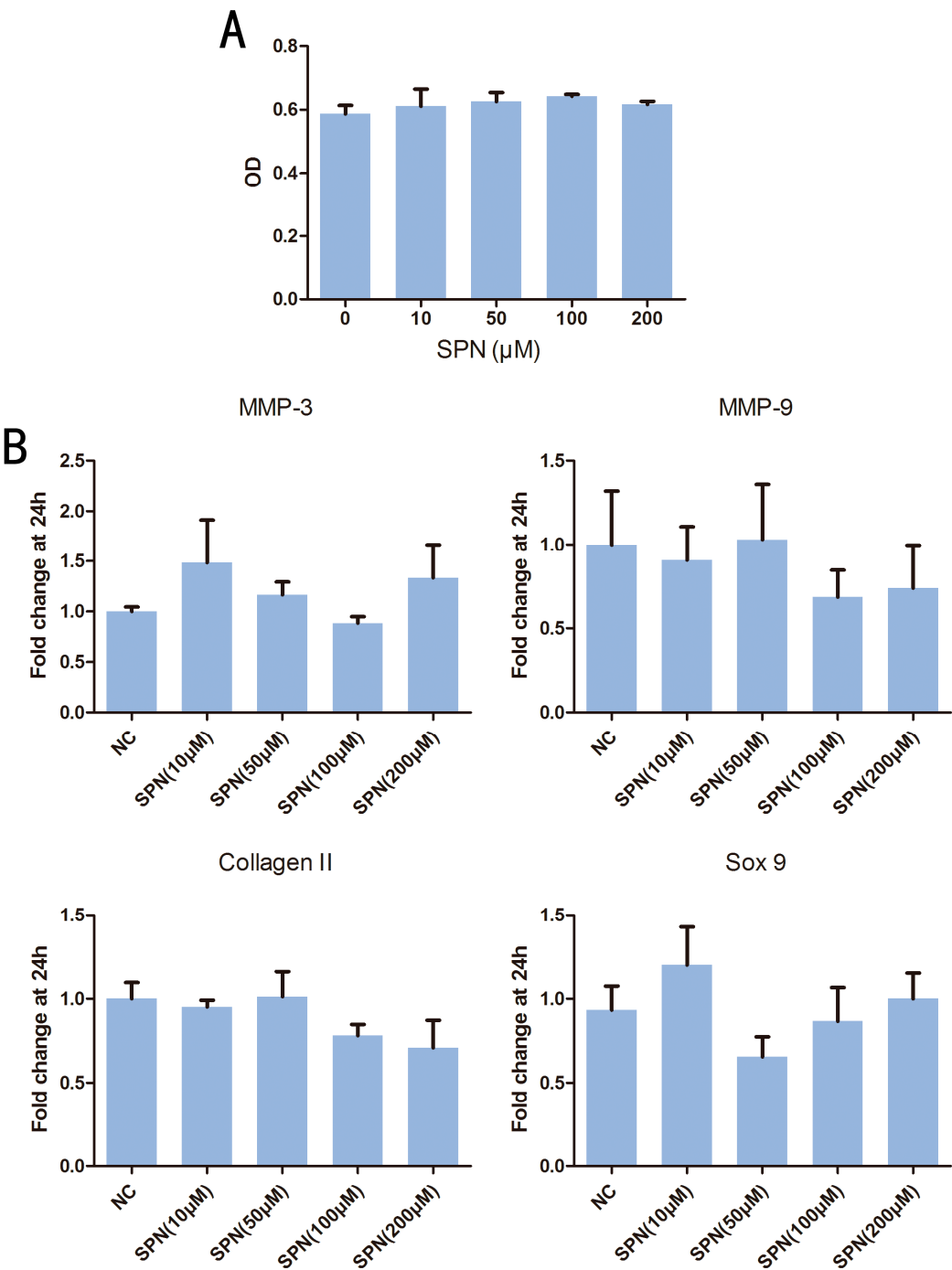


Fig 1. Effect of SPN on viability and gene expression of rat chondrocytes. Rat chondrocytes (5000/wel) were seed in 96-well plates for 24h at present of different concentrations of SPN. Cell viability was assessed by CCK-8 assay (A). Effect of SPN on expression of MMP3, MMP9, Collagen II, and sox9 was evaluated by real-time PCR (B). Significance was calculated by a one-way ANOVA with a post hoc Tukey's multiple comparisons test.

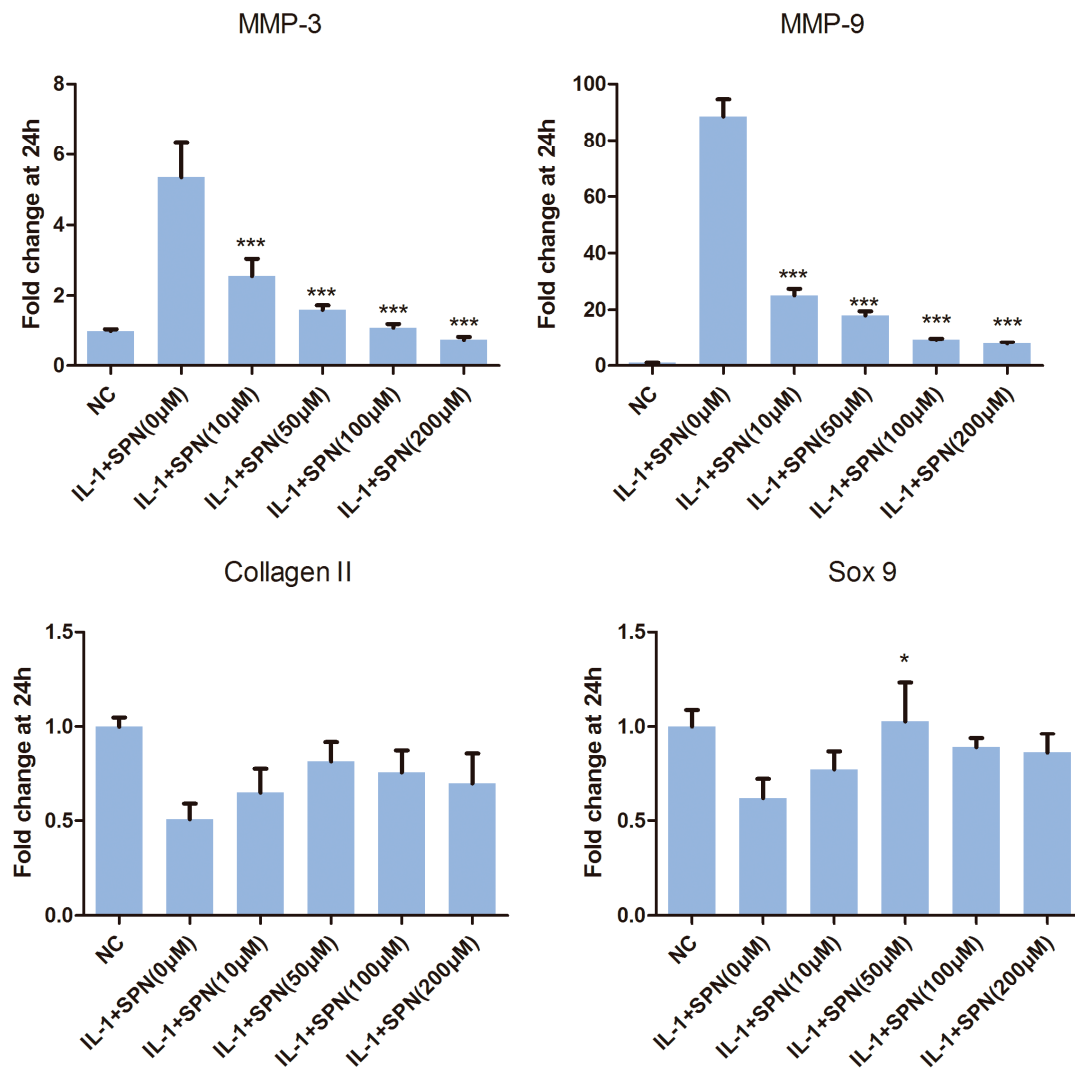


Fig 2. Effect of SPN on IL-1 $\beta$ -induced chondrocyte-specific gene degradation and cartilage matrix-degrading enzymes expression on rat chondrocytes. The chondrocytes were treated with different concentrations of SPN for 24 hours with or without presence of IL-1 $\beta$  (5ng/ml). The expression of chondrocyte-specific genes and matrix-degrading enzyme genes, MMP3, MMP9, Collagen II, and sox9 were evaluated by Real-Time PCR. Significance was calculated by a one-way ANOVA with a post hoc Tukey's multiple comparisons test. \* $p < 0.05$ , \*\*\* $p < 0.001$  versus IL-1+SPN (0 $\mu$ M) treated group.

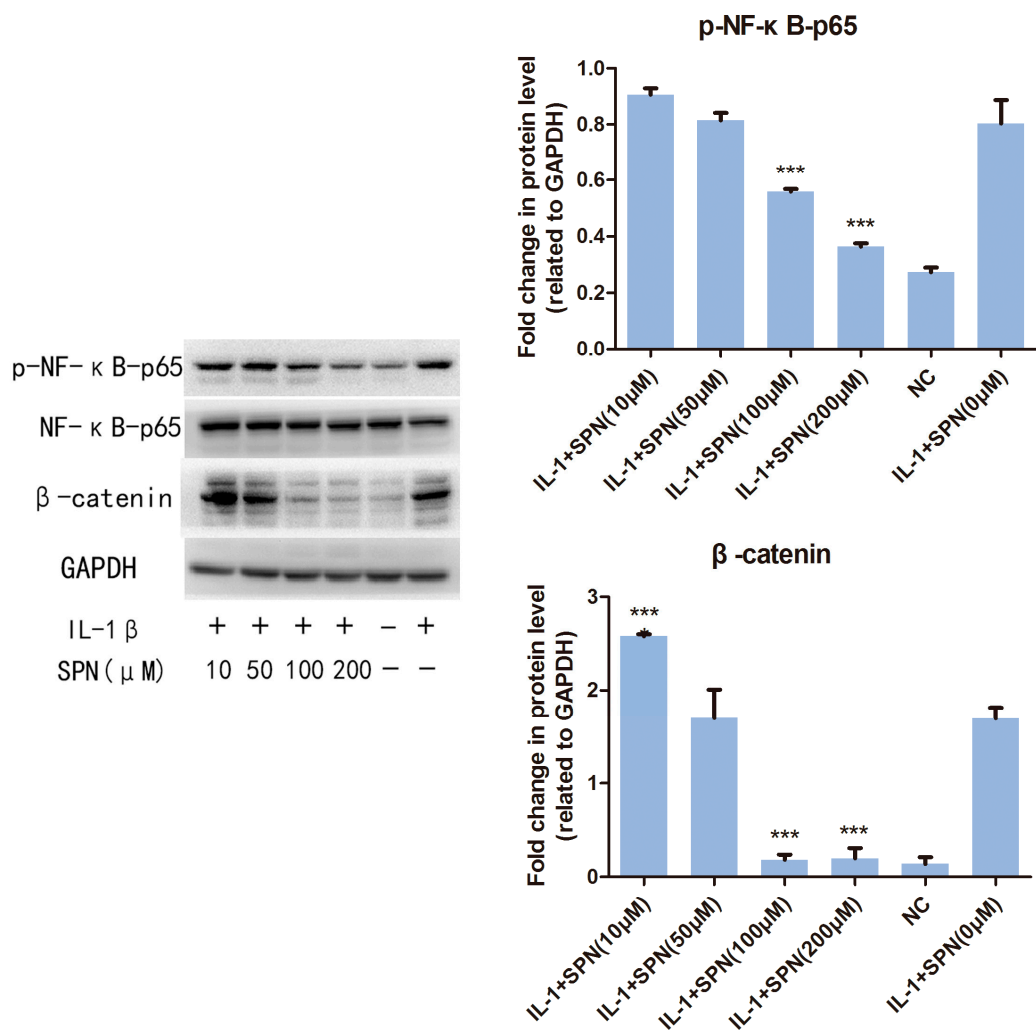


Fig 3. Effect of SPN on IL-1β-induced activation of NF-κB and wnt/β-catenin signaling on rat chondrocytes. The chondrocytes were treated with different concentrations of SPN for 24 hours with or without presence of IL-1β (5ng/ml). The level of β-catenin, NF-κB p65, GAPDH and phosphorylation level of NF-κB p65 was assessed by Western blot. GAPDH worked as endogenous controls here. Significance was calculated by a one-way ANOVA with a post hoc Tukey's multiple comparisons test. \*\*\*p<0.001 versus IL-1β+SPN (0μM) treated group.

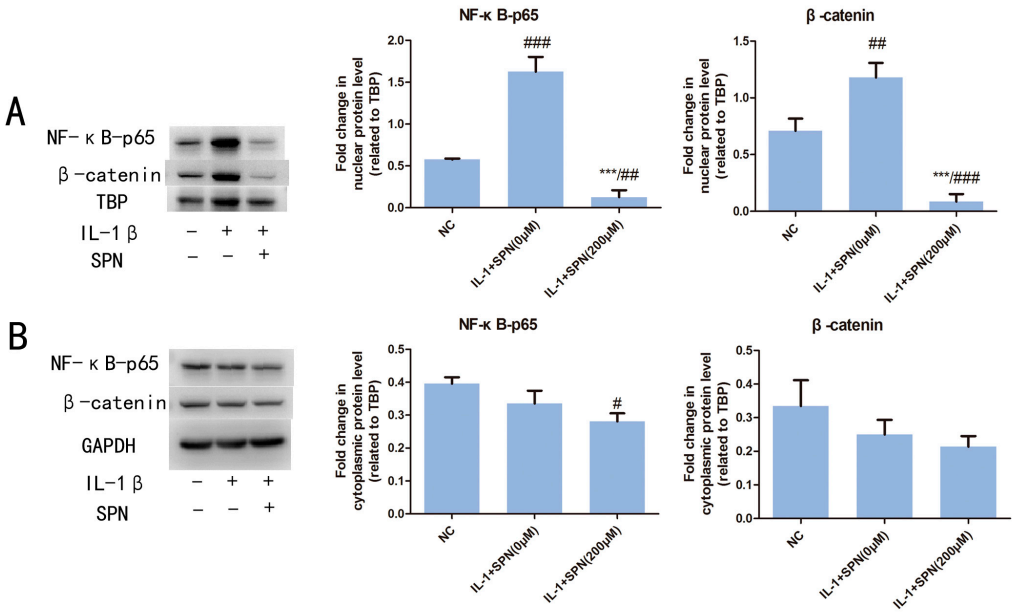


Fig 4. Effect of SPN on IL-1 $\beta$ -induced nucleus translocation of NF- $\kappa$ B p65 and  $\beta$ -catenin. Nuclear and Cytoplasmic Extraction Reagents were used to prepare nuclear and cytoplasmic extracts. Then, protein level of  $\beta$ -catenin, NF- $\kappa$ B p65, GAPDH in cytoplasm and  $\beta$ -catenin, NF- $\kappa$ B p65, TBP in nucleus was assessed by Western blot. GAPDH worked as endogenous control in cytoplasm, and TBP worked as endogenous control in nucleus. Significance was calculated by a one-way ANOVA with a post hoc Tukey's multiple comparisons test. \*\*\*p<0.001 versus IL-1 $\beta$ +SPN (0 $\mu$ M) treated group. #p<0.05, ##p<0.01, ###p<0.001 versus NC group.

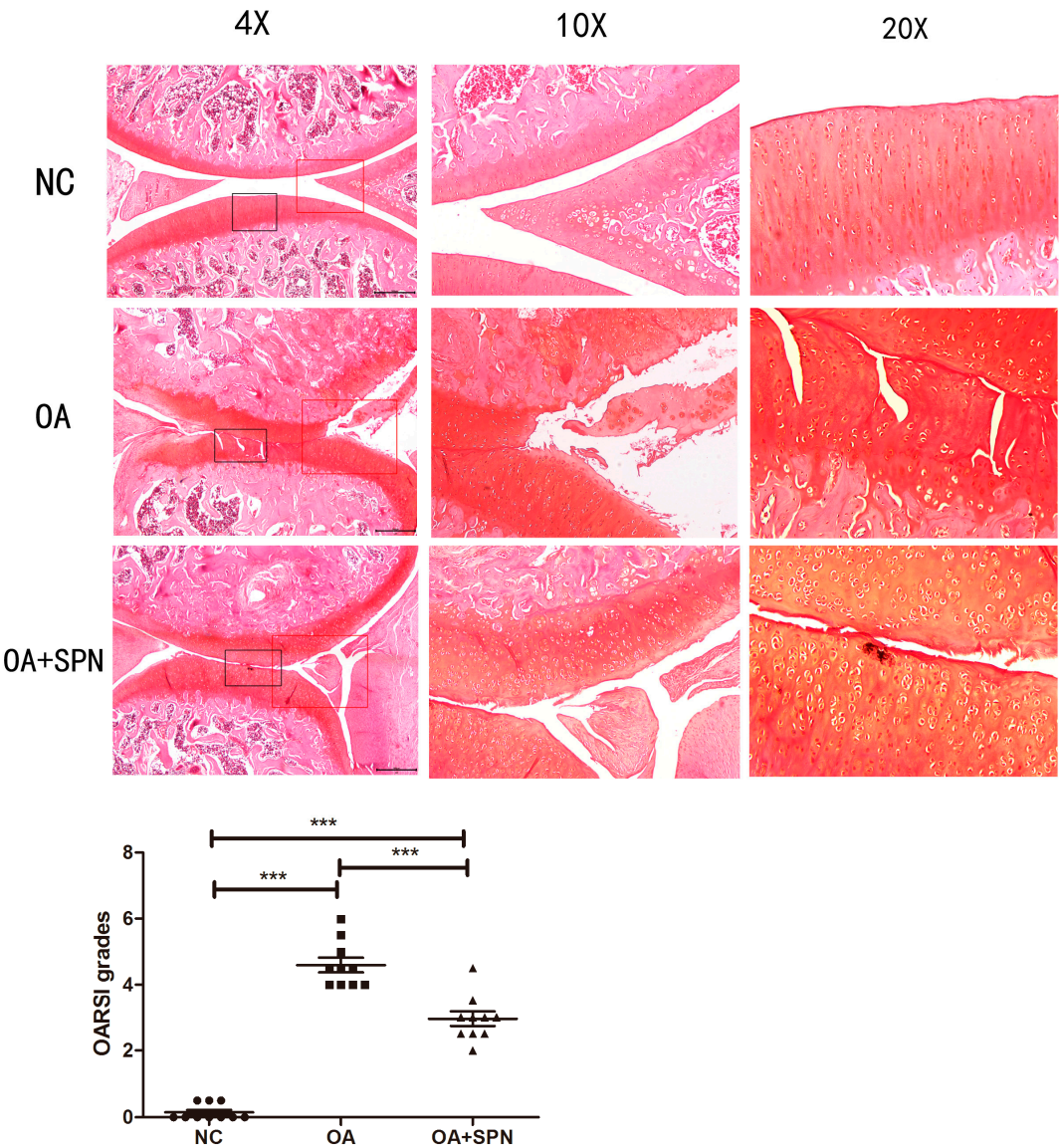


Fig 5. Effect of SPN treatment on cartilage degeneration in rat model of OA. ACL&MM-transected rats were injected intra-articularly with SPN (200μM) every 7 days. Rats were sacrificed to isolate the knees for analysis after 8 weeks' treatment. 4% paraformaldehyde solution fixed knees were decalcified and embedded in paraffin, sectioned at 5 μm thickness. The sections of interior joint were stained with Safranin O-fast green (SO). The OARSJ assessment system with grading method (0-6) was used to evaluate the sections. The red square illustrates 10X area, while black square illustrates 20X area. Significance was calculated by a one-way ANOVA with a post hoc Tukey's multiple comparisons test. \*\*\* means p<0.001.



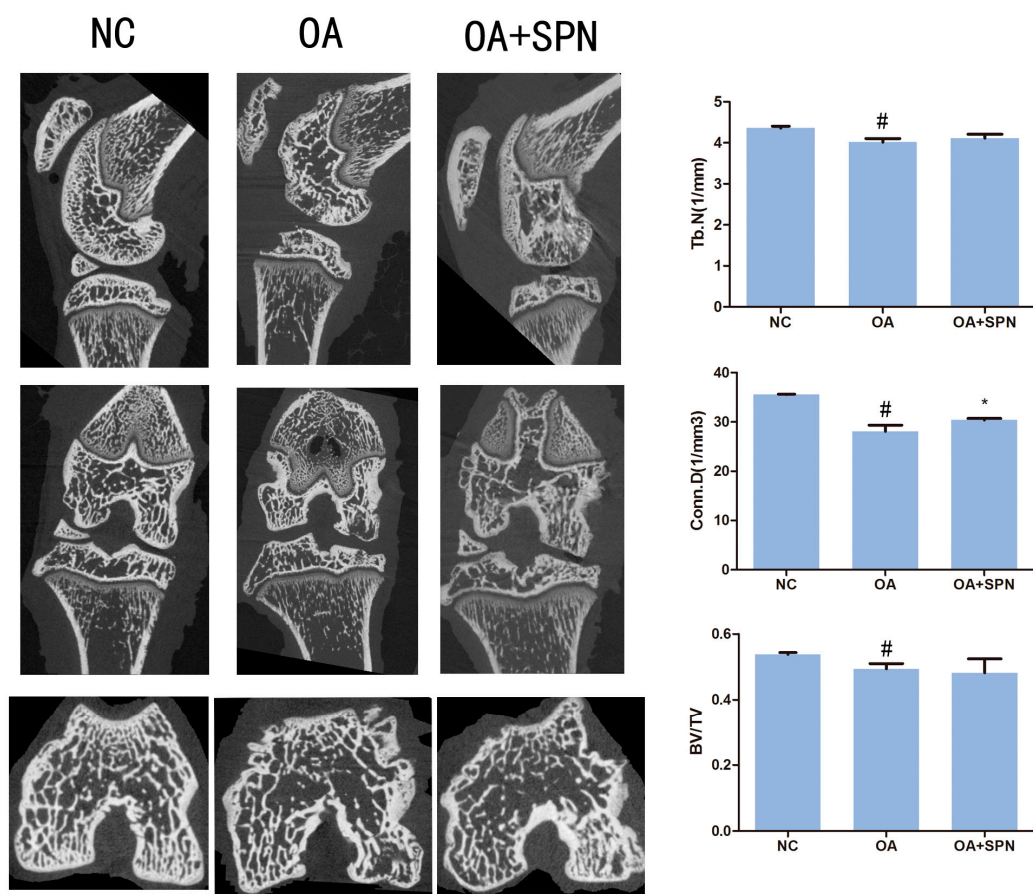


Fig 6. Effect of SPN treatment on degeneration of trabecular microarchitecture of subchondral bone in OA rats. The treatment was performed as described in Fig 5, and the knees were subjected to  $\mu$ CT for evaluation of subchondral bone. CT Analyzer software was used to evaluate BV/TV, Tb. N., and Conn.D. Significance was calculated by a one-way ANOVA with a post hoc Tukey's multiple comparisons test. \* $p < 0.05$  versus OA group; # $p < 0.05$  versus NC group.