

# Hydrogen sulfide attenuates IL-1 $\beta$ -induced inflammatory signaling and dysfunction of osteoarthritic chondrocytes

CHENGZHI HA<sup>1</sup>, SHAOQI TIAN<sup>1</sup>, KANG SUN<sup>1</sup>, DAWEI WANG<sup>2</sup>, JIANGTAO LV<sup>1</sup> and YUANHE WANG<sup>1</sup>

<sup>1</sup>Department of Joint Surgery, Affiliated Hospital of Qingdao University, Qingdao, Shandong 266071;

<sup>2</sup>Department of Joint Surgery, Liaocheng People's Hospital, Liaocheng, Shandong 252000, P.R. China

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**Abstract.** Inflammatory cytokines are crucial factors in the onset of osteoarthritis (OA). The pro-inflammatory cytokine, interleukin-1 $\beta$  (IL-1 $\beta$ ), is capable of stimulating a few cartilage degradation mediators and is of importance to the pathogenesis of OA. It has been demonstrated that hydrogen sulfide (H<sub>2</sub>S) exerts an inhibitory effect on inflammation. Thus, in the present study, we aimed to investigate the therapeutic effects of H<sub>2</sub>S in OA. For this purpose, an *in vitro* model of cartilage inflammation was created. Human OA chondrocytes were cultured and pre-treated with H<sub>2</sub>S (0.06-1.5 mM) with or without IL-1 $\beta$  (10 ng/ml) and then Griess reagent was used to quantify the production of nitric oxide (NO). Using enzyme-linked immunosorbent assay, we quantified the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and matrix metalloproteinase-13 (MMP-13). In addition, we determined the gene expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and MMP-13 using reverse transcription-quantitative polymerase chain reaction and the expression of signaling molecules related to mitogen-activated protein kinases (MAPKs) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) by western blot analysis. Our results revealed that H<sub>2</sub>S markedly reversed the effects of IL-1 $\beta$  on the gene expression of COX-2, MMP-13 and iNOS and on the production of MMP-13, PGE<sub>2</sub> and NO. In addition, H<sub>2</sub>S inhibited the activation of the extracellular signal-regulated kinase (ERK)/I $\kappa$ B $\alpha$ /NF- $\kappa$ B pathway which was induced by IL-1 $\beta$ . On the whole, the results of the present study suggest that H<sub>2</sub>S exerts chondroprotective effects. Thus, H<sub>2</sub>S may have potential for use in the treatment of patients suffering from OA.

## Introduction

Osteoarthritis (OA) is a prevalent degenerative disease of the joints affecting a large number of individuals worldwide. It is

estimated that 2/3 individuals older than 60 years of age suffer from OA and the number of individuals suffering from this disease is increasing (1). Among the clinical manifestations of the disease, those including joint pain are the most common. These manifestations appear with functional limitation at different levels, reducing the quality of life of affected patients. The pathological changes associated with OA are subchondral bone remodeling, synovial inflammation and the progressive degeneration of articular cartilage (2).

The cause of the cartilage damage involves the destruction of the shift which maintains the balance between chondrocyte anabolic and catabolic capacities. This balance, by means of producing various types of proteins associated with the matrix, enzymes, as well as cytokines, maintains the extracellular matrix (2). OA is triggered by the imbalance between chondrocyte catabolic and anabolic activities. For most individuals, in spite the fact that the etiology of OA has not yet been thoroughly elucidated, it is acceptable to say that the production of excess inflammatory cytokines is key to the development of OA (3). It has been demonstrated that interleukin-1 $\beta$  (IL-1 $\beta$ ), one of the cytokines with pro-inflammatory effects, is of particular importance to the development of the disease (4). By increasing the production of matrix metalloproteinases (MMPs), IL-1 $\beta$  facilitates the degradation of the cartilage matrix (4). At the same time, it may induce the expression of inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) [which contributes to the production of higher levels of NO and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)] (5). It has been found that NO is capable of upregulating the production of inflammatory cytokines and MMPs, as well as inducing chondrocyte apoptosis. PGE<sub>2</sub> plays a role in both joint pain and bone resorption. PGE<sub>2</sub> is also capable of mediating cartilage matrix degradation by promoting the activity of MMPs and other inflammatory cytokines (6).

To date, there is no available totally effective therapy for OA as a multifactorial degenerative joint disorder. Current treatment strategies for individuals suffering from this disease aim at controlling joint swelling and pain, and delaying the progression of the disease, as well as improving the quality of life of patients (5). Primarily, balneotherapy has been adopted as a treatment strategy. This ancient treatment method is applied by immersion in water with various chemical, thermal and mechanical properties (7). A previous study demonstrated that for rats with chronic experimental arthritis, therapy using

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**Correspondence to:** Professor Kang Sun or Dr Shaoqi Tian, Department of Joint Surgery, Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Qingdao, Shandong 266071, P.R. China  
E-mail: sunkang\_qy@aliyun.com  
E-mail: shaoqi99@aliyun.com

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a sulfur bath produced an anti-inflammatory result (8). This method has been adopted for the treatment of a number of patients with OA and rheumatic diseases; as previously demonstrated, patients spent 2-3 weeks being subjected to sulphurous mud-bath therapy at a spa in Italy (9). In addition, it has been discovered by some recently conducted research that hydrogen sulfide ( $H_2S$ ) is possibly a very important contributor to the positive effects of sulfur baths (10).

It is now considered that  $H_2S$ , carbon monoxide (CO) and NO belong to the same group of endogenously produced gas molecules. Nevertheless, studies on the biological function of  $H_2S$  are limited and fewer than those on CO and NO. Emerging evidence indicates that  $H_2S$  is crucial for a number of pathological and physiological processes, such as for example, in metabolic disorders including diabetes (11). Relevant studies have indicated that 30-100  $\mu M$  concentrations of physiological  $H_2S$  exert antinociceptive effects (12). In spite of the fact that relevant data have provided information regarding the mechanisms of action and precise sites of action of the inflammatory mediator,  $H_2S$ , these mechanisms and sites of action have not yet been fully elucidated. It seems that the upregulation of, for example, the production of CO, is capable of mediating some of the anti-inflammatory and antinociceptive effects of  $H_2S$ . This mediating process, by means of inflammatory stimuli, leads to the inhibition of the nuclear factor- $\kappa B$  (NF- $\kappa B$ ) pathway and the downregulation of iNOS expression (13).

In terms of the potential role of  $H_2S$  in OA, to the best of our knowledge, there is no relevant information currently available. According to our previous studies, the concentrations of  $H_2S$  contained in the synovial fluid aspirated from the knee joints of patients with OA are higher than those in the synovial fluid from individuals without OA (unpublished data). Analogously, other studies have demonstrated that human cartilage cells have the capacity to synthesize  $H_2S$ . This is observed as part of an acute response to the so-called pro-inflammatory mediators, including IL-1 $\beta$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and lipopolysaccharide (LPS). It has been suggested that cytokine-induced  $H_2S$  synthesis may be used to prevent oxidative injury to joint cells (14,15). According to previous findings, both the decrease in leukocyte velocity and synovial leukocyte adherence (independent of the dose used) may be caused by  $H_2S$  (16), indicating the anti-inflammatory effects of  $H_2S$  in OA. However, the anti-inflammatory mechanisms of action and effects of  $H_2S$  on chondrocyte inflammatory responses induced by IL-1 $\beta$  have not yet been fully clarified. In the process, NF- $\kappa B$  as an inducible transcription factor is important for the control of the transcription of inflammatory response genes (17,18). It is known that mitogen-activated protein kinases (MAPKs) function as upstream activators of NF- $\kappa B$ . It is evident that as regards NF- $\kappa B$  activity, extracellular signal-regulated kinase (ERK) is an important temporal regulator. When ERK1/2 is depleted using inhibitors, NF- $\kappa B$  activation is reduced, inhibiting NF- $\kappa B$ -dependent gene transcription (19). Therefore, based on these findings, the present study was conducted to determine the following: i) the effects of  $H_2S$  on the IL-1 $\beta$ -induced expression of chondrocyte catabolic factors and ii) whether  $H_2S$  protects chondrocytes from damage through the ERK-NF- $\kappa B$  channel in OA. To better conduct the evaluation of the chondroprotective effects of  $H_2S$  and to offer an advanced basis for the clinical treatment of

OA in the future, further knowledge of the underlying mechanisms may be required.

## Materials and methods

**Reagents.** Sodium hydrosulfide (NaHS), recombinant human IL-1 $\beta$ , collagenase type II, PD98058 (an inhibitor of ERK), Toluidine blue, Safranin O, and hematoxylin and eosin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin and streptomycin, fetal bovine serum (FBS), and 0.25% trypsin were obtained from Gibco-BRL (Grand Island, NY, USA). The cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). The Griess reagent assay kit was obtained from the Beyotime Institute of Biotechnology (Haimen, China). The enzyme-linked immunosorbent assay (ELISA) kit was provided by Boster Bio-Engineering Limited Company (Wuhan, China). TRIzol Reagent and the One-Step qPCR kit were purchased from Tiangen Biotech Co. Ltd. (Beijing, China). Antibodies against ERK1/2/phospho-ERK1/2 (Thr202/Tyr204), I $\kappa B\alpha$ /phospho-I $\kappa B\alpha$  (Ser32/36) and NF- $\kappa B$  p65/phospho-NF- $\kappa B$  p65 (Ser536) and  $\beta$ -actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The NF- $\kappa B$  (p65) transcription factor assay kit was purchased from Active Motif (Carlsbad, CA, USA). All other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

**Chondrocyte isolation and culture.** The purpose and nature of the study were clearly explained to the patients and written informed consent was obtained from all patients, and the study was approved by the Institutional Ethics Review Committee of the Affiliated Hospital of Qingdao University, Qingdao, China. Our obtained human cartilage samples were selected from OA sufferers who had undergone total knee arthroplasty. OA was diagnosed according to the Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Patients with the same grade of OA were included in our study. Patients who had received an intra-articular injection of steroids were excluded from our study.

After the cartilage was subjected to 30 min of digestion with 0.25% trypsin, it was then digested with 2 mg/ml collagenase II in DMEM for 6 h. The cells were suspended in DMEM with 15% FBS and antibiotics and then seeded in tissue culture flasks. Confluent cells were split at 1:3. An inverted phase contrast microscope (Olympus CK40, Germany) was used to observe the morphology of the chondrocytes cultured *in vitro*. The chondrocytic phenotype was confirmed by microscopic evaluation, by staining for glycosaminoglycan production (hematoxylin and eosin, Toluidine blue and Safranin O staining). Cells between passages 3 and 4 were used in the experiments.

**$H_2S$  donor.** NaHS is universally adopted and in this regard, it functions as an  $H_2S$  donor. NaHS is used to obtain  $H_2S$ , as well as to define the  $H_2S$  concentration in a solution. Compared with the defining method of measuring bubbling  $H_2S$  gas, this method is not only more reproductive, but also more accurate. Immediately prior to use from a solution of 200 mM NaHS stock, the solution containing NaHS was prepared. NaHS is dissociated to  $HS^-$  and  $Na^+$  (in solution) at first. It is then

dissociated to  $\text{HS}^-$  and binds to  $\text{H}^+$ ;  $\text{H}_2\text{S}$  is then formed. Under certain conditions, 18.5% of  $\text{H}_2\text{S}/\text{HS}^-$  exists (in a non-dissociated form). Without knowing the active form of  $\text{H}_2\text{S}$ , as regards the total number of the forms of  $\text{H}_2\text{S}$ , we used the terminology ' $\text{H}_2\text{S}$ '. Therefore, a solution of  $\text{H}_2\text{S}$  with a concentration at the level of approximately 1/3 of the NaHS original concentration was provided, as previously described (20).

**Cell viability assay.** We detected cell viability using the CCK-8 assay and cultured the chondrocytes in the exponential phase at  $1 \times 10^4$  cells/ml concentration in 96-well plates. Each group contained 4 duplicate wells. As soon as the cells reached 70-80% confluence, the cells were treated using conditioned medium, which contained various concentrations of  $\text{H}_2\text{S}$ .

Subsequently, 10  $\mu\text{l}$  of CCK-8 solution were added to each well and the cells were placed in an incubator for incubation. The incubation period lasted for 4 h. We performed measurements for absorbance at 450 nm, with the help of a microplate reader (Molecular Devices, Sunnyvale, CA, USA), and calculated the percentage cell viability in 4 wells, calculating the optical density (OD) value in specific groups.

The calculation was realized based on a relevant formula: cell viability percentage = (OD treatment group - OD blank group)/(OD control group - OD blank group)  $\times 100$ . The experiment was carried out in triplicate.

**Measurement of MMP-13,  $\text{PGE}_2$  and NO production in culture supernatants.** We cultured the human OA chondrocytes ( $1 \times 10^6$  cells/ml) in 6-well plates in complete DMEM with 10% FBS. The human OA chondrocytes (>85% confluent) were serum-starved before being treated with various doses of NaHS (0.06, 0.15, 0.3, 0.6 and 1.5 mM) for 30 min followed by stimulation with or without IL-1 $\beta$  (10 ng/ml) for 24 h. Twenty-four hours after the addition of IL-1 $\beta$ , we collected the cell conditioned medium and then stored it at  $-80^\circ\text{C}$  until analysis. The concentration of  $\text{PGE}_2$  and MMP-13 was quantified using MMP-13- and  $\text{PGE}_2$ -specific ELISA kits in accordance with the instructions of the manufacturer (Boster Bio-Engineering Limited Company). The plates were read at 450 nm. In order to measure NO production, we measured the nitrite concentration in the culture supernatants using Griess reagent. The absorbance was read at 540 nm.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for MMP-13, iNOS and COX-2.** Based on the instructions of Tiangen Biotech Co., Ltd. (Beijing, China) we used TRIzol reagent to extract the total RNA (1  $\mu\text{g}$ ), which was reverse transcribed into cDNA. We used the One-Step qPCR kit from the same company during amplification. We also used SYBR-Green detection to determine the mRNA expression of COX-2, iNOS and MMP-13 following the normalization of the values. During the performing of PCR, the 7300 sequence detection system was used. The primers used for PCR were as follows: MMP-13 forward, 5'-CGCCAGAAGAATCTGTCTAA-3' and reverse, 5'-CCAAATTATGGAGGAGATGC-3'; iNOS forward, 5'-CCTTACGAGCGAAGAAGGACAG-3' and reverse, 5'-CAGTTTGAGAGAGGAGGCTCCG-3'; COX-2 forward, 5'-TTCAAATGAGATTGTGGGAAA TTGCT-3' and reverse, 5'-AGATCATCTCTGCCTGAGTATCTT-3'; and GAPDH forward 5'-GGTATCGTCAAGGA

CTCATGAC-3' and reverse, 5'-ATGCCAGTGAGCTTCCCG TTCAGC-3'. The thermal cycling conditions for quantitative PCR were  $95^\circ\text{C}$  for 2 min followed by 45 cycles (at different temperatures and for different periods of time). We used the temperature of  $68^\circ\text{C}$  for data collection. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. We quantified the quantitative PCR data using the  $\Delta\text{CT}$  method with the formula:  $n=2^{-(\Delta\text{CT}_{\text{targeted gene}} - \Delta\text{CT}_{\text{GAPDH}})}$ .

**Western blot analysis.** Western blot analysis was performed to determine the effects of NaHS on IL-1 $\beta$ -dependent ERK1/2-phosphorylation, I $\kappa$ B $\alpha$ -phosphorylation, I $\kappa$ B $\alpha$  degradation, NF- $\kappa$ B p65 translocation and NF- $\kappa$ B p65 activity. Whole cell lysates, the chondrocyte monolayer nuclear and cytoplasmic extracts were washed with Hanks' solution 3 times and whole cell proteins were extracted using lysis buffer on ice for half an hour. Centrifugation (700  $\times$  g) was performed to remove the cell debris. The supernatants were stored at  $-80^\circ\text{C}$  until use. We measured the total protein concentration in the cytoplasmic, nuclear and whole cell extracts using the BCA protein assay kit (Uptima; Interchim, Montlucon, France). During the process, BSA was used as a standard. When the adjustments for equal amounts of total protein were made, we separated the proteins under reducing conditions. The separated proteins were then transferred onto PVDF membranes. The membranes were then blocked for 30 min at room temperature in fresh blocking buffer [0.1% Tween-20 in Tris-buffered saline (TBS-T) containing 5% fat-free milk] and then incubated with either anti-ERK1/2 (1:1,000 dilution), anti-p-ERK1/2 (1:1,000 dilution), anti-I $\kappa$ B $\alpha$  (1:1,000 dilution), anti-p-I $\kappa$ B $\alpha$  (1:1,000 dilution), anti-NF- $\kappa$ B p65 (1:1,000 dilution), anti-p-NF- $\kappa$ B p65 (1:1,000 dilution), or anti- $\beta$ -actin antibodies (1:5,000 dilution) in freshly prepared TBS-T with 3% skimmed milk overnight with gentle agitation at  $4^\circ\text{C}$ . Following 3 washes with TBS-T, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:3,000 dilution; Kangchen Biotech, Shanghai, China) in TBS-T with 3% skimmed milk for 1.5 h at room temperature. The membranes were rinsed 3 times with TBS-T, developed in ECL solution and visualized using X-ray film. All the experiments were repeated for no less than 3 times. ImageJ 1.47 software was used to analyze and scan the films for quantification.

**NF- $\kappa$ B activity assay.** Based on relevant instructions provided by the manufacturer (Active Motif), the TransAM<sup>TM</sup> NF- $\kappa$ B p65 transcription factor assay kit was used to analyze the DNA-binding activity of NF- $\kappa$ B. In brief, the kit with an ELISA format as its basis is used to perform the analysis in a 96-well plate. During this process, in the wells, the oligonucleotide which contained the NF- $\kappa$ B consensus-binding sequence (5'-GGGACTTCC-3') was immobilized. We incubated the nuclear extracts in the wells and then used a primary antibody to perform the detection of bound NF- $\kappa$ B p65. We then utilized an HRP-conjugated secondary antibody for the detection of the bound primary antibody. The HRP-conjugated secondary antibody also laid the foundation for colorimetric quantification. Using a microplate reader (Molecular Devices), we took measurements for the enzymatic product at 450 nm. For the purpose of monitoring the specificity of the assay, we added (mutated) competitive control into the wells with the wild-type

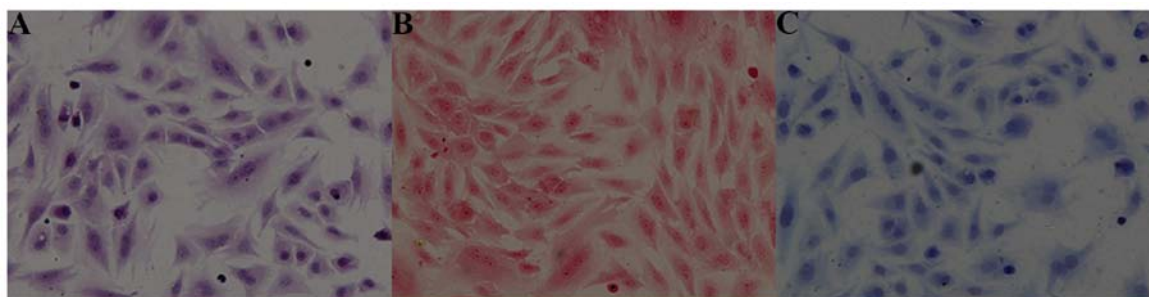


Figure 1. Identification of the chondrocytic phenotype. The chondrocytic phenotype was confirmed by hematoxylin and eosin staining and staining for glycosaminoglycan production (Toluidine blue and Safranin O staining). (A) Hematoxylin and eosin staining (x200 magnification); (B) Safranin O staining (x200 magnification); (C) Toluidine blue staining (x200 magnification).

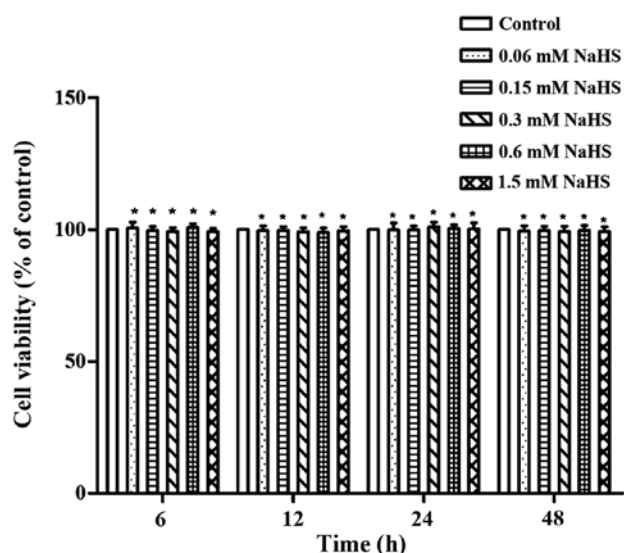


Figure 2. Effects of hydrogen sulfide ( $H_2S$ ) on the viability of articular chondrocytes. The cell counting kit-8 (CCK-8) was used for the determination of cell viability. No significant cytotoxicity to the osteoarthritic (OA) chondrocytes was observed at any of the  $H_2S$  concentrations used. \* $P > 0.05$  vs. control.

(mutated) NF- $\kappa$ B consensus oligonucleotide before adding the nuclear extracts.

**Statistical analysis.** All experiments reported in this study were performed independently at least 3 times and the data are expressed as the means  $\pm$  SD. Statistical significance was assessed by one-way analysis of variance (ANOVA) using SPSS 13.0 software. Differences were considered statistically significant at  $P < 0.05$ .

## Results

**Chondrocyte morphology and cytochemical characteristics.** The chondrocytes were allowed to adhere to the bottom of dishes for 4 to 12 h. In all monolayer cultures, the chondrocytes were typically flattened and elongated in shape. Images which were obtained following staining with hematoxylin and eosin revealed that the nuclei were stained an amethyst color and that the cytoplasm was stained pink (Fig. 1A). We used both Safranin O and Toluidine blue staining for the purpose of identifying the chondrocytes. It was found that the nuclei

were stained a crimson color (Fig. 1B), or dark blue (Fig. 1C). Under the influence of chondroitin and glycosaminoglycan staining, the cellular cytoplasm was stained light blue or light red. Therefore, as shown by these results, the OA chondrocytic phenotype was evident.

**Effects of  $H_2S$  on articular chondrocyte viability.** From the various  $H_2S$  concentrations we used in this study, no major cytotoxic effects on the OA chondrocytes were observed. This was demonstrated by the assessment of cell viability using CCK-8 assay ( $P > 0.05$ ; Fig. 2).

**$H_2S$  suppresses the IL-1 $\beta$ -induced secretion of NO, PGE<sub>2</sub> and MMP-13 by OA chondrocytes.** As shown in Fig. 3, we determined the effects of  $H_2S$  on the generation of MMP-13, PGE<sub>2</sub> and NO induced by IL-1 $\beta$ . Various concentrations of NaHS (0, 0.06, 0.15, 0.3, 0.6 or 1.5 mM) were used to treat the human OA chondrocytes for 30 min prior to stimulation for 24 h with IL-1 $\beta$  (10 ng/ml). It was observed that stimulation with IL-1 $\beta$ , compared with the unstimulated controls, markedly promoted the production of PGE<sub>2</sub>, NO, and MMP-13 ( $P < 0.05$ ). However, treatment of the OA chondrocytes with NaHS (0.06, 0.15, 0.3 or 0.6 mM) prior to stimulation with IL-1 $\beta$  led to a significant decrease in the production of MMP-13, PGE<sub>2</sub> and NO ( $P < 0.05$ ), with the most prominent effects being observed with the dose of 0.3 mM NaHS. CCK-8 assay revealed that cell viability was not affected by  $H_2S$ ; the inhibitory effects of  $H_2S$  did not actually result in reduced cell viability, even though the IL-1 $\beta$ -induced production of NO, PGE<sub>2</sub> and MMP-13 in the chondrocytes was not inhibited by treatment with NaHS at the dose of 1.5 mM ( $P > 0.05$ ).

**The induction of the expression of COX-2, MMP-13 and iNOS by IL-1 $\beta$  in OA chondrocytes is attenuated by  $H_2S$ .** The expression and production of COX-2, MMP-13 and iNOS is crucial to the degradation of cartilage and human OA chondrocytes (stimulated with IL-1 $\beta$ ). We used RT-qPCR to determine the effects of  $H_2S$  on the gene expression levels of COX-2, MMP-13 and iNOS induced by IL-1 $\beta$ . The results revealed that the mRNA expression of COX-2, MMP-13 and iNOS was markedly inhibited by NaHS (0.06, 0.15, 0.3 and 0.6 mM) ( $P < 0.05$ ; Fig. 4). The maximum response was observed at the dose of 0.3 mM NaHS, in spite of the fact that no obvious inhibitory effect on the mRNA expression levels was observed at the dose of 1.5 mM NaHS ( $P > 0.05$ ).



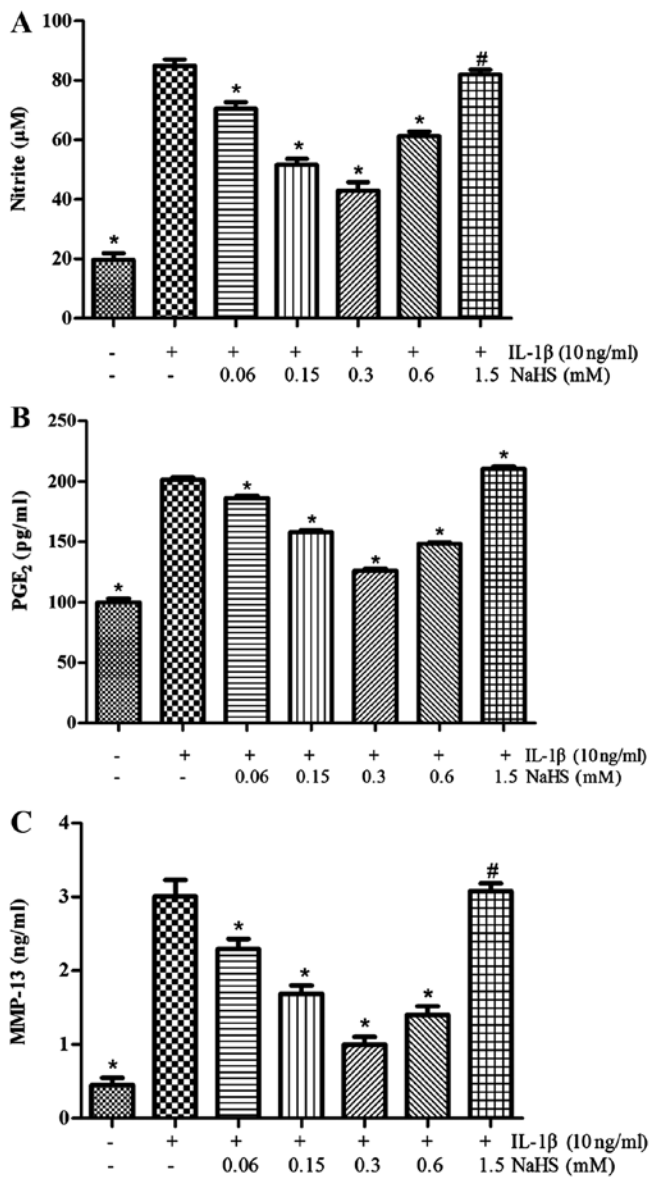


Figure 3. Concentration-dependent effects of H<sub>2</sub>S on the expression of inducible nitric oxide synthase (iNOS), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and matrix metalloproteinase-13 (MMP-13) induced by interleukin-1β (IL-1β) in osteoarthritic (OA) chondrocytes stimulated with IL-1β. The cells were pre-treated with sodium hydrosulfide (NaHS) at the dose of 0, 0.06, 0.15, 0.3, 0.6 or 1.5 mM for 30 min. The cells were then stimulated with IL-1β for 24 h. (A) Griess reaction was used to assess the nitrite levels in the culture medium. (B and C) The MMP-13 and PGE<sub>2</sub> expression levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit. Values are the means ± standard deviation. \*P<0.05 vs. IL-1β group; #P>0.05 vs. IL-1β group.

**Effect of IL-1β on NF-κB signaling and ERK1/2 activation.** The activation the MAPK and NF-κB signaling pathways induced by IL-1β is of great importance to the regulation cytokine expression. We investigated the effects of IL-1β on the phosphorylation of ERK1/2, IκBα and NF-κB p65, as well as on the NF-κB binding activity in the OA chondrocytes at different time points. In the control (unstimulated) OA chondrocytes, no detectable phosphorylation of ERK1/2, IκBα and NF-κB p65 was observed. The phosphorylation of NF-κB p65, ERK1/2 and IκBα in the OA chondrocytes was markedly induced by stimulation with IL-1β within a very short period of time

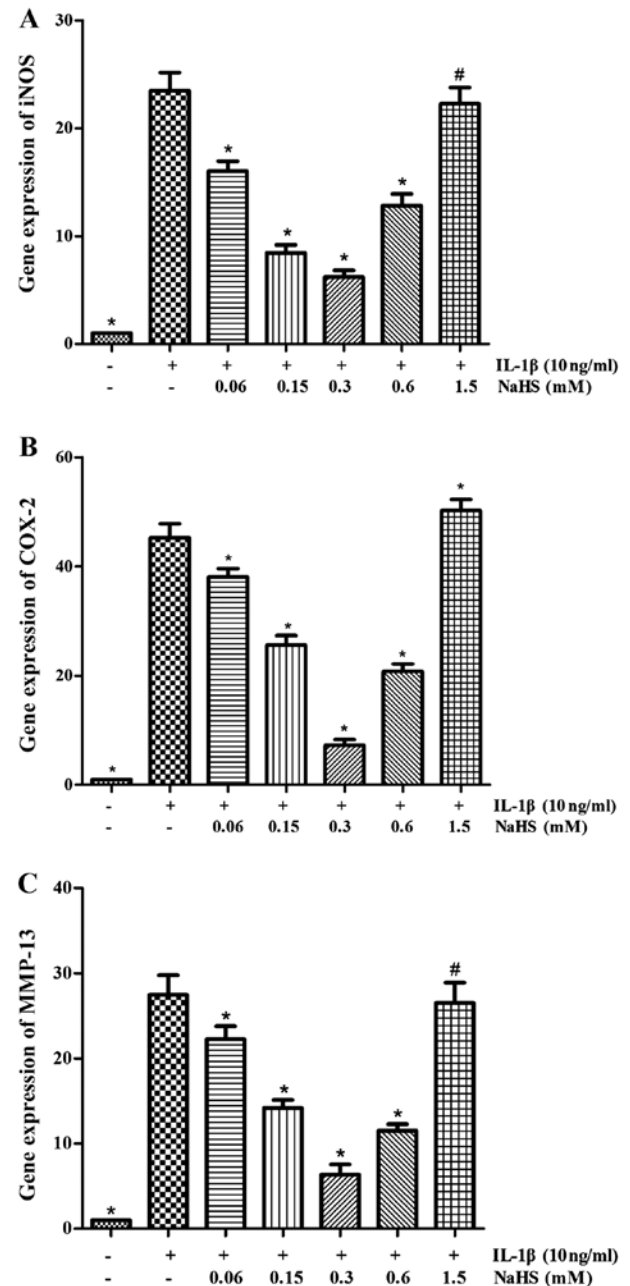


Figure 4. Concentration-dependent effects of hydrogen sulfide (H<sub>2</sub>S) on the expression of (A) inducible nitric oxide synthase (iNOS), (B) cyclooxygenase-2 (COX-2) and (C) matrix metalloproteinase-13 (MMP-13) induced by interleukin-1β (IL-1β) in osteoarthritic (OA) chondrocytes stimulated with IL-1β. The human OA chondrocytes were pre-treated with sodium hydrosulfide (NaHS) at the dose of 0.06, 0.15, 0.3 0.6 or 1.5 mM for 30 min. The cells were then stimulated with IL-1β (10 ng/ml) for 24 h. RT-qPCR was used for the analysis of mRNA expression. The normalization of relative gene expression to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was conducted. The expression levels were compared to those of the controls (unstimulated cells). Values are the means ± standard deviation. \*P<0.05, #P>0.05 compared with IL-1β group.

(following treatment for 0.5, 1 and 2 h). The phosphorylation levels reached peak levels at 2 h and then decreased to normal levels. However, stimulation with IL-1β for longer periods of time (6 or 24 h) did not increase the phosphorylation levels of ERK1/2, IκBα and NF-κB p65 (Fig. 5A-C). Moreover, as regards the NF-κB binding activity, which was in accordance with the changes occurring during ERK1/2 activation, even

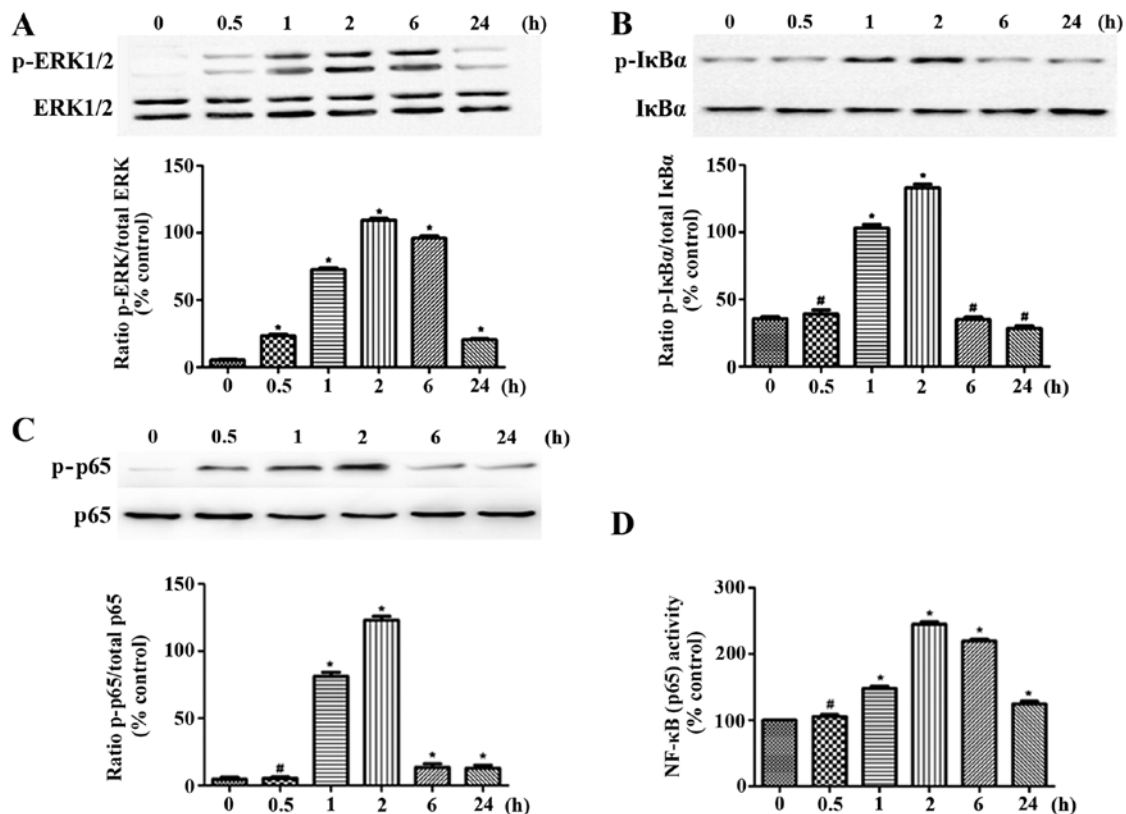


Figure 5. Interleukin-1 $\beta$  (IL-1 $\beta$ )-induced ERK1/2 and nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling in human osteoarthritic (OA) chondrocytes at different time points. The chondrocytes ( $1 \times 10^6$  cells/ml) were stimulated with IL-1 $\beta$  (10 ng/ml) for different periods of time. The collection of cell lysates was performed. Western blot analysis was used to determine the levels of phosphorylated (A) ERK1/2, (B) I $\kappa$ B $\alpha$  and (C) NF- $\kappa$ B p65. After the data were analyzed by densitometry, they were represented as ratios of phosphorylated protein to total protein in 3 independent experiments. The data are presented as the means  $\pm$  SD. The extraction and use of nuclear fractions was used for NF- $\kappa$ B DNA binding assay. The transcription assay kit for NF- $\kappa$ B (p65) was used to measure NF- $\kappa$ B activity. (D) A spectrophotometric plate reader at wavelengths of 450 nm was used to quantify NF- $\kappa$ B activity. Values are presented as the means  $\pm$  SD of 3 independent experiments. \* $P < 0.05$  vs. untreated group; # $P > 0.05$  vs. untreated group.

though an increase was observed within a short period of time, the activity was reduced as time progressed (Fig. 5D). Based on the time course, the 2 h time period was selected for use in the subsequent experiments.

**Effect of exposure time of OA chondrocytes to  $H_2S$  on ERK1/2 and NF- $\kappa$ B signaling.** Without the involvement of IL-1 $\beta$ , we performed 120-min time-course experiments using 0.3 mM NaHS, and then conducted another 2-h stimulation process with IL-1 $\beta$  (10 ng/ml). The cells were lysed, and the phosphorylation levels of NF- $\kappa$ B p65, I $\kappa$ B $\alpha$  and ERK1/2 were determined by western blot analysis. The NF- $\kappa$ B binding activity was determined with the use of an NF- $\kappa$ B p65 transcription assay kit. The brief exposure of the OA chondrocytes to NaHS led to the inhibition of the activation of NF- $\kappa$ B and ERK1/2 signaling induced by IL-1 $\beta$  (Fig. 6). The most prominent inhibitory effect of  $H_2S$  was observed in the cells pre-treated with  $H_2S$  for half an hour. Thus, pre-treatment for 30 min was used in the subsequent experiments.

**Effect of PD98059 (as an ERK pathway inhibitor) on the suppressive effects of  $H_2S$  on the IL-1 $\beta$ -induced activation of NF- $\kappa$ B signaling.** PD98059, as one of the effective inhibitors of ERK was used by us in an effort to determine the involvement of ERK in the suppressive effects of  $H_2S$  on the IL-1 $\beta$ -induced activation of NF- $\kappa$ B signaling. The cells were pre-incubated

with PD98059 (50  $\mu$ M) for 1 h. The cells were then treated for half an hour with NaHS (0.1 mM). Subsequently, the cells were stimulated for 2 h with IL-1 $\beta$  (10 ng/ml). Following the collection of the cell conditioned medium, the NF- $\kappa$ B p65 transcription assay kit was used to perform NF- $\kappa$ B activity assay. In addition, the levels of PGE $_2$  and MMP-13 generated in the culture medium were quantified with the use of specific ELISA kits. We also used Griess reagent to measure the NO production. The inhibitory effects of  $H_2S$  on the IL-1 $\beta$ -induced activation of NF- $\kappa$ B signaling were suppressed by PD98059 (Fig. 7A). The inhibitory effects of  $H_2S$  on the secretion of PGE $_2$ , MMP-13 and NO (induced by IL-1 $\beta$ ) were also abolished by PD98059 (Fig. 7B-D).

## Discussion

Although the molecular biology of cartilage damage in OA has been extensively investigated (21-23), no single causative factor for joint damage in OA has been identified to date. Generally speaking, when the development of OA begins, synovium-produced inflammatory mediators and cytokines are released. After being activated, chondrocytes produce PGE $_2$ , MMPs, TNF- $\alpha$  and NO. Those molecules facilitate catabolic activity and cause cartilage structural changes (24). IL-1 $\beta$  is not only important in the pathogenesis of OA, but also acts as a powerful mediator of catabolic processes in articular chondrocytes (25).

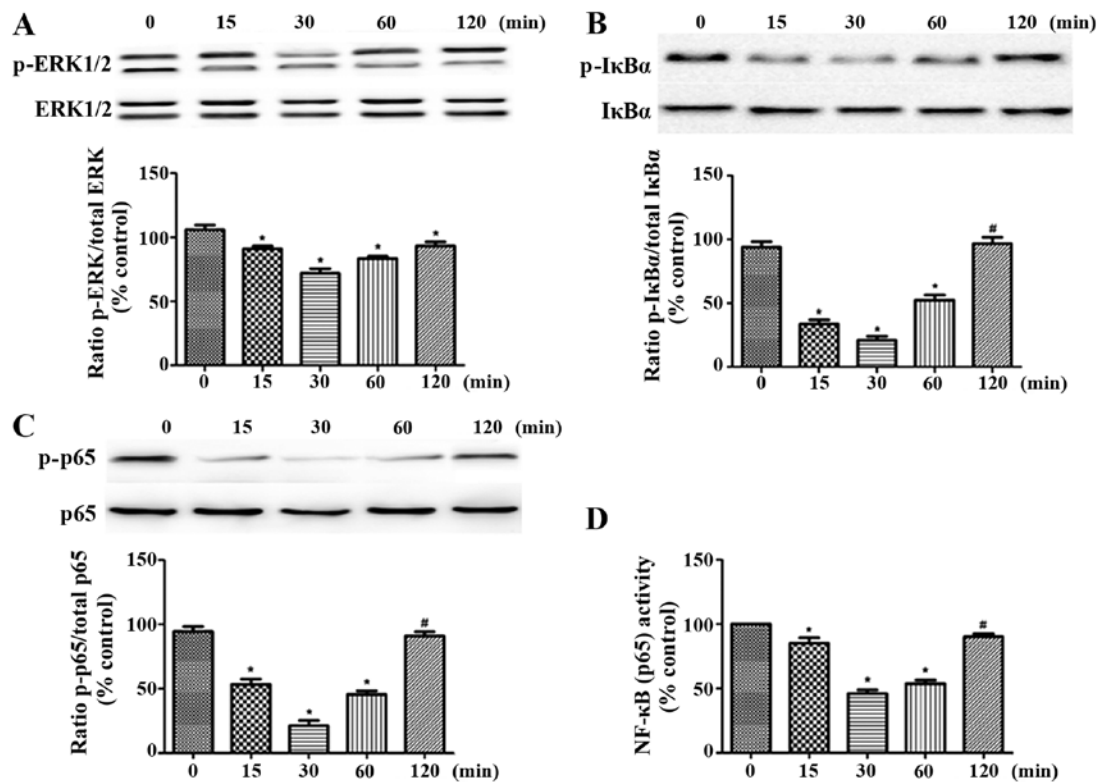


Figure 6. Effect of exposure time of osteoarthritic (OA) chondrocytes to hydrogen sulfide ( $\text{H}_2\text{S}$ ) on ERK1/2 and nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) signaling. The OA chondrocytes were incubated with 0.3 mM sodium hydrosulfide (NaHS) for 0-120 min. The cells were then stimulated with interleukin-1 $\beta$  (IL-1 $\beta$ ) (10 ng/ml) for a further 2 h. Western blot analysis was used to determine the levels of phosphorylated (A) ERK1/2, (B) I $\kappa\text{B}\alpha$  and (C) NF- $\kappa\text{B}$  p65. After the data were analyzed by densitometry, they were represented as ratios of phosphorylated protein to total protein in 3 independent experiments. Data are presented as the means  $\pm$  SD. The extraction and use of nuclear fractions was used for NF- $\kappa\text{B}$  DNA binding assay. Transcription assay kit for NF- $\kappa\text{B}$  (p65) was used to measure NF- $\kappa\text{B}$  activity. (D) A spectrophotometric plate reader at wavelengths of 450 nm was used to quantify NF- $\kappa\text{B}$  activity. Values are presented as the means  $\pm$  SD of 3 independent experiments. \* $P < 0.05$  vs. the group treated with IL-1 $\beta$ ; # $P > 0.05$  vs. the group treated with IL-1 $\beta$ .

This study used IL-1 $\beta$  to mimic the pathophysiology of OA. As we had hypothesized, in response to stimulation with IL-1 $\beta$ , an increase in the production of NO, MMP-13 and PGE $_2$  and in the expression of COX-2 and iNOS was observed. Our data demonstrate the effects of IL-1 $\beta$  on the progression of OA.

A recent study demonstrated that pro-inflammatory cytokines induce CSE (one of the  $\text{H}_2\text{S}$  synthesizing enzymes) expression and its activation through p38-ERK-NF- $\kappa\text{B}$ -dependent pathways in chondrocytes, thus increasing  $\text{H}_2\text{S}$  synthesis (14). Nevertheless, to the best of our knowledge, no investigation on the effects of  $\text{H}_2\text{S}$  on human OA chondrocytes has been conducted to date. Therefore, this study has a huge significance in this regard. We demonstrated that exogenous  $\text{H}_2\text{S}$  at the doses used in this study attenuated the IL-1 $\beta$ -induced inflammatory responses *in vitro*. Our results demonstrate that  $\text{H}_2\text{S}$  exerts an anti-inflammatory effect by inhibiting iNOS/NO and COX-2/PGE $_2$  expression (or production), as well as MMP-13 production by suppressing ERK1/2 and NF- $\kappa\text{B}$  signaling.

Although  $\text{H}_2\text{S}$  tissue production and the levels of  $\text{H}_2\text{S}$  in the blood have been the subject of much controversy, certain studies have identified generous basal plasma  $\text{H}_2\text{S}$  levels in the 50-150  $\mu\text{M}$  range according to the spectrophotometric approach based on the indicator dye, methylene blue (26). The  $\text{H}_2\text{S}$  concentration range in this study was decided according to the  $\text{H}_2\text{S}$  physiological level in the human body. A previous study also revealed the cytoprotective effects of  $\text{H}_2\text{S}$  at

micromolar concentrations (27). Certain cellular effects are linked with the modulation of kinase pathways or intracellular caspases. It was proven in this study that NaHS at the dose of 0.3 mM had the most prominent cytoprotective effects. Previous data indicated that exposure to  $\text{H}_2\text{S}$  (millimolar) at higher concentrations may be cytotoxic to cells. This is caused by many factors, such as the generation of free radicals (28). According to our data, at the concentration of 1.5 mM NaHS,  $\text{H}_2\text{S}$  loses its cytoprotective effects. It should be noted that we did not observe any cytotoxic effects of  $\text{H}_2\text{S}$ . The reason for this difference in results between our study and other studies may be due to the different inflammatory models used, or different  $\text{H}_2\text{S}$  donor doses used.

Among the pro-inflammatory factors, NO is a major toxic substance with a rapid, as well as spontaneous reaction [reacts with a superoxide anion ( $\text{O}_2^{\cdot-}$ )]. Through this reaction, it forms both peroxynitrous acid (ONOOH; the conjugate acid of peroxynitrite) and a peroxynitrite anion. Currently 3 isoforms of NOS have been identified. Among these isoforms, iNOS is capable of inducing high levels of NO production, apart from its being synthesized for response to inflammatory stimuli (29). In our study, we demonstrated that  $\text{H}_2\text{S}$  is capable of preventing the production of NO induced by IL-1 $\beta$ , as well as the expression of iNOS at the mRNA level in OA chondrocytes. Our results revealed that the inhibitory effects of  $\text{H}_2\text{S}$  on the IL-1 $\beta$ -induced iNOS protein expression were mediated through the inhibition

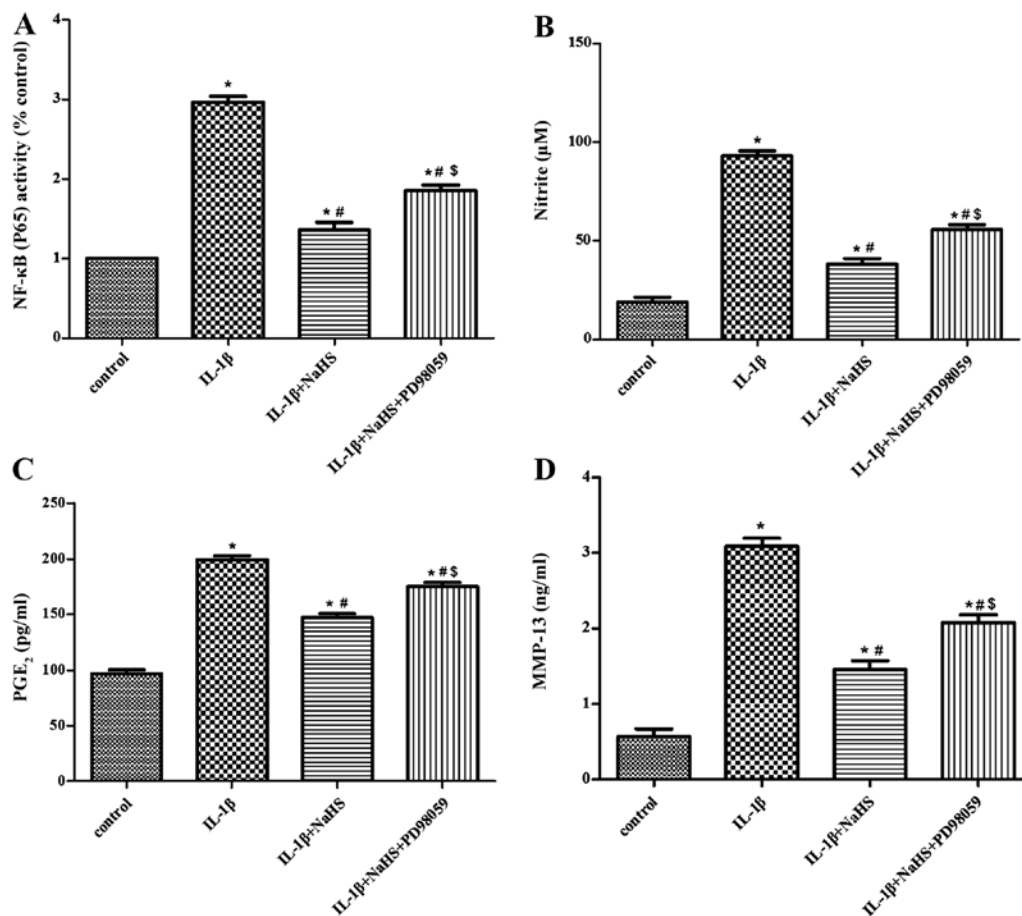


Figure 7. Effect of PD98059, a specific ERK pathway inhibitor, on the inhibitory effects of hydrogen sulfide ( $H_2S$ ) on the activation of nuclear factor- $\kappa B$  (NF- $\kappa B$ ) signaling induced by interleukin- $1\beta$  (IL- $1\beta$ ). Before being treated with sodium hydrosulfide (NaHS) (0.3 mM) for 30 min, the cells were incubated with PD98059 (50  $\mu M$ ) for 1 h. The cells were then stimulated for 2 h with IL- $1\beta$  (10 ng/ml). (A) NF- $\kappa B$  (p65) transcription assay kit was utilized for the determination of the activity of NF- $\kappa B$  in the nucleus. (B) Griess reagent was used to measure NO production in the cell conditioned medium. (C and D) The specific enzyme-linked immunosorbent assay (ELISA) kit was used for the quantification of the production of prostaglandin  $E_2$  (PGE<sub>2</sub>) and matrix metalloproteinase-13 (MMP-13) in the culture medium. Results are the means  $\pm$  SD of triplicate measurements and from 3 separate experiments. \* $P < 0.05$ , compared with the value in unstimulated cells (controls); \*\* $P < 0.05$ , compared with the value in cells treated with IL- $1\beta$  alone; \*\*\$ $P < 0.05$ , compared with the value in cells treated with IL- $1\beta$  and NaHS.

of NO production. Our results were concurrent with those of Hu *et al* (30). According to their findings,  $H_2S$  is capable of inhibiting inflammation in microglia stimulated with LPS, due to the fact that exogenous  $H_2S$  and endogenous  $H_2S$  markedly suppress NO production. The suppression of iNOS following stimulation with LPS is involved in the mechanisms behind the anti-inflammatory effects of  $H_2S$ .  $H_2S$ -NO interactions seem to be more and more possible. Another study revealed that  $H_2S$ , by certain means, promotes both iNOS expression and NO production (31).  $H_2S$  biosynthesis in LPS-induced endotoxemia has been shown to be reduced by nitro-flurbiprofen-provided NO. The reason for this may lie in transduction inhibition through the NF- $\kappa B$  pathway (31). It is possible that NO is capable of controlling endogenous  $H_2S$  biosynthesis and regulating CSE expression in a positive manner. It seems that gaseous mediator interactions are crucial; thus further studies are warranted to investigate these interactions.

Although joint pain is one of the symptoms of OA, information regarding the mechanisms responsible for OA-induced pain is limited. It has been demonstrated that knee nociceptors may be sensitized by peripheral pro-inflammatory mediators, such as PGE<sub>2</sub> and COX-2 (32). IL- $1\beta$  stimulates

the production of COX-2 as a major inducer of pain inflammation and a mediator in osteoarthritic joints, rather than that of COX-1. In addition, PGE<sub>2</sub> exerts catabolic effects on chondrocytes (33,34). In our study, in spite of the fact that only traces of COX-2 and PGE<sub>2</sub> expression were observed in the unstimulated human chondrocytes, the expression of PGE<sub>2</sub> and COX-2 was enhanced following stimulation with IL- $1\beta$ . We demonstrated that the addition of  $H_2S$  suppressed PGE<sub>2</sub> production and elevated COX-2 gene expression in the IL- $1\beta$ -stimulated cells. Therefore, it is possible that  $H_2S$  exerts an anti-nociceptive effect, similar to that of NSAIDs. This effect, by means of suppressing COX-2 and PGE<sub>2</sub> expression, seems to ameliorate OA symptoms. In the study by Distrutti *et al*,  $H_2S$  was shown to be a promising analgesic. The systemic administration of different  $H_2S$  donors led to the opening of  $K^+_{(ATP)}$  channels, inhibiting visceral nociception (35). Nevertheless, it was demonstrated by Kawabata *et al* that either endogenous or exogenous  $H_2S$  has a peripheral activity that may promote pain. It seems that the mechanisms of  $H_2S$ -induced hyperalgesia rely on the adjustment of T-type  $Ca^{2+}$  channel activity, but are not dependent on  $K^+_{(ATP)}$  channels (36). Apart from  $H_2S$ , CO and NO also show nociceptive-related ambiguous activity (23).



MMPs are synthesized in articular joints by synovial cells and chondrocytes. MMP-13 is considered a main cause for the degradation of collagens and aggrecan in cartilage. It is considered that OA chondrocytes express MMP-13 (37). Thus, MMP-13 has become a therapeutic target with the most potential. In the present study, we wished to determine whether H<sub>2</sub>S exerts chondroprotective effects through the suppression of MMP-13 in chondrocytes. Our results revealed that H<sub>2</sub>S inhibited MMP-13 expression induced by IL-1 $\beta$ . This inhibition occurred at the protein and mRNA level and was independent of the dose used in human OA chondrocytes. The results obtained in the study by Vacek *et al* were also similar. Their study demonstrated that H<sub>2</sub>S inhibited collagen helix destruction and MMP activation by suppressing oxidative stress (38).

The underlying cellular targets of the protective effects of H<sub>2</sub>S on IL-1 $\beta$ -induced OA remain to be elucidated. In this study, we analyzed the potential molecular mechanisms responsible for the inhibitory effects of H<sub>2</sub>S on inflammatory mediators in response to IL-1 $\beta$  in chondrocytes. Among the downstream effects of IL-1 $\beta$  stimulation, many of these effects are regulated by the activation of NF- $\kappa$ B signaling (39,40). NF- $\kappa$ B is retained in the cytoplasm with I $\kappa$ B $\alpha$  in an inactive state. IL-1 $\beta$  has the capacity to activate NF- $\kappa$ B, resulting in the change of the position of NF- $\kappa$ B p65 so as to regulate gene expression (17). Our results revealed that IL-1 $\beta$  induced I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65 phosphorylation, which was reduced by an exogenous H<sub>2</sub>S donor (NaHS) at the concentration of 0.3 mM in short-term treatment (15-30 min). However, the phosphorylation of I $\kappa$ B $\alpha$  and NF- $\kappa$ B p65 was not further inhibited by long-term H<sub>2</sub>S treatment (60-120 min) following stimulation with IL-1 $\beta$ . This reveals that the effects of H<sub>2</sub>S may be produced rapidly, a fact having been confirmed in a previous study, which demonstrated that the inhibitory effects of H<sub>2</sub>S are not determined by the release rate (41). Our results are consistent with the findings of a previous study on the protective role of GYY4137 (13). On the whole, we found new evidence to support the hypothesis that the NO-, PGE<sub>2</sub>- and MMP-13-mediated inflammation and cytotoxicity are regulated by the activation of NF- $\kappa$ B and that H<sub>2</sub>S protects against inflammation induced by IL-1 $\beta$  by inhibiting NF- $\kappa$ B signaling.

It has also been suggested by previous findings that the regulatory role of H<sub>2</sub>S on MAPK phosphorylation may exist and that the regulation contributes to different types of pathological and physiological roles in all types of tissues and cells (42,43). The fact that the IL-1 $\beta$ -induced phosphorylation of ERK1/2 was decreased in fibroblast-like synoviocytes (FLS) of OA was also previously demonstrated (42,44). Since it is known that ERK1/2 is an upstream activator of NF- $\kappa$ B signaling, we conducted investigations on the relevant functions of ERK1/2. Therefore, we used the PD98059 inhibitor to block the activation of ERK1/2. The inhibition of ERK1/2 by pre-treatment with PD98059 abolished the inhibitory effects of H<sub>2</sub>S on cytokines following stimulation with IL-1 $\beta$ , while attenuating its inhibitory effects on the activation of NF- $\kappa$ B signaling induced by IL-1 $\beta$ . These findings, in a straightforward manner, indicate that the cytoprotective effects of H<sub>2</sub>S in OA may involve the ERK-NF- $\kappa$ B signaling pathway. Nevertheless, attention should be paid to the fact that pre-treatment with PD98059 did not completely abolish the cytoprotective effects of H<sub>2</sub>S.

This means that H<sub>2</sub>S may regulate the activation of NF- $\kappa$ B signaling through mediators apart from ERK1/2. According to previous research, the inhibitory effects of H<sub>2</sub>S on inflammation are exerted by suppressing the channels for p38 and NF- $\kappa$ B signaling and by upregulating HO-1 expression (45). It is possible that the different stimuli and different types of cells used may attribute to the different results obtained by different studies. Nevertheless, the regulatory mechanisms of H<sub>2</sub>S in chondrocyte inflammation remain to be fully elucidated. Further studies are warranted to determine the precise channels of signaling involved in this process.

In conclusion, this study proves that a cytoprotective effect against OA induced by IL-1 $\beta$  is conferred by H<sub>2</sub>S, and that the process is realized by suppressing the channel for ERK-NF- $\kappa$ B signaling in chondrocytes, which is activated by IL-1 $\beta$ . This study has provided new insight into the cytoprotective effects of H<sub>2</sub>S in OA. The data from our study also suggest that H<sub>2</sub>S may prove to be a potential therapeutic agent for the treatment of OA.

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