Hyperbaric Oxygen Treatment Prevents Nitric Oxide-Induced Apoptosis in Articular Cartilage Injury via Enhancement of the Expression of Heat Shock Protein 70

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ABSTRACT: Heat shock proteins (HSPs), inflammatory cytokines, nitric oxide (NO), and localized hypoxia-induced apoptosis are thought to be correlated to the degree of cartilage injury. We investigated the effect of hyperbaric oxygen (HBO) on (1) interleukin-1β (IL-1β)-induced NO production and apoptosis of rabbit chondrocytes and (2) healing of articular cartilage defects. For the in vitro study, RT-PCR and Western blotting were performed to detect mRNA and protein expressions of HSP70, inducible NO synthase (iNOS), and caspase 3 in IL-1β-treated chondrocytes. To clarify that the HSP70 was necessary for anti-iNOS and anti-apoptotic activity by HBO, we treated the cells with an HSP70 inhibitor, KNK437. For the in vivo study, cartilage defects were created in rabbits. The HBO group was exposed to 100% oxygen at 2.5 ATA for 1.5 h a day for 10 weeks. The control group was exposed to normal air. After sacrifice, specimen sections were sent for examination using a scoring system. Immunohistochemical analyses were performed to detect the expressions of iNOS, HSP70, and caspase 3. Our results suggested that HBO upregulated the mRNA and protein expressions of HSP70 and suppressed those of iNOS and caspase 3 in chondrocytes. KNK437 inhibited the HBO-induced downregulation of iNOS and casapase 3 activities. The histological scores showed that HBO markedly enhanced cartilage repair. Immunohistostaining showed that HBO enhanced HSP70 expression and suppressed iNOS and caspase 3 expressions in chondrocytes. Accordingly, HBO treatment prevents NO-induced apoptosis in articular cartilage injury via enhancement of the expression of heat shock protein 70. © 2012 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 31:376–384, 2013

Keywords: hyperbaric oxygen; heat shock protein 70; nitric oxide; caspase 3; apoptosis

Articular chondrocytes are specialized cells that can survive in an avascular environment and obtain oxygen via the diffusion pathway.[1] The normal synovium is highly vascularized to satisfy the metabolic demands of the avascular cartilage[2]; however, in the case of joint disease, the oxygen gradient could be disturbed.[3] Apoptosis of chondrocytes can be induced by localized hypoxia[4] and NO production.[5] Oxygen appears to play an important role in improving matrix production and osteochondral regeneration after cartilage injury.[3] Oxidant conditioning protects the cartilage from mechanically induced damage.[6] Suppression of NO production after chondral trauma, or during the early stage of osteoarthritis (OA), may inhibit the initiation or progression of OA.[7]

Heat shock proteins (HSPs) are a family of highly conserved proteins that are synthesized in cells after stress loading. HSP70 expression levels in chondrocytes are correlated with the histological severity of OA.[8] However, the expression level of HSP70 may not be high enough to accelerate metabolic activity in the damaged cartilage, and this may cause progression of the disorder.[9] Over-expression of HSP70 results in the promotion of the metabolic activity of chondrocytes[10] and protection of chondrocytes from NO-induced apoptosis.[9,11]

Studies have shown close associations among hyperbaric oxygen (HBO), IL-1β, HSP70, NO, and caspase. HBO treatment has shown to increase tissue/microvascular partial pressure of oxygen,[12] decrease IL-1β secretion from monocytes[13] and degenerated disc cells,[14] suppress apoptosis of degenerated disc cells,[15] reduce caspase 3 activities in rat brain,[16] and suppress iNOS expression and apoptosis of chondrocytes in rabbit cartilage defects.[17] HBO treatment upregulated the expression of HSP70.[18] Because HSP70 prevents NO-induced apoptosis in articular chondrocytes,[9] we propose that upregulation of HSP70 expression in the articular cartilage by HBO may provide a novel therapeutic strategy for cartilage repair.

In this present study, we first examined the effects of normal baric 100% oxygen, hyperbaric normal air, and HBO on the protein expressions of HSP70, iNOS, and caspase 3 in IL-1β-treated chondrocytes. In addition, we further examined the effects of HBO on the mRNA and protein expressions of HSP70, NOS, and caspase 3 in IL-1β-treated chondrocytes. To clarify whether the decrease in the expressions of iNOS and caspase 3 after HBO treatment was caused by increased HSP70 expression, we treated the cells with HSP70 inhibitor, KNK437. Finally, we examined the effects of HBO therapy in a rabbit model for full-thickness articular defect.

MATERIALS AND METHODS

All rabbits were cared for in accordance with the regulations of the National Institutes of Health of the Republic of China, under the supervision of a licensed veterinarian.

Steve W. N. Ueng, Li-Jen Yuan, and Song-Shu Lin contributed equally to this work.

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Cell Isolation and Cell Culture

Rabbit chondrocytes were released from the articular cartilage by digestion using 1 mg/mL collagenase (Sigma, St. Louis, MO) in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Grand Island, NY) containing 2% fetal bovine serum (FBS). The isolated chondrocytes were seeded in T-75 flasks in 15 mL of DMEM/F12 supplemented with 10% FBS and incubated in a humidified atmosphere of 5% CO_2/95% air.

Induction of NO Production and Apoptosis

The cells were plated at 5×10^5 cells per 100 mm dish in 10 mL of DMEM/F12 containing 5% FBS. Cell apoptosis was induced using IL-1β (10 ng/mL, R&D System, Minneapolis, MN) which generated NO.

Exposure to Different Treatments

At 36 h after IL-1β treatment, the cells were divided into control group (C group), normal baric 100% oxygen group (O group), hyperbaric normal air group (HBA group), and HBO groups. The control cells were maintained in 5% CO_2/95% air throughout the experimental period. The O group was exposed to 100% O_2 for 25 min and then to 5% CO_2/95% air for 5 min at 1 atmospheres absolute (ATA) in a hyperbaric chamber over a total treatment time of 90 min. The HBA group was exposed to 5% CO_2/95% air at 2.5 ATA over 90 min. The HBO group was exposed to 100% O_2 for 25 min and then to 5% CO_2/95% air for 5 min at 2.5 ATA over 90 min.

Protein Extraction and Western Blot Analysis for HSP70, iNOS, and caspase 3

At 24 h after each treatment, cells were washed with PBS and extracted using M-PER mammalian protein extraction reagent (Thermo, Rockford, IL). The protein was separated by 10% SDS–PAGE for HSP70 and iNOS and by 15% SDS–PAGE for caspase 3. After blocking, the membranes were incubated overnight at 4°C with 1,000-fold diluted mouse antibodies to HSP70 (Stressgen, BC, Canada), iNOS (R&D System), and caspase 3 (Calbiochem, San Diego, CA) or with 2,000-fold diluted mouse antibodies to β-actin (Chemicon, Temecula, CA). After washing, the membranes were further incubated for 2 h with 10,000-fold goat anti-mouse IgG conjugated to horseradish peroxidase (Calbiochem). The band images were photographed using ECL Hyperfilm (Amersham, Little Chalfont, UK). The intensity of the staining was quantified by the use of an image-analysis system (Image-Pro plus 5.0, Media Cybernetics, Silver Spring, MD).

RNA Isolation and RT-PCR for HSP70, iNOS, and Caspase 3

At 8 h after HBO treatment, total mRNA was isolated from the chondrocytes using TRIzol (Invitrogen) and reverse transcribed using ImProm-II reverse transcription system (Promega Corporation, Madison, WI). The obtained cDNA was amplified by polymerase chain reaction (PCR) using a PCR Master Mix system (Promega Corporation), with the following primers: caspase 3 (5′-CCGAATTCCCATGGAGAACAACGAAACCT C-3′; 5′-CCGGATCCCATTTCTTTAGTGATAAATA-3′), GAPDH (5′-GCCTGGTCACCAGGGCTGC-3′; 5′-TGCTAAGCAGTTGGTGGTGCA-3′), iNOS (5′-CGCCCTTCCGCACTTTCT-3′; 5′-TCCAGGAGGACATGCAGCAC-3′), HSP70 (5′-CTCCAGCATCCGACAAGAAGC-3′; 5′-ACGGTGTTGTGGGGGTTCAGG-3′). Amplification of GAPDH messages was performed for 30 cycles at 94°C for 1 min, 58°C for 30 s, and 72°C for 30 s after initial denaturation at 94°C for 2 min. Amplification of iNOS messages was performed for 30 cycles at 94°C for 45 s, 59°C for 45 s, and 72°C for 1 min after initial denaturation at 94°C for 5 min. Amplification of Hsp70 messages was performed for 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min following initial denaturation at 95°C for 3 min. Amplification of caspase 3 messages was performed for 30 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 1.5 min following initial denaturation at 94°C for 5 min. An aliquot of the reaction mixture was subjected to electrophoresis on a 2% agarose gel. The intensity of the staining was quantified by the use of an image-analysis system (Image-Pro plus 5.0).

Protein Extraction and Western blot Analysis for HSP70, iNOS, and caspase 3

At 12 and 24 h after HBO treatment, protein extraction and Western blotting were performed as previous description.

Exposure to KNK437 and HBO Treatment

Cells were treated with and without an HSP70 inhibitor, 100 μM KNK437 (*N*-formyl-3, 4-methylenedioxy-benzylidenec-butyrolaetam, Calbiochem) for 2 h after IL-1β treatment, and then exposure to HBO treatment. At 12 h after HBO treatment, proteins were extracted and analyzed for HSP70, iNOS, and caspase 3 as previous description.

Animal Operation

New Zealand rabbits weighing 3 kg were anesthetized by intravenous injection of a mixture of 5 mL ketamine (Parke-Davis, Chungli, Taiwan) and Rompum (Bayer) mixture. Under aseptic condition, a drill to create a 4-mm diameter cartilage defect passing through the subchondral bone on the medial femoral condyle of the weight-bearing surface. After creation of the cartilage defect, the knee joint was irrigated and the wound was closed.

Exposure to HBO Treatment

Ten rabbits were used and divided into two groups: (I) HBO group, which included five rabbits exposed to HBO with 100% oxygen at 2.5 ATA for 1.5 h daily, 5 days in a week after surgery and (II) Control group, housed in cages containing normal air.

Tissue Processing and Safranin-O Staining

The animals were sacrificed at 10 weeks after surgery. After decalcification, the blocks were cut in half through the defect area and embedded in paraffin. Five-micron sections were cut and stained with safranin-O.

Scoring System for Histological and Histochemical Analysis

The sections were examined blindly and independently by five authors, who selected the most typical section from each specimen, and then scored up according to the International Cartilage Repair Society (ICRS) histological assessment scale (Table 1).[19]

Immunohistochemistry for iNOS and HSP70 Detection

The tissue sections were deparaffinized in xylene, passed through decreasing gradations of ethanol for HSP70 detection, and further incubated with chondroitinase ABC (0.25 U/ml, Sigma) in PBS (pH 7.4) for 60 min for iNOS detection. Endogenous peroxidase activity was blocked with 1% H_2O_2. The presence and distribution of iNOS and HSP70

Table 1. ICRS Visual Histological Assessment Scale

Feature	Score
I. Surface	
Smooth/continuous	3
Discontinuities/irregularities	0
II. Matrix	
Hyaline	3
Mixture: hyaline/fibrocartilage	2
Fibrocartilage	1
Fibrous tissue	0
III. Cell distribution	
Columnar	3
Mixed/columnar-clusters	2
Clusters	1
Individual cells/disorganized	0
IV. Cell population viability	
Predominantly viable	3
Partially viable	1
<10% viable	0
V. Subchondral bone	
Normal	3
Increased remodeling	2
Bone necrosis/granulation tissue	1
Detached/fracture/callus at base	0
VI. Cartilage mineralization (calcified cartilage)	
Normal	3
Abnormal/inappropriate location	0

were determined by incubation with using anti-iNOs Ab (1:40, R&D System) and anti-HSP70 Ab (1:200, Stressgen) for 2 h at room temperature. A peroxidase conjugated 2° Ab (dilution 1:1,000, Jackson Res, West Grove, PA) was used for 20 min. Bound immunoglobulin was detected with a DAB peroxidase substrate kit (Vector Lab, Burlingame, CA) and 0.1% methyl green was used for counter staining.

Immunohistochemistry for Caspase 3 Detection

The tissue sections were deparaffinized, dehydration, and put in 100 mM citrate buffer (pH 6) and boiling for 15 min and then cooling in TBS for 20 min. Endogenous peroxidase activity was blocked with 3% H_2O_2. The presence and distribution of caspase 3 was determined using anti-caspase 3 Ab (1:200 dilution, Lab VISION, Fremont, CA) at 4°C overnight. A peroxidase conjugated 2° Ab (1:1,000 dilutions, Jackson Res) was used for 20 min. Bound immunoglobulin was detected with a DAB peroxidase substrate kit (Vector Lab) and 0.1% methyl green was used for counterstaining.

Statistical Analysis

Variations between the control and HBO group regard to mRNA and protein expression of HSP70, iNOS, and caspase 3 were compared by Student's *t*-test. Histological score results were analyzed using the Mann–Whitney test. The values were expressed as the mean ± SD for *t*-test and as median and range for Mann–Whitney test; $p < 0.05$ was considered statistically significant.

RESULT

Effect of IL-1β, Normal Baric 100% Oxygen, HBA, and HBO on Protein Expressions of HSP70, iNOS, and Caspase 3 in the Chondrocytes

IL-1β-induced protein expressions of HSP70, iNOS, pro-caspase 3 (~32 kDa), and active form of caspase 3 (~18 kDa). Normal baric 100% oxygen (O group), HBA, and HBO upregulated protein expressions of HSP70 (Fig. 1A; HSP70/β-actin ratio: control 29.8 ± 2.2%, O group 45.4 ± 3.4%, HBA group 49.5 ± 3.8%, HBO group 65.6 ± 5.9%, $n = 3$) but downregulated

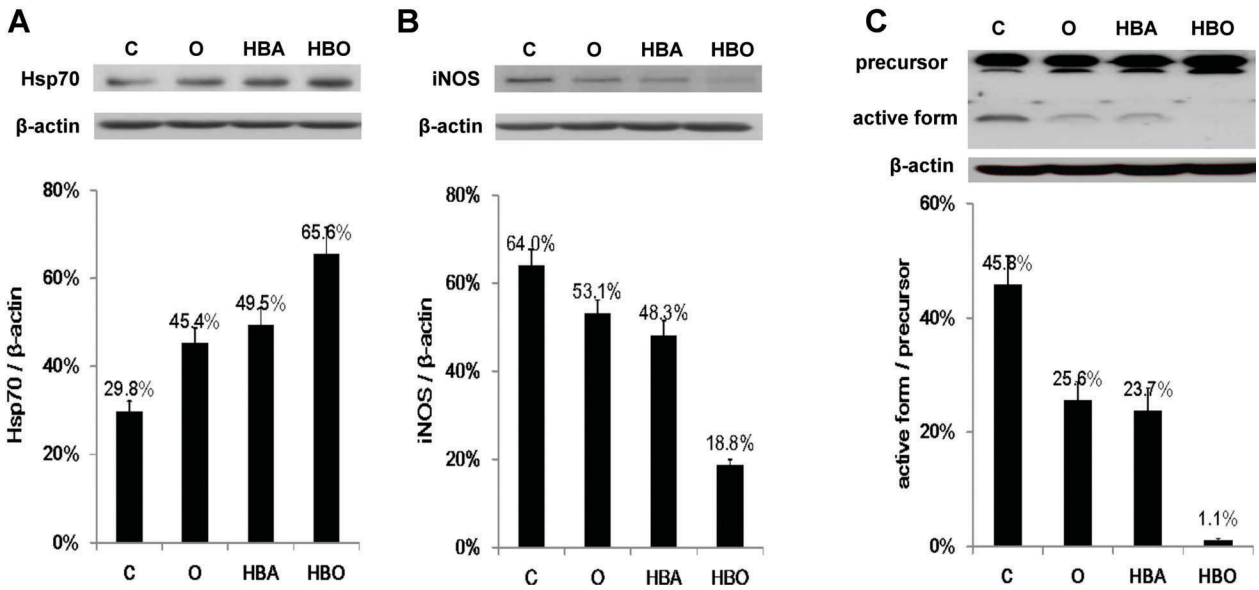

Figure 1. Effect of IL-1β, normal baric 100% oxygen, hyperbaric normal air, and HBO treatments on protein expressions of HSP70, iNOS, and caspase 3 in chondrocytes. IL-1β-induced protein expressions of HSP70, iNOS, pro-caspase 3 (~32 kDa), and active form of caspase 3 (~18 kDa). Normal baric 100% oxygen, HBA, and HBO upregulated protein expressions of HSP70 (A) but downregulated that of iNOS (B) and active form of caspase 3 (C) in chondrocytes. C, control group; O, normal baric 100% oxygen group; HBA, hyperbaric normal air group; HBO, hyperbaric oxygen group.

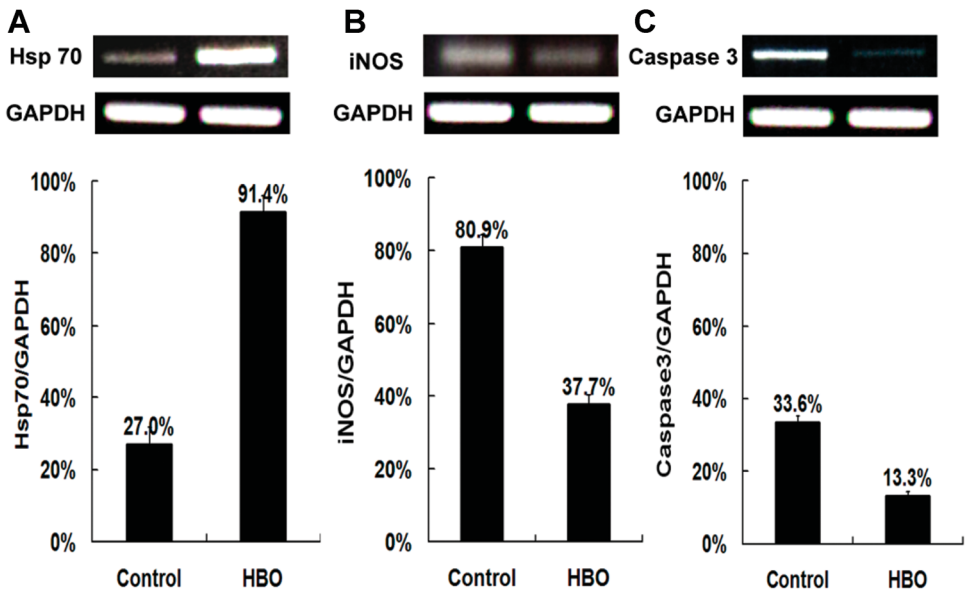

Figure 2. Effect of IL-1β and HBO treatments on mRNA expressions of HSP70, iNOS, and caspase 3 in chondrocytes. IL-1β treatment induced mRNA expression of HSP70, iNOS, and caspase 3. HBO treatment upregulated mRNA expression of HSP70 (A; $p < 0.01$) but downregulated those of iNOS (B; $p < 0.01$) and caspase 3 (C; $p < 0.01$) in chondrocytes.

that of iNOS, (Fig. 1B; iNOS/β-actin ratio: control 64.0 ± 3.7%, O group 53.1 ± 3.1%, HBA group 48.3 ± 3.2%, HBO group 18.8 ± 1.2%, $n = 3$) and active form of caspase 3 (Fig. 1C; active form/precursor ratio: control 45.8 ± 5.0%, O group 25.6 ± 3.3%, HBA group 23.7 ± 4.0%, HBO group1.1 ± 0.3%, $n = 3$) in chondrocytes.

Effect of IL-1β and HBO on mRNA Expressions of HSP70, iNOS, and Caspase 3 in the Chondrocytes

IL-1β treatment induced mRNA expressions of HSP70, iNOS, and caspase 3 (Control group). HBO treatment upregulated the mRNA expression of HSP70 (Fig. 2A; HSP70/GAPDH ratio: control 27.0 ± 4.9% vs. HBO 91.4 ± 4.6%; $p < 0.01$, $n = 3$) but downregulated those of iNOS (Fig. 2B; iNOS/GAPDH ratio: control 80.9 ± 3.6% vs. HBO 37.7 ± 2.7%; $p < 0.01$, $n = 3$) and caspase 3 (Fig. 2C; caspase 3/GAPDH ratio: control 33.6 ± 1.6% vs. HBO13.3 ± 1.3%; $p < 0.01$, $n = 3$).

Effect of IL-1β and HBO on Protein Expressions of HSP70, iNOS, and Caspase 3 in the Chondrocytes

HBO upregulated protein expressions of HSP70 (Fig. 3A; HSP70/β-actin ratio: control 27.3 ± 1.6% vs. H_{12} 43.7 ± 1.6%, $p < 0.01$, $n = 3$; control 27.3 ± 1.6% vs. H_{24} 71.8 ± 2.4%, $p < 0.01$, $n = 3$) but downregulated that of iNOS (Fig. 3B; iNOS/β-actin ratio: control 78.2 ± 4.1% vs. H_{12} 42.6 ± 2%, $p < 0.01$, $n = 3$; control 78.2 ± 4.1% vs. H_{24} 12.8 ± 0.8%, $p < 0.01$, $n = 3$) and active form of caspase 3 (Fig. 3C; active form/precursor ratio: control 79.6 ± 4.4% vs. H_{12} 13.5 ± 1.7%, $p < 0.01$, $n = 3$; control 79.6 ± 4.4% vs. H_{24} 0.3 ± 0.1%, $p < 0.01$, $n = 3$) in chondrocytes.

Effect of KNK437 Treatment on Protein Expressions of HSP70, iNOS, and Caspase 3 in the Chondrocytes

HBO treatment upregulated protein expression of HSP70 but downregulated those of iNOS and active caspase 3. Upregulation of HSP70 (Fig. 4A), downregulation of iNOS (Fig. 4A) and caspase 3 activities (Fig. 4B) by HBO were inhibited under the influence of KNK437.

Effect of HBO Treatment on Cartilage Defect Repair

In the control group (Fig. 5A,B), the repaired tissue was fibrous and irregular. The thickness of the repaired cartilage was approximately 50% of that of the normal adjacent cartilage. In the HBO group (Fig. 5C,D), the articular cartilage surface was smooth and intact with both nest and columnar arrangement of chondrocytes. The histological score results are summarized in Table 2. HBO markedly enhanced cartilage repair.

Effect of HBO Treatment on HSP70 Expression

In the repaired cartilage of the HBO group, an increased staining intensity of HSP70 was observed (Fig. 6A) as compared to the control group (Fig. 6B). Immunostaining showed that HBO treatment significantly increased HSP70 expression in chondrocyte.

Effect of HBO Treatment on iNOS Expression

In the repaired cartilage of the control group (Fig. 7A), an increased staining intensity of iNOS was observed as compared to the HBO group (Fig. 7B). HBO treatment markedly suppressed iNOS expression in chondrocytes.

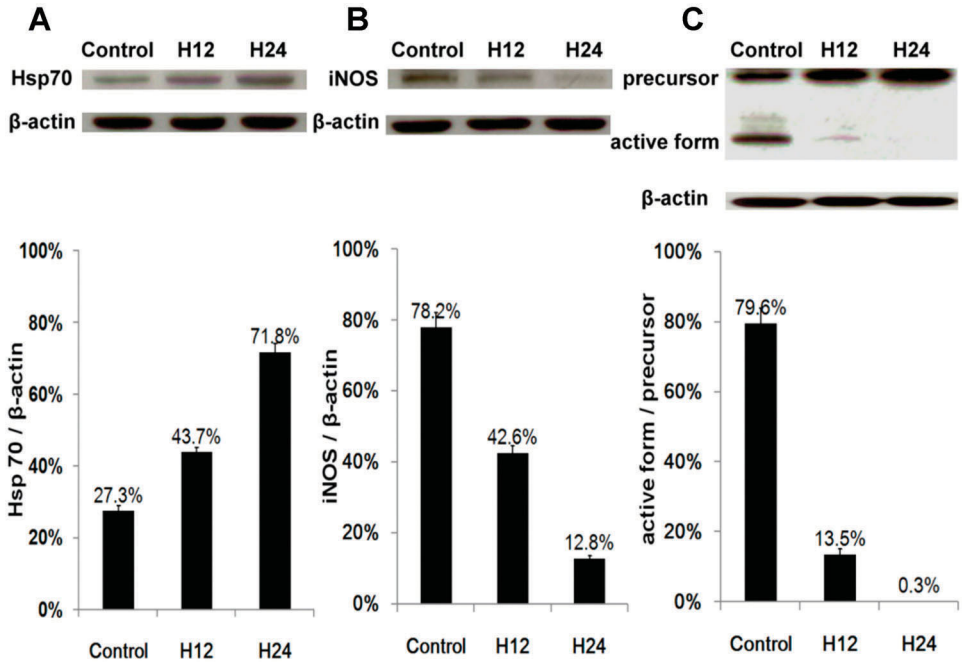

Figure 3. Effect of IL-1β and HBO treatment on protein expressions of HSP70, iNOS, and caspase 3 in chondrocytes. IL-1β treatment induced protein expressions of HSP70, iNOS, pro-caspase 3 (~32 kDa), and active form of caspase 3 (~18 kDa). HBO upregulated protein expressions of HSP70 (A; $p < 0.05$) but downregulated those of iNOS (B; $p < 0.05$) and active form of caspase 3 (C) in chondrocytes.

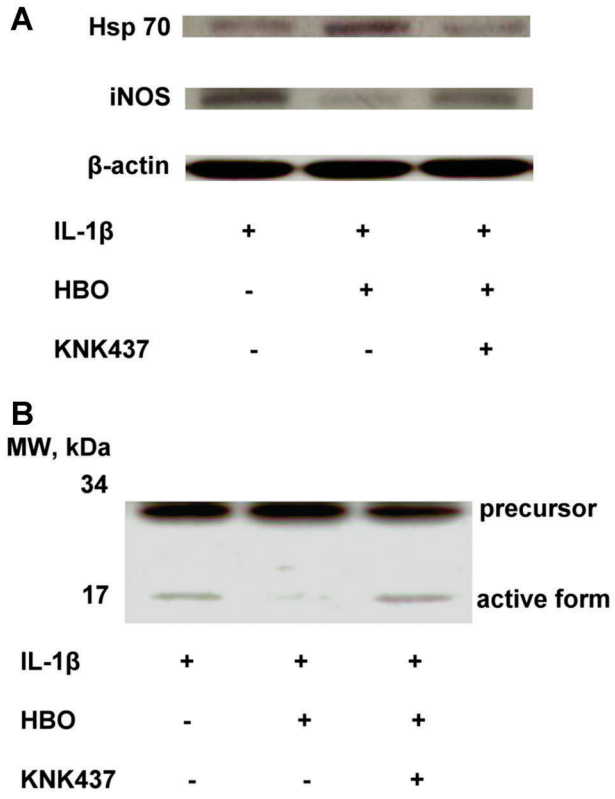

Figure 4. Effect of KNK437 treatment on protein expressions of HSP70, iNOS, and caspase 3 in chondrocytes. KNK437 treatment inhibited the upregulation of HSP70 (A), downregulation of iNOS (A) and caspase 3 activities (B) in the HBO group.

Effect of HBO Treatment on Apoptosis

In the repaired cartilage of the control group, an increased staining intensity of caspase 3 was observed (Fig. 8A) as compared to the HBO group (Fig. 8B). Immunostaining of the cartilage sections showed HBO treatment significantly decreased caspase 3 expression in chondrocytes. HBO treatment markedly suppressed apoptosis of chondrocytes.

DISCUSSION

Although articular cartilage is an avascular tissue that functions in a low oxygen tension environment, a further decrease in synovial fluid oxygen tension and environmental changes in traumatic injury or degenerative diseases of articular cartilage have been reported.[2,20] Previous study showed that HBO treatment increased oxygen tension in rabbit synovial fluid and suppressed the iNOS expression and apoptosis of chondrocytes.[17] In the present study, we further demonstrated that upregulation of HSP70 expression in the articular cartilage by HBO may provide a therapeutic strategy for cartilage repair.

In the first part of this study, we demonstrated that normal baric 100% oxygen (O group), hyperbaric normal air (HBA group), and HBO upregulated the protein expressions of HSP70 (Fig. 1A) and suppressed those of iNOS (Fig. 1B) and caspase 3 (Fig. 1C) in chondrocytes. Although normal baric 100% oxygen and HBA treatment both increased the oxygen dissolved in culture medium, the oxygen tension in culture medium was lower than that after HBO treatment (estimated by the color change of phenol red). Because of this, the

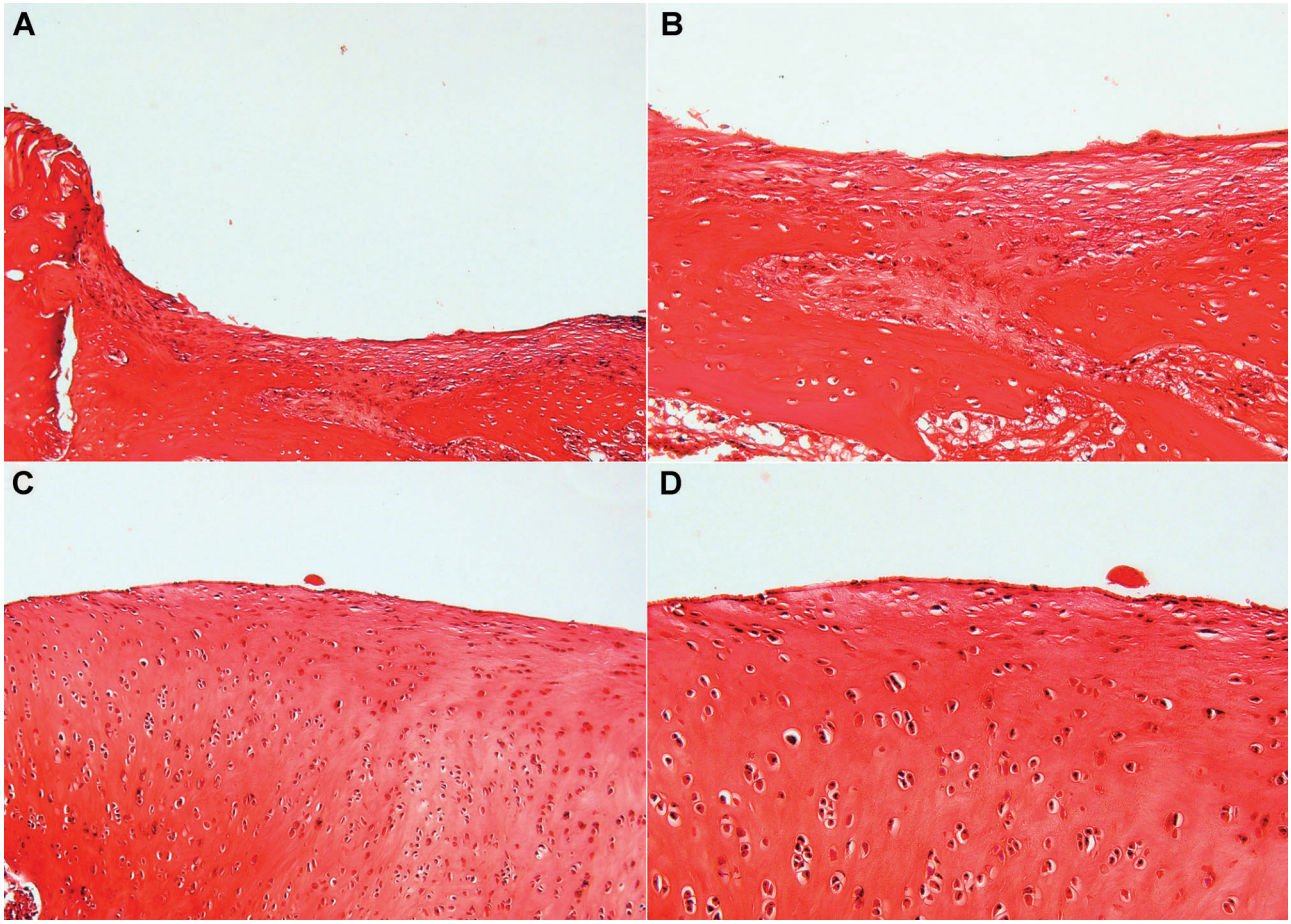

Figure 5. Effect of HBO treatment on cartilage defect repair. In the control group (A,B), the repaired tissue was fibrous and irregular. The thickness of the repaired cartilage was approximately 50% of that of the normal adjacent cartilage. In the HBO group (C,D), the articular surface was smooth and intact with both nests and columnar arrangement of the chondrocytes (original magnification, A: ×100, B: ×200, C: ×100, and D: ×200).

increased ratio of Hsp70 and decreased ratio of iNOS and caspase 3 caused by HBA or 100% oxygen in chondrocytes were lower than that by HBO treatment. The effects of HBA were little higher than 100% oxygen treatment but both were far away from HBO treatment (Fig. 1). The major limitation in this study was that we were not able to measure the actual oxygen tension in culture medium after each treatment. In addition, the increased oxygen content in culture medium came from the increased normal air (containing 21% oxygen) supplement in HBA treatment. It is better for us to investigate the effects of hydrostatic pressure to find the role of pure pressure on culture cells.

Table 2. Histological Score Results

	Control (1, 2, 1, 1, 1)	HBO (11, 10, 8, 11, 10)	p-Value
Median	1	10	$p < 0.01$
Range	1	3	

Mann–Whitney test.

Because the beneficial effects of HBA or 100% oxygen treatment were lower than that of HBO treatment. In the following study, we main focused on the HBO effects on the mRNA and protein expressions of HSP70, iNOS, and caspase 3 in IL-1β-treated chondrocytes. Our results suggested that HBO upregulated the mRNA (Fig. 2) and protein (Fig. 3) expressions of HSP70 and suppressed those of iNOS and caspase 3 in chondrocytes.

Under normal conditions, HSP70 functions as an ATP-dependent molecular chaperone by assisting the folding of newly synthesized polypeptides, assembly of multi-protein complexes, and transport of proteins across cellular membranes.[21] Under various stress conditions the synthesis of stress-inducible HSP70 enhances the ability of the stressed cells to cope with increased concentrations of unfolded or denatured proteins.[22] HSP70 can inhibit apoptosis and thereby increase the survival of cells exposed to a wide range of lethal stimuli.[23] In the present study, HBO treatment upregulated mRNA (Fig. 2A) and protein expressions (Fig. 3A) of HSP70 thus suppressed iNOS expression (Figs. 2B and 3B) in the articular chondrocytes.

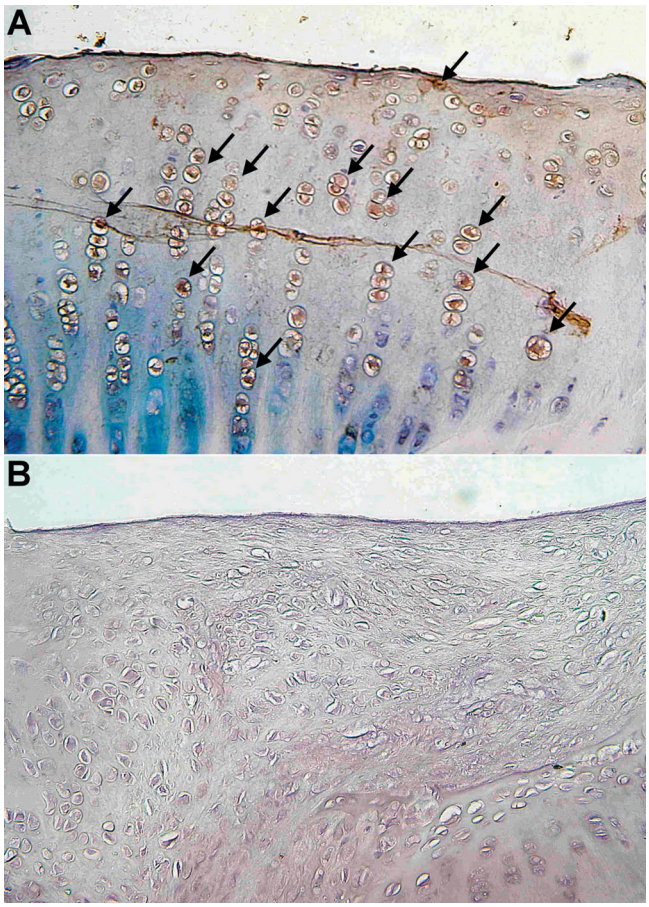

Figure 6. Effect of HBO treatment on HSP70 expression. In the repaired cartilage of the HBO group (A), an increased staining intensity of HSP70 was observed as compared to the control group (B) (original magnification, ×200).

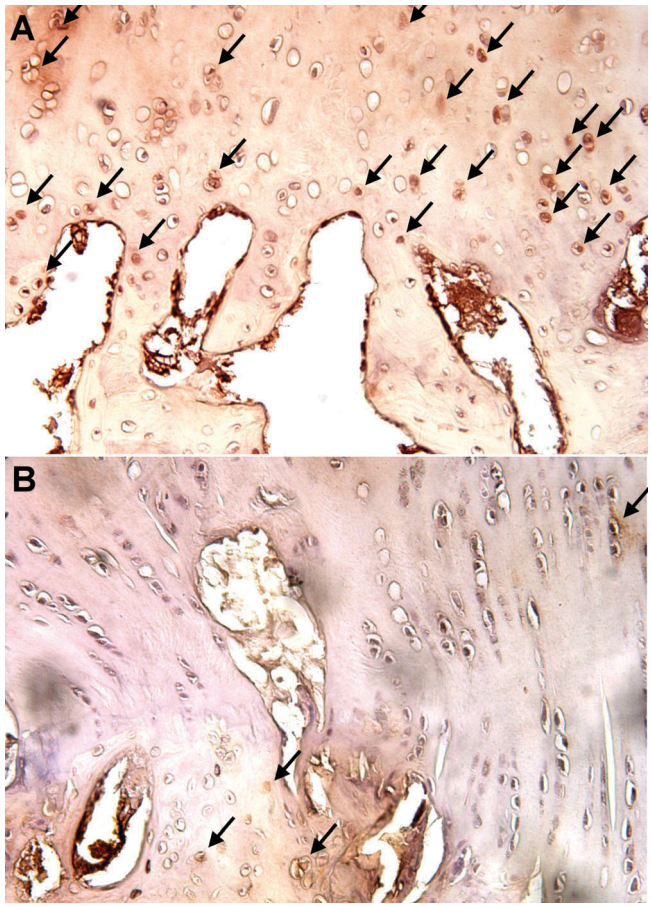

Figure 7. Effect of HBO treatment on iNOS expression. In the repaired cartilage of the control group (A), an increased staining intensity of iNOS was observed as compared to the HBO group (B) (original magnification, ×200).

Inhibition of HSP70 production by KNK437 in HBO-treated cells reversed iNOS expression (Fig. 4A). These results confirm that prevention of IL-1β-induced cell damage of HBO-treated cells was mediated by HSP70 expression.

Previous studies have shown that stress-induced activation of HSPs not only protects cells from stress but also suppresses apoptosis in various cells.[24,25] Similar to these studies, our results showed that increased HSP70 expression in chondrocytes after HBO treatment enabled the chondrocytes to tolerate stress and protected them from apoptosis (Figs. 2 and 3). Furthermore, HSP70 was observed to be involved in quality control of proteoglycan synthesis[26] and promoted cartilage metabolism[12] and these findings suggest that HSP70 promotes cartilage repair (Table 2). Based on these findings, it is reasonable to postulate that administration of HBO to defected cartilage, in which stress response via HSP70 is present, induces a sufficient increase in HSP70 for therapeutic purposes in vivo.

In addition to HSP70, caspase 3 is another key molecule involved in apoptosis signal transduction. The activation of caspase 3 is associated with the enzymatic cleavage of the precursor protein, pro-caspase 3. HSP70 prevents NO-induced activation of caspase 3 in articular chondrocytes.[9] Our previous study showed a decrease in the numbers of iNOS-positive and TUNEL-positive cells in rabbit cartilage repair model after HBO treatment.[17] In the present study, not only the mRNA expression of iNOS (Fig. 2B) and caspase 3 (Fig. 2C) were downregulated, and additionally the contents of iNOS protein (Fig. 3B) and active form of caspase 3 protein (Fig. 3C) had also drastically decreased in the cells after HBO treatment. These results suggested that NO-induced activation of caspase 3 was blocked by HSP70 expression in the chondrocytes after HBO treatment and the role of HSP70 in blocking the NO-induced activation of caspase 3 was confirmed by KNK437 treatment (Fig. 4B). In addition, the beneficial effects of HBO treatment were also evident in our rabbit cartilage repair model (Figs. 5–8).

Long-term and repeated HBO treatment may increase oxidative stress.[12] Tolerance to HBO treatment can be extended by intermittent exposure to it. The authors used a clinical HBO protocol in this study. Because exposure to hyperoxia in clinical protocols is

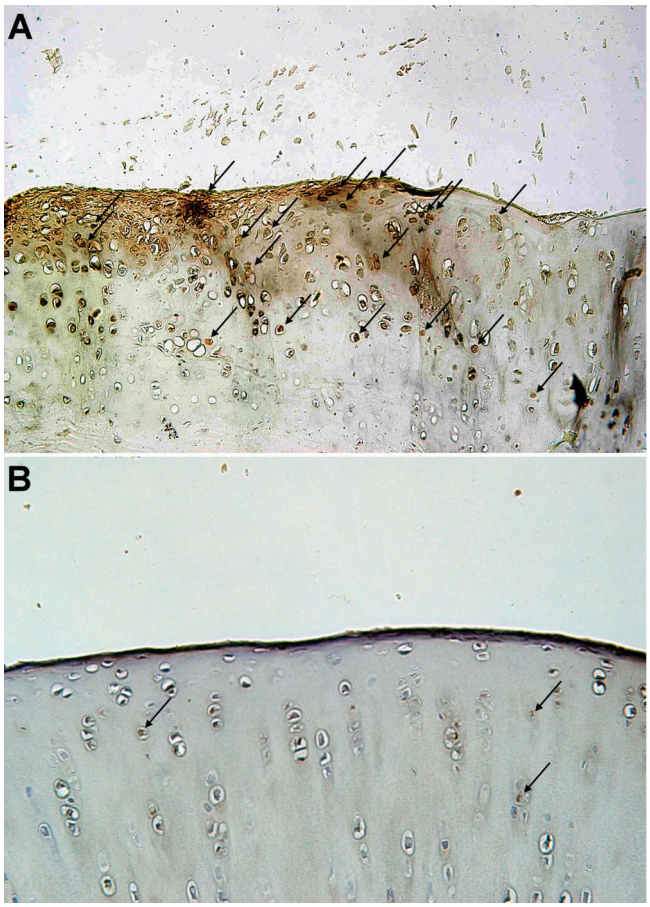

Figure 8. Effect of HBO treatment on chondrocyte apoptosis. In the repaired cartilage of the control group (A), an increased staining intensity of caspase 3 was observed as compared to the HBO group (B) (original magnification, ×100).

rather brief (typically <2 h/day), studies show that antioxidant defenses are adequate so that biochemical stresses related to increases in ROS are reversible.[12] The present study showed that HBO enhances the expression of HSP70 and prevents NO-induced apoptosis in vitro and in vivo. HBO therapy may offer a potential method for clinical cartilage repair however the effects of HBO on human OA chondrocytes have to be determined to verify this.

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