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Attenuation of apoptosis and enhancement of proteoglycan synthesis in rabbit cartilage defects by hyperbaric oxygen treatment are related to the suppression of nitric oxide production

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Abstract

Proinflammatory cytokine, nitric oxide (NO) and localized hypoxia-induced apoptosis and proteoglycan (PG) degradation are thought to be correlated to the degree of cartilage injury. This study evaluated hyperbaric oxygen (HBO)-induced changes in joint cavity oxygen tension, antigenickeratan sulfate (KS) content, inducible nitric oxide synthase (iNOS) expression, PG synthesis, and cell apoptosis in full-thickness defects of rabbit cartilage. The HBO group was exposed to 100% oxygen at 2.5 atm for 2 h daily, 5 days per week. Meanwhile, the control group was kept in housing cages with normal air. The joint cavity oxygen tension was determined with an oxygen sensor. Blood serum KS was quantified by competitive indirect enzyme-linked immunosorbent assay (ELISA). After sacrifice, specimen sections were sent for histological and histochemical examination with a standardized scoring system. In situ analysis of iNOs expression and apoptosis detection were performed using immunostaining and TUNEL staining, respectively and quantified by a computerized imagine analysis system. This study demonstrated that HBO treatment increased joint cavity oxygen tension but decreased blood KS content. Histological and histochemical score results showed that HBO treatment significantly increased the cartilage repair. Moreover, immunostaining and TUNEL staining showed that HBO treatment suppressed the iNOs expression and apoptosis of chondrocytes, respectively. Accordingly, HBO offers a potential treatment method for cartilage injury.

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Keywords: Hyperbaric oxygen; Full-thickness defects; Nitric oxide; Apoptosis; Proteoglycan; Keratan sulfate

Introduction

Although articular cartilage normally exists in a steady state of matrix degradation and generation, it has limited capacity for repair after blunt trauma injury. Larger full-thickness articular cartilage defects cannot be repaired and may initiate progressive degenerative changes in the remaining cartilage similar to those seen with osteoarthritis (OA) [36]. Surgical removal of damaged cartilage and penetration into the subchondral bone to allow population of the defect with progenitor cells can lead to the defect being filled with repair tissue. However, the resulting fibrocartilage often degenerates over time [34]. Articular chondrocytes are specialized to survive in an avascular environment and rely on diffusion pathway to obtain O_2 [20]. The normal synovium is highly vascularized to satisfy the metabolic demands of the avascular cartilage [35], but the oxygen gradient is likely to be disturbed during a joint disease state [9]. Oxygen seems to play an important role in improving matrix production and osteochondral regeneration after cartilage injury [9].

The remaining cartilage of full-thickness defected model or OA cartilage spontaneously produces nitric oxide (NO) [10,22,36], which is believed to mediate the suppression of cartilage proteoglycan (PG) synthesis induced by interleukin-1 [1,36,37]. Moreover, NO was demonstrated to reduce the synthesis of IL-1 receptor

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antagonist (IL-1 Ra) in chondrocytes [29], thus enhancing the suppression effect on these cells. Apoptosis of chondrocytes can be induced by NO [1,3,10] and by localized hypoxia [17,21]. Suppression of NO production after chondral trauma, or during the early stage of OA, may inhibit the initiation or progression of OA [36]. Keratan sulfate (KS) is a major component of cartilage PG. Because the degradation of cartilage PGs in a single joint caused a dramatically rise in levels of serum KS, measurement of KS levels in blood serum may provide useful information about PG degradation occurring in an injured or OA joint [31,38].

Close associations have been reported among hyperbaric oxygen (HBO), proinflammatory cytokines, PG, and NO synthesis. HBO treatment increases the tissue/microvascular pO_2 [16], suppresses the IL-1 β secretion [11,43], decreases the NO production [5,18], improves the epiphysial growth cartilage transplantation results [8], accelerates bone healing [41,42], increases glycosaminoglycan synthesis [32], and stimulates fibroblast proliferation [40]. The present study examines the effects of HBO treatment on the process of cartilage repair by measuring joint cavity oxygen tension, antigenic KS content, PG synthesis, iNOs expression, and cell apoptosis in a rabbit cartilage full-thickness defect model.

Materials and methods

Animal operation

New Zealand rabbits weighing 3 kg were anesthetized by intravenous injection of 5 ml ketamine hydrochloride (Ketalar, ParkeDavis, Taiwan) and Rompum (Bayer, Leverkusen, Germany) mixture. An aseptic method was employed, using a drill to create a 4-mm diameter cartilage defect passing through the subchondral bone on the right side of the medial femoral condyle of the weight-bearing surface. The full-thickness defect was made, as demonstrated by the bleeding. A straight midline incision was made with a medial parapatella arthrotomy. After creation of the cartilage defect, the knee joint was irrigated and the wound was closed with 3-0 nylon sutures at the arthrotomy wound and skin. The animals were allowed to feed freely in their cages.

Exposure to intermittent HBO

Twenty-four rabbits were used in this study, randomly divided into two groups: (I) HBO group, containing 12 rabbits exposed to HBO with 100% oxygen at 2.5 atm for 2 h daily, 5 days in a week after surgery. (II) Control group, containing 12 rabbits housed cages with normal air.

Joint cavity oxygen tension detection

Mean joint cavity oxygen tension was determined with an oxygen sensor (Oxylite multi-channel system, Oxford Optronix Ltd., UK). The animal was anesthetized and small skin incision was made over lateral side of knee joint with dissection of soft tissue till exposure of joint capsule. The sensor was then inserted into the joint to detection the oxygen tension. Pre-HBO oxygen tension was measured in six rabbits of HBO group. Continuous in vivo measurements of oxygen tension in the joint cavities lasted for 30 min. After HBO treatment, variation of oxygen tension in the joint cavities was quantified.

Antigenic KS detection in blood serum

At intervals of 7, 14, 21, 28, and 35 days after operation, venous blood was obtained from the rabbit ears in both the control and HBO groups. Blood serum was obtained after centrifugation and analyzed for agKS (antigenic KS, bearing the 1/20/5-D-4 epitope) content by competitive indirect ELISA [31,38]. Briefly, after an inhibition step in which the 1/20/5-D-4 monoclonal antibody was allowed to interact with agKaS in a diluted sample of serum, the mixture was placed in the well of a microtiter plate coated with chondroitinase ABC treated, agKS-containing aggrecan. After rinsing, the mixture was incubated with a solution of peroxidase coupled antimouse IgG. Then, after further rinses, the new mixture was incubated with a solution containing the substrate for peroxidase. The absorbance of the colored product was read at 490 nm. agKS concentration was calculated by comparing this absorbency value with that generated using serial dilutions of an international standard of purified bovine KS (Sigma, St. Louis, MO, USA). The KS standard was used at concentrations ranging from 6.25 to 200 ng/ml.

Tissue processing and Toluidine blue staining, Safranin-O staining

Animals were sacrificed at 5 and 10 weeks after surgery. The defect area was sent for histological and immunohistochemical assessments. Tissue blocks were fixed in 10% neutral buffered formalin for 2 weeks, and decalcified with 20% EDTA in PBS. After decalcification, the blocks were cut in half through the defect and embedded in paraffin. Five-micron sections were cut and stained with Toluidine blue and Safranin-O.

Scoring system for histological and histochemical analysis

The sections from each specimen were examined blindly and independently by six authors, who selected the most typical section from each specimen, and then examined it to assess cellular morphology, affinity of the matrix for the safranin-O stain, surface regularity, structural integrity, thickness of the graft, bonding to the adjacent articular cartilage, cellular changes of degeneration, and the condition of the adjacent cartilage. Each of these characteristics was given a score according to the Salter's standard scoring system (Table 1), which had been previously found to be reliable [27].

Immunohistochemistry examination for iNOs

Tissue sections were deparaffinized in xylene, passed through decreasing gradations of ethanol, and incubated with chondroitinase ABC (0.25 U/ml, Sigma, St. Louis, MO, USA) in phosphate buffered saline (PBS, pH 7.4) for 60 min at 37 °C. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide. The presence and distribution of iNOs was determined using anti-iNOs antibody (1 mg/ml, 1:40 dilution, R&D system, MN, USA) for 2 h at room temperature. A peroxidase conjugated secondary antibody (0.8 mg/ml, 1:1000 dilution, Jackson Immuno Res., PA, USA) was used for 20 min at room temperature. Bound immunoglobulin was detected with a DAB peroxidase substrate kit (Vector Lab., CA, USA) and 0.1% methyl green was used for counter staining. Each image was captured by a digital camera (DP 50; Olympus, Shibuya-ku, Tokyo) and the positive staining cells were quantified by Image-Pro Plus 4.0 image analysis software (Media Cybernetics, Silver Spring, MD).

TUNEL staining for apoptosis detection

In situ analysis of apoptosis was performed using a cell death detection kit, POD (Roche, Mannheim, Germany) according to the manufacturer's instructions with slight modification. Briefly, 5 μm cartilage sections were floated onto poly-L-lysine-coated slides, deparaffinized in xylene, passed through decreasing gradations of ethanol, treated with proteinase K (20 μg/ml, Sigma, St. Louis, MO, USA) for 15 min, and quenched of endogenous hydrogen peroxidase activity in 3% hydrogen peroxide. After a series of rinse, the sample was labeled with TUNEL reaction mixture for 60 min at 37 °C. The color was developed with a DAB peroxidase substrate kit (Vector Lab., CA, USA) and 0.1% methyl green was used as a counter stain. Each image

Table 1
Scoring system for histological and histochemical results

Characteristic	Points
Nature of the predominant tissue	
Cellular morphology	
Hyaline articular cartilage	4
Incompletely differentiated	2
Fibrous tissue or bone	0
Safranin-O staining of the matrix	
Normal or near normal	3
Moderate	2
Slight	1
None	0
Structural characteristics	
Surface regularity	
Smooth and intact	3
Superficial horizontal lamination	2
Fissures, 25–100% of the thickness	1
Severe disruption, or fibrillation	0
Structural integrity	
Normal	2
Slight disruption, including cysts	1
Severe disintegration	0
Thickness	
100% of normal adjacent cartilage	2
50–100% of normal cartilage	1
0–50% of normal cartilage	0
Bonding to the adjacent cartilage	
Bonded at both ends of graft	2
Bonded at one end, or partially at both ends	1
Not bonded	0
Freedom from cellular changes of degeneration	
Hypocellularity	
None	3
Slight	2
Moderate	1
Severe	0
Chondrocyte clustering	
None	2
<25% of cells	1
25–100% of cells	0
Freedom from degenerative changes in adjacent cartilage	
Normal cellularity, no clusters, normal staining	3
Normal cellularity, mild clusters, moderate staining	2
Mild or moderate hypocellularity, slight staining	1
Severe hypocellularity, poor or no staining	0
Perfect score	24

was captured by a digital camera (DP 50; Olympus, Shibuya-ku, Tokyo) and the positive staining cells were quantified by Image-Pro Plus 4.0 image analysis software (Media Cybernetics, Silver Spring, MD).

Statistical analysis

Pre- and post-HBO oxygen tension were compared using the paired Student's t test. Serum KS levels between the control and HBO groups were compared using the Student's t-test. Histological and histochemical score results were analyzed using the Mann–Whitney U test, a non-parametric t-test and necessary for categorical data. Immunohistochemical results for iNOs staining and TUNEL staining between the two groups were compared using the Mann–Whitney U test. Values were expressed as mean ± SD for Student's t-test and as median and range for Mann–Whitney U test; $p < 0.05$ was considered statistically significant.

Results

Effect of HBO on joint cavity oxygen tension

The traces for continuous in vivo measurements of oxygen tension in the joint cavities are shown in Fig. 1. The mean joint cavity oxygen tensions were 11.62 ± 2.51 pre-HBO treatment and 25.02 ± 4.34 post-HBO treatment. The oxygen tension significantly increased in the joint cavity after HBO treatment (Table 2, $p < 0.01$).

Effect of HBO on agKS secretion

The serum KS levels in both groups are shown in Fig. 2 and Table 3. No significant differences were noted in preoperative serum KS levels between the control and HBO groups ($p > 0.05$). After surgery, serum KS levels rose sharply on week 1, but had almost returned to preoperative levels by week 4 in the control group. Serum KS levels were slightly increased in the HBO group, but were significantly lower than that of the control group in 1, 2, and 3 weeks after surgery ($p < 0.01$, $p < 0.01$, $p < 0.05$).

Effect of HBO on cartilage defect repair

The gross appearance of the defects and repaired cartilage were examined. At 5 weeks after operation, early formation of woven bone spicule was noted in the depth of the defect in the control group, but the superficial layer of the repair tissue was still fibrous and irregular (Fig. 3A). In the HBO group, the thickness of the repair cartilage was about 50% that of the normal adjacent cartilage (Fig. 3B). Safranin-O staining of the matrix was evident in the area of the hypertrophic chondrocytes at the base of the defect. At 10 weeks after surgery, the gross appearance of the defects demonstrated about 40% repair in the control group (Fig. 3C). The repair tissue consisted of a mixture of mostly hypocellular fibrous tissue in the superficial layer and a little fibrocartilage in the basal layer. In the HBO group, the defects were completely healed and the tidemark was partly reconstituted (Fig. 3D). Safranin-O staining was evident throughout the hyaline-like repair cartilage, with vertical column formation of chondrocytes in the radial zone (a typical feature of normal articular cartilage) (Fig. 3E). The histological and histochemical score results are summarized in Table 4. The total scores were also better for the HBO groups than control groups ($p < 0.01$) in each time point show.

Effect of HBO on iNOs expression

In the repaired cartilage of control group, a strong staining for iNOs was observed (Fig. 4A and C) when

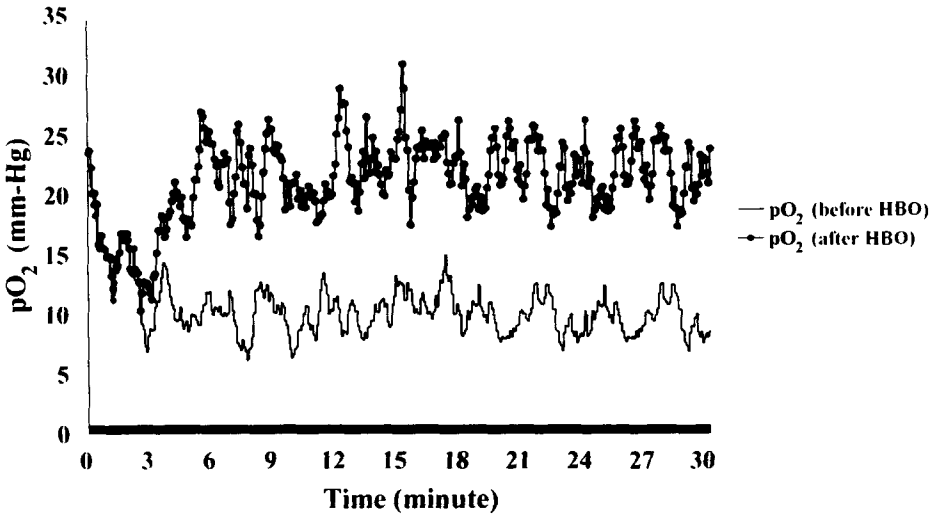

Fig. 1. Oxygen sensor tracings of pO_2 obtained in one of the animals. HBO treatment significantly increased the oxygen tension in the joint cavity in continuous in vivo measurements.

Table 2
Effect of HBO on joint cavity oxygen tension (mm Hg)

Animal	Pre-HBO treatment	Post-HBO treatment
1	10.47	21.21
2	9.98	22.19
3	14.98	30.96
4	12.67	26.31
5	13.44	28.89
6	8.17	20.58
Mean ± SD	11.62 ± 2.51	25.02 ± 4.34**

Paried t-test, $^{**}p < 0.01$.

Table 3
Effect of HBO on agKS secretion (ng/ml)

Weeks after OP	Control groups	HBO groups	p-value
0	15.5 ± 2.4	15.4 ± 4.7	$p > 0.05$
1	154.5 ± 30.1	46.9 ± 10.7	$p < 0.01$
2	102.5 ± 20.2	30.4 ± 7.2	$p < 0.01$
3	50.4 ± 10.7	15.9 ± 3.2	$p < 0.01$
4	20.3 ± 7.9	16.2 ± 5.2	$p > 0.05$
5	16.7 ± 6.5	15.8 ± 4.7	$p > 0.05$

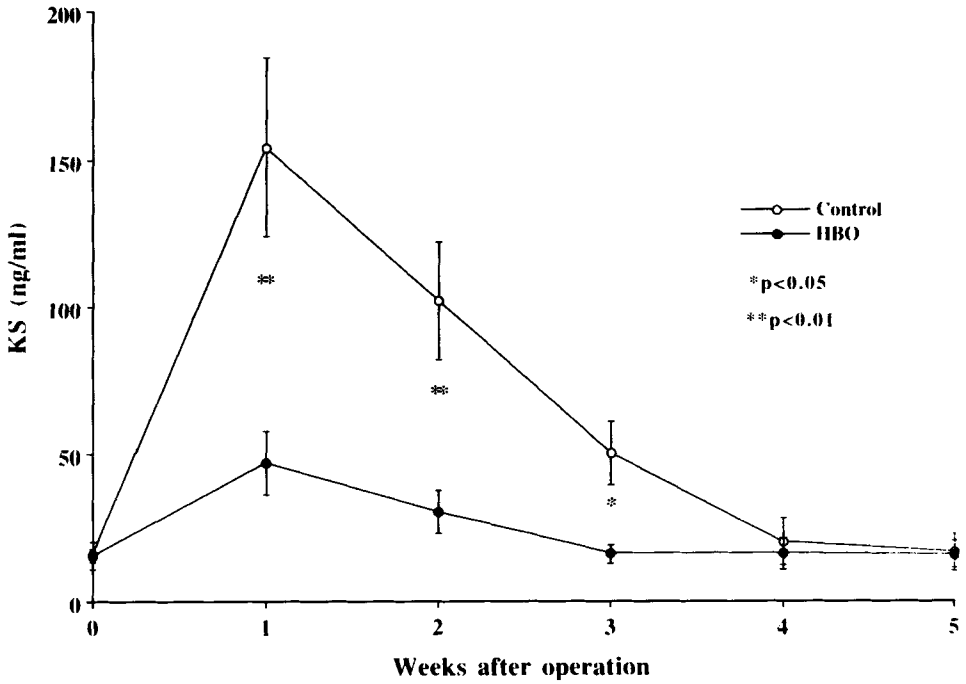

Fig. 2. Increased serum keratan sulfate levels in the rabbit cartilage injury model. Serum KS level was slightly increased after surgery in the HBO group, but still significantly lower than in the control group. Data were expressed as mean ± SD of findings in six rabbits in each group; $p < 0.05$ was considered statistically significant.

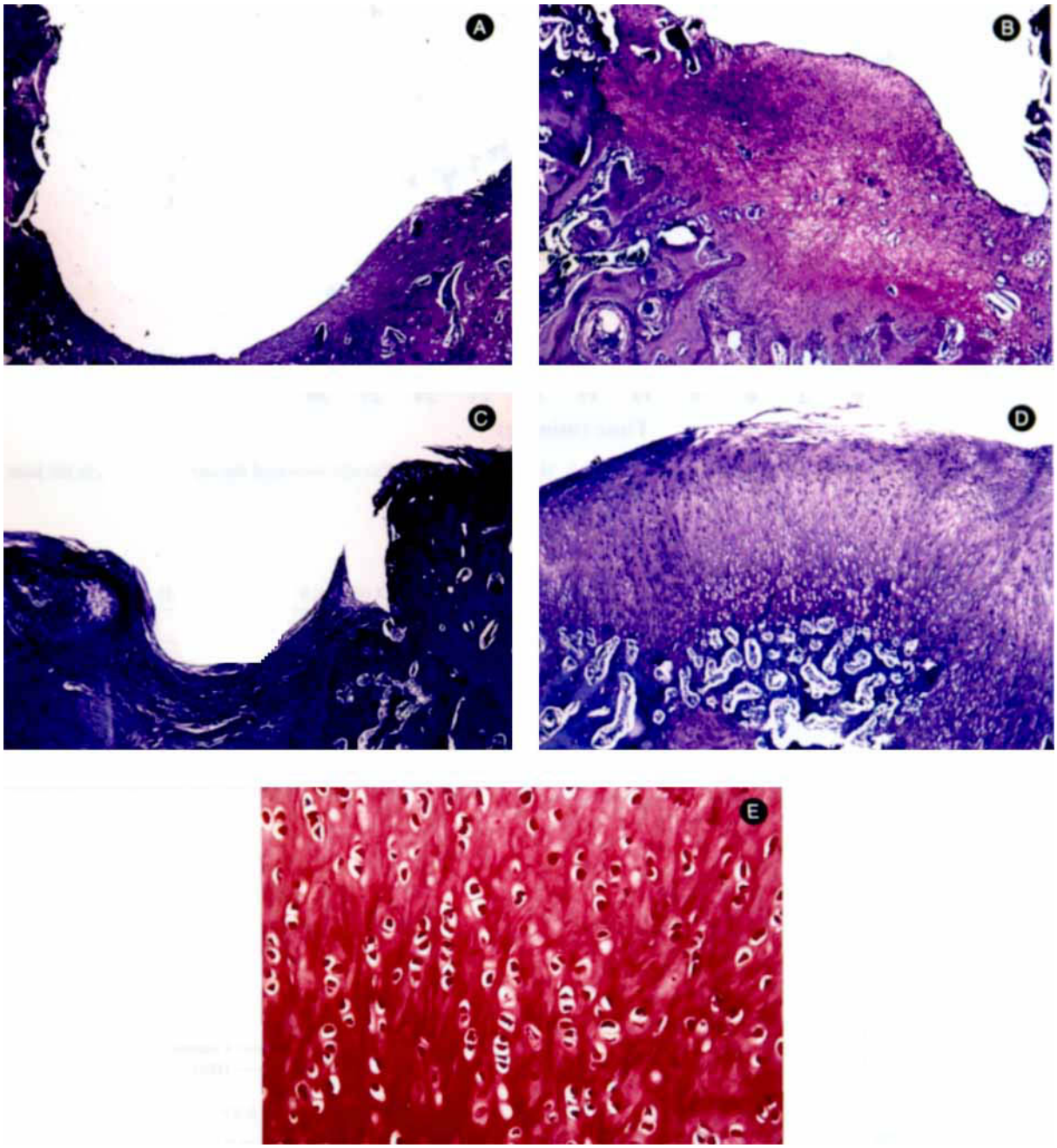

Fig. 3. The gross appearance of the defects and the repaired cartilage were examined. At 5 weeks after surgery: (A) the defects were almost empty in the control group (Toluidine blue stain, ×200); (B) the thickness of the repair cartilage was about 50% of the normal adjacent cartilage in the HBO group (Toluidine blue stain, ×200). At 10 weeks after operation, (C) the gross appearance of the defects was about 40% repaired in control group (Toluidine blue stain, ×200); (D) the defects were completely healed and the tidemark was partly reconstituted in the HBO group (Toluidine blue stain, ×200); (E) Safranin-O staining was evident throughout the hyaline-like repair cartilage with vertical column formation of chondrocytes in the radial zone in the HBO group (Safranin-O stain, ×200).

compared to the HBO group (Fig. 4B and D). The quantitative results for iNOs expression of chondrocyte are summarized in Table 5. Immunostaining demonstrated that HBO treatment significantly suppressed iNOs expression of chondrocyte ($p < 0.01$) in each time point show.

Effect of HBO on apoptosis of chondrocyte

In the repaired cartilage of the control group, numerous of TUNEL-labeled cells were observed (Fig. 5A and C) when compared to the HBO group (Fig. 5B and D). The quantitative results for

Table 4
Histological and histochemical score results

	Control groups		HBO groups		p-value
	Median	Range	Median	Range	
5 weeks	1	2	7.5	2	$p < 0.01$
10 weeks	8	2	21.5	5	$p < 0.01$

Mann–Whitney U test.

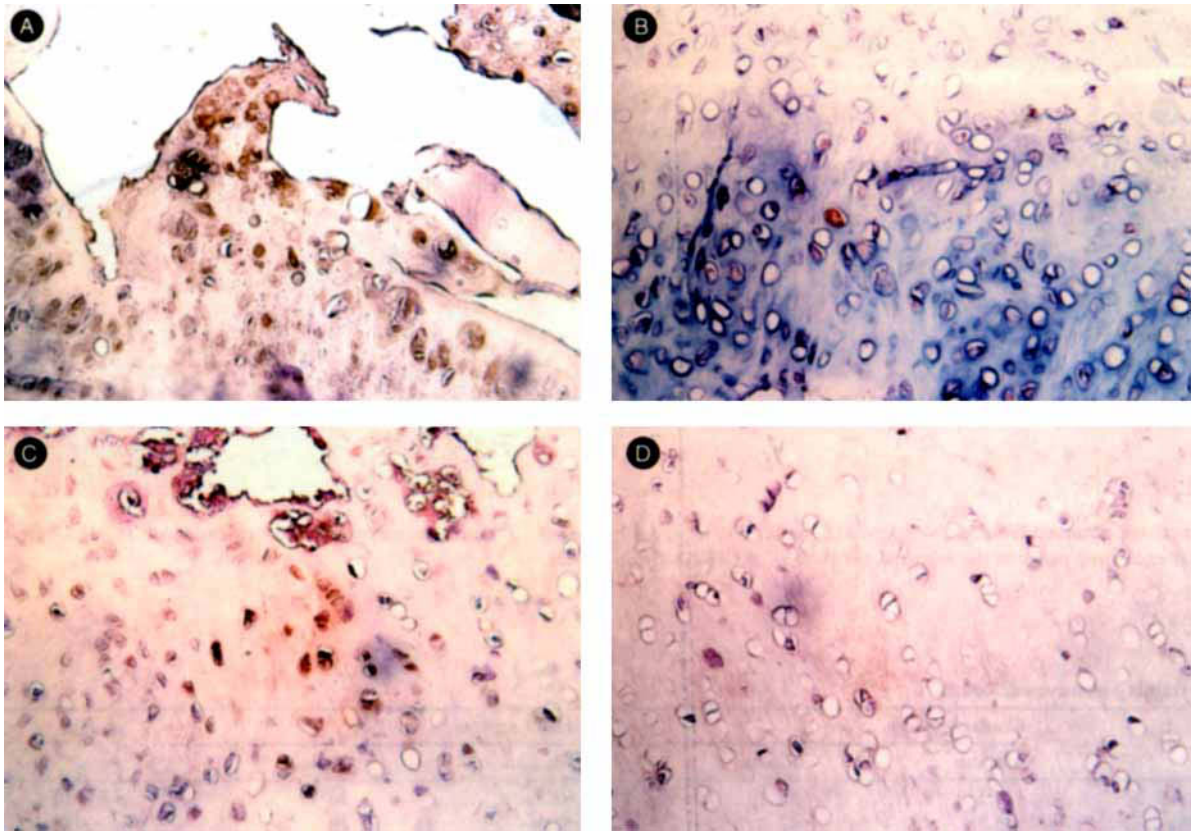

Fig. 4. Cartilage obtained from both groups at 5 weeks (A, B) and 10 weeks (C, D) after surgery. Reduced expression of iNOs is revealed at each time point in the repaired cartilage of the HBO group (B, D).

Table 5
iNOs staining positive cell count

	Control groups		HBO groups		p-value
	Median	Range	Median	Range	
5 weeks	166.5	74	32	42	$p < 0.01$
10 weeks	69	56	14	27	$p < 0.01$

Mann–Whitney U test.

apoptotic chondrocytes are summarized in Table 6. HBO treatment significantly suppressed the apoptosis of chondrocyte ($p < 0.01$) in each time point show.

Discussion

Although articular cartilage is an avascular tissue that functions in a low oxygen tension environment, a

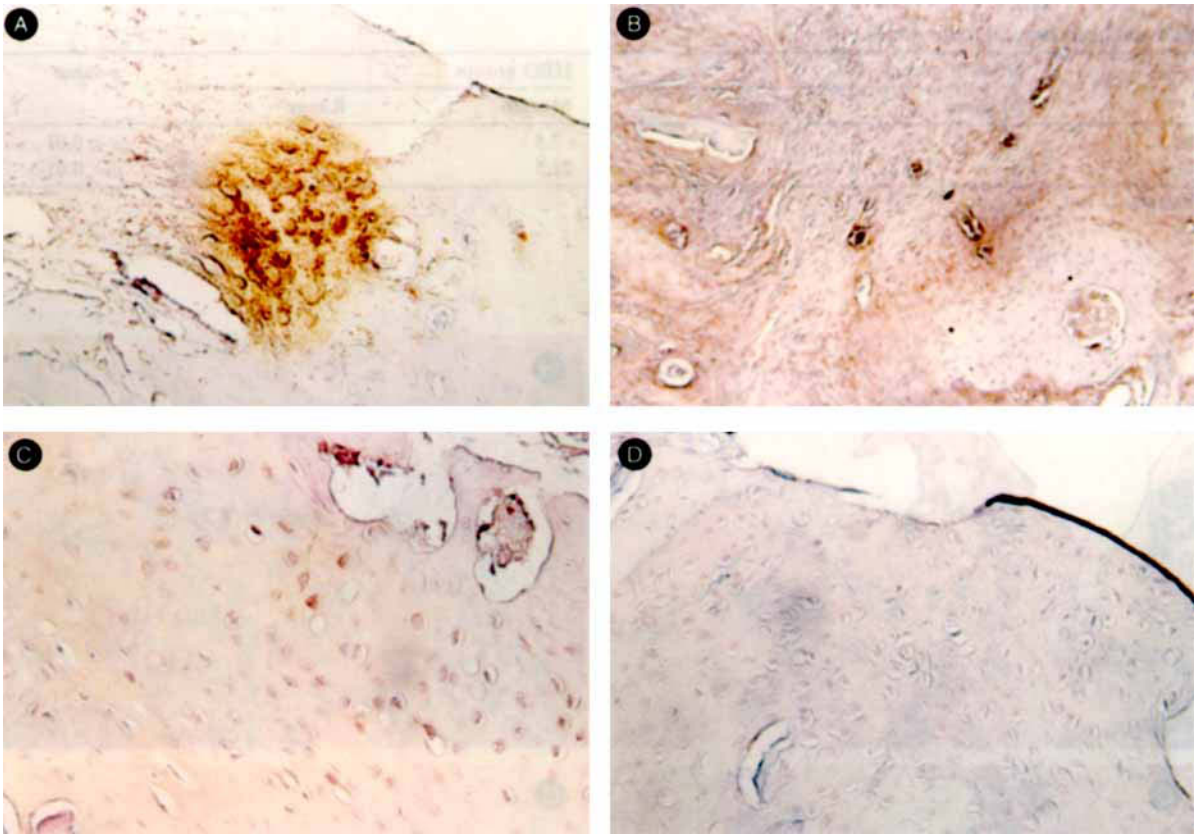

Fig. 5. Cartilage obtained from both groups at 5 weeks (A, B) and 10 weeks (C, D) after surgery. Reduced expression of TUNEL-labeled cells is revealed at each time point in the repaired cartilage of the HBO group (B, D).

Table 6
TUNEL staining positive cell count

	Control groups		HBO groups		*p*-value
	Median	Range	Median	Range	
5 weeks	121.5	54	63.5	36	$p < 0.01$
10 weeks	65.5	47	12.5	10	$p < 0.01$

Mann–Whitney *U* test.

further decrease in synovial fluid oxygen tension and environmental changes in traumatic injury or degenerative diseases of articular cartilage have been reported [2,15,35]. In vivo, hypoxia has been suggested to be playing central in the development of tissue damage in OA [14,15]. In vitro, the glycosaminoglycan (GAG) biosynthesis rate in cartilage explants cultured at oxygen tensions of 78–180 mm Hg was twice that in those cultured at 45 mm Hg [45]. Additionally, more cartilaginous matrix formed in rabbit periosteal explants cultured at oxygen tension of 91–114 mm Hg than in those cultured at 8–38 mm Hg [28]. Further studies demonstrated marked inhibition of chondrocyte proliferation and extracellular matrix synthesis under hypoxic conditions [4,19]. Moreover, significant enhancement of the NO-induced depression of chondrocyte mitochondrial oxidative phosphorylation was also reported under hypoxic conditions [39]. These findings indicate that hypoxic conditions comparable with those present in vivo are not optimal for osteochondral regeneration after cartilage injury or in vitro chondrogenesis.

The repair mechanisms of articular cartilage are poorly understood but there were some important implications in previous investigations. Salter et al. used the full-thickness defect and the chymopapain-induced injury models to investigate the effects of continuous passive motion (CPM) on cartilage repair [13,23]. They hypothesized that CPM of synovial joints should have the following beneficial effects in vivo: (1) enhance the nutrition and metabolic activity of articular cartilage, (2) protect

and stimulate repair of the articular matrix after chymopapain-induced injury, and (3) accelerate healing of both articular cartilage and periarticular tissues, such as tendons and ligaments [33]. Thonar et al. used the anterior cruciate ligament transection (ACLT) model to investigate the changes in serum levels of KS and suggested that injury to a single synovial joint gives KS rise in local at first but become systemic with time [39]. Tanaka et al. used the cartilage defected model and reveals an increase in the NO production which can affect the homeostasis of the cartilage extracellular matrix [36]. Hashimoto et al. [10] and Pelletier et al. [30] used the ACLT model and suggest that NO production may lead to chondrocyte apoptosis, and that both events contribute to the cartilage degradation. Selective inhibition of iNOs reduces progression of experimental OA in vivo [30]. In the present study, a static defect model created by drilling of holes through the subchondral bone-plate and have similar findings in vivo: (1) The post-operative rise in the serum KS level is great and lasts for several weeks suggest that the trauma to bone and cartilage has systemic effects on the turnover of aggrecan throughout the body [39]. Our data showed slightly raised serum KS level after surgery in the HBO group, but still lower than that of the control group (Fig. 2). The systemic mechanisms of HBO effects on all cartilages are not clear. At least in part, HBO increases oxygen supply to anoxic areas of local injured cartilage (Fig. 1), which increases chondrocyte proliferation and extracellular matrix synthesis. Histological and histochemical evidences were also confirmed by the score analysis system (Fig. 3 and Table 4), (2) HBO reduced the PG degradation of injured cartilage and this beneficial effect may be related to the suppression of iNOs expression and apoptosis of chondrocyte (Figs. 4 and 5). Other possible mechanism for cartilage destruction is that NO may stimulate matrix metalloprotease (MMP) activity in chondrocyte [25], but the effect of HBO on MMP activity is not clear, (3) HBO accelerate healing of both articular cartilage and periarticular tissues, such as ligaments [12], which may improve the joint instability caused by ligament transection in ACLT model.

Critical evaluation of methods intended to restore cartilaginous articular surfaces requires consideration of the limitations of used experimental models and their relationships to clinical disorders. In the present study, acute experimental chondral and osteochondral defects created by drilling of holes simulates replicate acute chondral and osteochondral injuries that occur in humans as a result of joint trauma and may provide appropriate models for some osteochondritis dissicans and traumatic lesions. This kind of defect is similar to the full thickness osteochondral injury after trauma such as comminuted tibial plateau fracture induced focal cartilage defect. But this defect was done under well control and made by drilling which would have some heat effect to the bone and cartilage. In the real situation, there is only impact but no such heat effect on the bone and cartilage and the defect is usually irregular and uneven depth. Those defects commonly occur in adolescents and young adults who wish to maintain a high level of activity, and in some of these individuals they cause joint pain, effusion, and mechanical dysfunction [6,7,24]. Inhalation of HBO is now an established mode of therapy for a variety of medical conditions and provides a clear mechanism of the inhibition of inflammatory reaction by HBO. Weisz et al. reported that HBO treatment in Crohn's disease (a perianal inflammatory bowel disease) decreased tumor necrosis factor-α (TNF-α), IL-1 and IL-6 secretion by monocytes [43]. Yin et al. reported that the neuroprotective effect of HBO might lead to an inhibition of cyclooxygenase-2 (COX-2) over expression [44]. Tolerance to HBO can be extended by intermittent exposure when HBO treatments are required in humans. The authors used a clinical HBO therapy protocol described in the United States Navy Treatment [26] in this study. From our experience, this HBO therapy protocol causes no oxygen toxicity in rabbits [41,42]. Furthermore, the joint cavity oxygen tension ranged from 20.58 to 30.96 mm Hg after HBO treatment (Table 1). This is a safe oxygen tension range as compared with that of normal joint cavities in rabbits in previous studies [15]. The present study demonstrated that HBO treatment could suppress apoptosis and increases cell growth and PG synthesis of defect cartilage, and moreover suggested that this beneficial effect is related to the suppression of NO production. HBO therapy may offer a potential method for cartilage repair clinically, although the optimal timing, duration and frequency of HBO treatment have to be determined to verify this.

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