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BASIC SCIENCE

Effect of Hyperbaric Oxygenation on Intervertebral Disc Degeneration

An In Vitro *Study With Human Lumbar Nucleus Pulposus*

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Study Design. An *in vitro* study with degenerated human lumbar intervertebral disc specimens cultured under hyperbaric oxygenation (HBO).

Objective. To observe the changes in interleukin (IL)-1β, prostaglandin (PG)-E2, nitric oxide (NO), cell growth, and apoptosis of the human nucleus pulposus cell (NPC) after HBO.

Summary of Background Data. Intervertebral disc degeneration has been demonstrated as related to IL-1β, PG-E2, NO, and O_2 concentration but the actual mechanism is not clear. HBO also has also been reported in the literature to influence changes in IL-1β, prostaglandin E2, NO, and O_2 concentration. However, the direct effect of HBO on the disc cells has not been previously reported.

Methods. We collected 12 human lumbar degenerated disc specimens and evaluated the effects of HBO on the cultured NPCs. The amounts of IL-1β, PG-E2, and NO in the conditioned medium were quantified by enzyme-linked immunosorbent assay and high performance liquid chromatography. Cell growth was measured by increase in cell number. Cell viability and proteoglycan content were evaluated by histologic study using safranin O staining. *In situ* analysis of apoptosis was performed using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining.

Results. Our data indicated that HBO treatment inhibited IL-1β, PG-E2, and NO production but increased cell number and matrix synthesis of cultured NPCs. TUNEL staining showed that HBO treatment suppressed the apoptosis of cultured NPCs.

Conclusion. HBO provides a potential treatment modality for disc degeneration.

Key words: disc degeneration, hyperbaric oxygenation, apoptosis.

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Degenerative disc disease is believed to be a major etiology in low back pain. There has been a lot of effort to explain the process of intervertebral disc degeneration. Possibly as part of the aging process, the structural changes in disc degeneration markedly alter the microenvironment. The impacts on the microenvironment, including vessel depletion[1,2] and oxygen concentration change,[3–5] are thought to be related to the loss of nutrient supply and play an important role in disc degeneration.[6,7] From the biochemical perspective, some processes of disc degeneration, including inhibition of nuclear proteoglycan synthesis and enhanced matrix degradation, have been found to be related to chemical mediators such as interleukin (IL)-1, IL-6, prostaglandin E2 (PG-E2), nitric oxide (NO), and matrix metalloproteinases (MMPs)[8–10]; however, the actual mechanism has not been well investigated.

As a frequently used medical modality, the effects of hyperbaric oxygenation (HBO) on new vessel formation[11] and increasing tissue oxygen concentration are well known.[12,13] Several reports have also revealed a close relationship between HBO and cell cycle alteration, apoptosis, collagen, proteoglycans, and NO. The proven effects of hyperbaric oxygenation include suppression of IL-1β secretion,[14,15] decreased NO production,[16,17] increased glycosaminoglycan synthesis,[17,18] acceleration of bone healing,[19,20] stimulation of fibroblasts,[21,22] and increase of microvascular pO_2 in tissue.[13,23]

Reports in the English literature regarding HBO and disc degeneration are rare. In this study, we collected 12 degenerated disc specimens from human spinal surgery. Using disc cell culture, high performance liquid chromatography (HPLC), and flow cytometry, we investigated the effect of HBO on degenerated disc cells by measuring IL-1β concentration, PG-E2 concentration, NO concentration, cell growth, matrix formation, and cell apoptosis. We hoped to use the results of this *in vitro* study to establish a therapeutic model for degenerative disc disease.

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MATERIALS AND METHODS

Our experimental protocol was approved by the human subjects institutional review board at the Chang Gung Memorial Hospital.

In brief, we collected 12 degenerated disc specimens and evaluated the effects of HBO treatment on the degenerated intervertebral discs. Nuclei pulposi were collected from degenerated disc specimens after enzyme digestion and cultured in alginate beads. All hyperoxic cells were exposed to 100% O_2 at 2.5 atmospheres absolute in a hyperbaric chamber. Cell growth was measured by increase in cell number. The amounts of IL-1β, PG-E2, and NO in the conditioned medium were quantified by enzyme-linked immunosorbent assay and HPLC. Cell viability and proteoglycan content were evaluated by histologic study using safranin O staining. *In situ* analysis of apoptosis was performed using TUNEL staining.

Nucleus Pulposus Cell Isolation and Culture

Fresh disc tissue was harvested from the lumbar intervertebral discs of patients undergoing spinal surgery. Nuclei pulposi were separated from the nucleus tissue by performing sequential enzymatic digestion, first with 0.4% pronase (Sigma, St Louis, MO) for 1 hour and subsequently with 0.025% collagenase P (Boehringer Mannheim, Germany) and 0.004% DNase II (Sigma) at 37°C overnight. After digestion, the cells were washed extensively with DMEM/F-12 cell culture medium (Gibco BRL, Grand Island, NY), seeded in 3 fresh flasks at a density of 5000 cells/cm^2, and incubated in a humidified atmosphere of 5% CO_2 and 95% air until the cells attained confluence. Nucleus pulposus cells (NPCs; 4×10^6 cells) were resuspended in 1.2% low-viscosity alginate (Sigma) in 0.15 mol/L sodium chloride. The cell suspension was passed gently through a 22-gauge needle connected to a 5-mL syringe into a 102-mmol/L calcium chloride solution, where each drop was immediately transformed into a semisolid microspheric bead. After 10 minutes of incubation at 37°C to facilitate further polymerization, the newly formed beads were washed 3 times with DMEM/F-12 to remove the excess calcium chloride. About 10 beads (4×10^6 cells) were placed in each well of a 24-well plate (Corning, Corning, NY) and then cultured in 0.4 mL DMEM/F-12 supplemented with 10% FBS. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, with the medium being replaced every 2 days.

Exposure to Intermittent HBO

The cells of the control group were maintained in 5% CO_2/95% Air throughout the experimental protocol. All hyperoxic cells were exposed to 100% O_2 at 2.5 atm in a hyperbaric chamber (Sigma II, Perry Baromedical Corporation, Riviera Beach, FL) for 120 minutes per 48 hours. A series of 3 HBO treatments was used. At 24 hours after each treatment, conditioned media (CM) were collected and centrifuged at 1200*g* for 5 minutes to remove debris, and then stored at –70°C until analysis.

IL-1β Enzyme-Linked Immunosorbent Assay

The levels of IL-1β in the CM after hyperbaric or normobaric treatments were determined using a commercial immunoassay kit (Quantikine Human IL-1β; R&D System). At 24 hours after each treatment, 200 μL of CM was sampled and analyzed according to the manufacturer's instructions. The measurements were performed in duplicate and results were normalized to 10^6 cells.

PG-E2 Enzyme-Linked Immunosorbent Assay

The levels of PG-E2 in the CM after hyperbaric or normobaric treatments were determined using a commercial immunoassay kit (Quantikine Human PG-E2; R&D System). At 24 hours after each treatment, 200 μL of CM was sampled and analyzed according to the manufacturer's instructions. The measurements were performed in duplicate. The results were normalized to 10^6 cells.

Nitric Oxide Assay

Cell production of NO was assessed as nitrite content of the CM. The levels of NO in the CM after hyperbaric or normobaric treatments were determined using the HPLC method. At 24 hours after each treatment, 1 mL of CM was diluted with 3.5 mL of double distilled, deionized water, deproteinated with 0.25 mL of Correz II solution (30% $ZnSO_4.7H_2O$), and 0.25 mL of Correz I solution (15% $K_4Fe(CN)_6{\cdot}3H_2O$) and then centrifuged at 11,000*g* for 5 minutes. Ten microliters of supernatant was injected into the HPLC device for analysis. The HPLC analysis was conducted using a Waters 600 Multisolvent Delivery System (Waters Asia Ltd., Taipei, Taiwan Branch). The guard column was a Hypersil ODS C18 column (150 mm 5 ID 4.6 mm, 3 μm [Alltech, Deerfield, MI]). Separation was achieved using a Lichrospher 100 RP-18 column (125 mm 5 I.D. 4 mm, 5 μm [Merck, Darmstadt, Germany]). The mobile phase was 0.01 M n-octylamine added to 2% acetonitrile. The pH was adjusted to 6 using sulfuric acid. The absorbance was monitored at 205 nm and the flow rate was 1.2 mL/min. The measurements were performed in duplicate. The results were normalized to 10^6 cells.

Cell Growth Assays

Cell growth was measured by increase in cell number after treatment. At intervals of 48, 96, and 144 hours, the alginate beads from each well were solubilized by incubating them at 37°C for 20 minutes in a dissolving buffer (pH 6.8) containing 55 mmol/L sodium citrate, 30 mmol/L disodium EDTA, and 0.15 mol/L sodium chloride. After mild centrifugation, the cell pellet was washed twice with the dissolving buffer, collected by centrifugation, resuspended in PBS, and then the cells were counted using a hemocytometer.

Safranin O Staining for Proteoglycan Detection

At intervals of 48 and 144 hours, the alginate beads were collected and fixed in 4% paraformaldehyde for 4 hours at room temperature and subsequently dehydrated in a graded series of ethanol, washed with xylene, and embedded in paraffin. Samples were cut to a thickness of 5 μm using a microtome (Leica Inc., Deerfield, IL). Four sample sections of each group (control and HBO) were stained with Safranin O with a dye concentration of 0.1% for 6 minutes at pH 5.7. Positive staining was detected

TABLE 1. Effect of Hyperbaric Oxygenation on IL-1β Concentration (pg/mL, n = 4) at Three Time Points

	Control Group	HBO Group	*P*
48 h	1.775 ± 0.32	1.110 ± 0.14	<0.01
96 h	2.283 ± 0.34	1.350 ± 0.23	<0.01
144 h	2.881 ± 0.26	1.540 ± 0.31	<0.05

TABLE 2. Effect of Hyperbaric Oxygenation on PG-E2 Concentration (pg/10^6 Cells, n = 4) at Three Time Points

	Control Group	HBO Group	*P*
48 h	2196 ± 173	1844 ± 112	<0.05
96 h	2383 ± 116	2012 ± 103	<0.05
144 h	2167 ± 172	1803 ± 124	<0.05

during the incubation, indicating the presence of proteoglycan. All staining procedures were performed at room temperature.

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. Five-micrometer sample sections of each group (control and HBO) were floated onto poly-L-lysine-coated slides, deparaffinized in xylene, passed through decreasing gradations of ethanol, treated with proteinase K (20 Lg/mL, Sigma, St. Louis, MO) for 15 minutes, and quenched of endogenous hydrogen peroxidase activity in 3% hydrogen peroxide. After a series of rinses, the sample was labeled with TUNEL reaction mixture for 60 minutes at 37°C. The color was developed with a DAB peroxidase substrate kit (Vector Lab., CA) and 0.1% methyl green was used as a counter stain. Each image was captured by a digital camera (DP 50; Olympus, Shibuya-ku, Tokyo) and the positive stained cells were quantified using Image-Pro Plus 5.0 image analysis software (Media Cybernetics, Silver Spring, MD).

STATISTICAL ANALYSIS

Data are given as mean ± standard error of the noted in the results. *P* value for the Student *t* test was calculated and a *P* value of less than 0.05 defined statistically significant differences.

RESULTS

Effect of HBO on Expression of IL-1β

In the control group, the average concentration of IL-1β detected in the CM at each time point was 1.775 pg/mL (48 hours), 2.283 pg/mL (96 hours), and 2.881 pg/mL (144 hours). In the HBO group, the average concentration of IL-1β detected in the CM after each HBO treatment was 1.110 pg/mL (48 hours), 1.350 pg/mL (96 hours), and 1.540 pg/mL (144 hours [Table 1]). The results showed that the HBO group had a lower level of IL-1β and the difference between the control group and HBO group at each time point was statistically significant. ($P < 0.01$ at 48 and 96 hours, $P < 0.005$ at 144 hours; Figure 1).

Effect of HBO on Expression of PG-E2

In the control group, the level of PG-E2 in the CM detected at each time point was 2196 pg/10^6 cells (48 hours), 2383 pg/10^6 cells (96 hours), and 2167 pg/10^6 cells (144 hours). The level of PG-E2 detected in the CM of the HBO group after each HBO treatment was 1844 pg/10^6 cells (48 hours), 2012 pg/10^6 cells (96 hours), and 1803 pg/10^6 cells (144 hours; Table 2). It

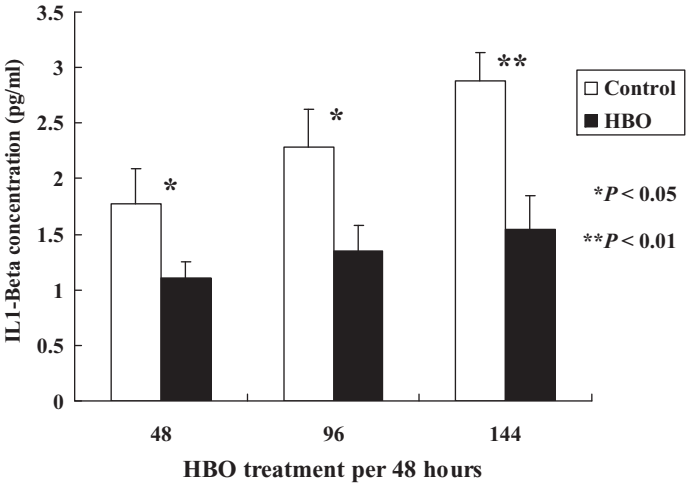

Figure 1. The average IL-1β concentration of the control group and HBO group after each HBO treatment. The HBO group had a lower IL-1β concentration at each time point and the differences between the groups were statistically significant.

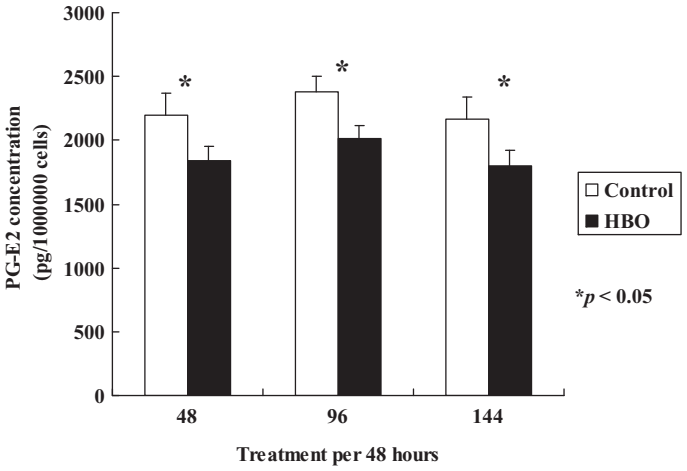

Figure 2. The average PG-E2 concentration of the control group and HBO group after each HBO treatment. The HBO group has a lower PG-E2 concentration at each time point and the differences between the groups were statistically significant.

TABLE 3. Effect of Hyperbaric Oxygenation (HBO) on NO Concentration (μM/10^6 Cells, n = 4) at Three Time Points

	Control Group	HBO Group	*P*
48 h	90.8 ± 13.2	90.4 ± 10.7	>0.05
96 h	121.4 ± 21.4	71.0 ± 11.3	<0.05
144 h	190.3 ± 18.5	30.9 ± 5.3	<0.01

TABLE 4. Effect of Hyperbaric Oxygenation (HBO) on Nucleus Pulposus Cell No. (10^6 Cells/per Well, n = 4) at Three Time Points

	Control Group	HBO Group	*P*
48 h	1.440 ± 0.114	1.680 ± 0.108	>0.05
96 h	1.700 ± 0.145	1.920 ± 0.161	>0.05
144 h	1.820 ± 0.186	2.460 ± 0.152	<0.05

was obvious that the HBO group had a lower level of PG-E2 than the control group and the differences were statistically significant ($P < 0.05$; Figure 2).

Effect of HBO on Expression of NO

In the control group, the concentration of NO detected in the CM at each time point was 90.8 μmol/L/10^6 cells (48 hours), 121.4 μmol/L/10^6 cells (96 hours), and 190.3 μmol/L/10^6 cells (144 hours). In the HBO group, the concentration of NO detected in the CM after each HBO treatment was 90.4 μmol/L/10^6 cells (48 hours), 71 μmol/L/10^6 cells (96 hours), and 30.9 μmol/L/10^6 cells (144 hours; Table 3). There was a trend of decreasing NO concentration after each HBO treatment and it was statistically significant at 96 hours ($P < 0.05$) and 144 hours ($P < 0.01$; Figure 3).

Effect of HBO on Cell Growth and Matrix Synthesis of Nucleus Pulposus

In the control group, the average number of cells per well at different time points was 1.44×10^6 cells/well (48 hours), 1.7×10^6 cells/well (96 hours), and 1.82×10^6 cells/well (144 hours). In the HBO group, the average number of cells per well after each HBO treatment was 1.68×10^6 cells/well (48 hours), 1.92×10^6 cells/well (96 hours), and 2.46×10^6 cells/well (144 hours; Table 4). The results showed that the HBO group had more cells than the control group after each HBO treatment; the difference between control group and HBO group was statistically significant at 144 hours ($P < 0.05$; Figure 4). Safranin O staining was performed for both groups at the time points of 48 and 144 hours; the HBO group also demonstrated a more cellular appearance with abundant matrix formation (Figure 5).

Effect of HBO on Apoptosis of Nucleus Pulposus

In the control group, numerous of TUNEL-labeled cells were observed (Figure 6A) when compared to the HBO group (Figure 6B). The quantitative results for apoptotic NPCs are summarized in Table 5. HBO treatment significantly suppressed the apoptosis of NPCs ($P < 0.01$).

DISCUSSION

Most physicians believe that intervertebral disc degeneration is a source of chronic pain. Being a natural part of the aging process, the pathophysiology of disc degeneration is still being investigated. As the largest avascular tissue in the body, the intervertebral disc has a relatively low oxygen status, but the actual mechanism and the impact of change on oxygen concentration is still unclear.[24] With regard to anatomy, the aging or degenerated disc undergoes structural change with vascular depletion, endplate calcification, and disc size increase that

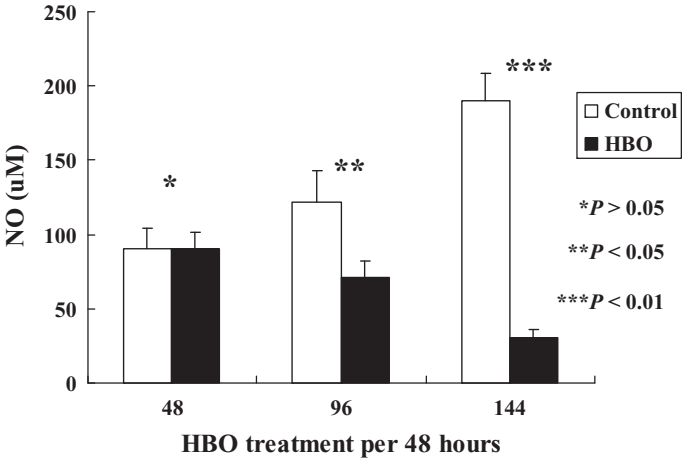

Figure 3. The average NO concentration of the control group and HBO group after each HBO treatment. At the time points of 96 and 144 hours, the HBO group had a significantly lower NO level ($P < 0.05$ and $P < 0.01$, respectively)

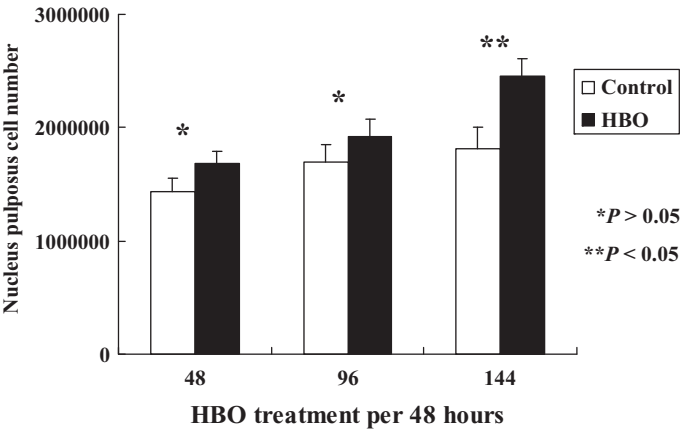

Figure 4. The average nucleus pulposus cell number of the control group and HBO group after each HBO treatment. The HBO group had a trend of lower nucleus pulposus cell number. At the time point of 144 hours, the difference was statistically significant ($P < 0.05$)

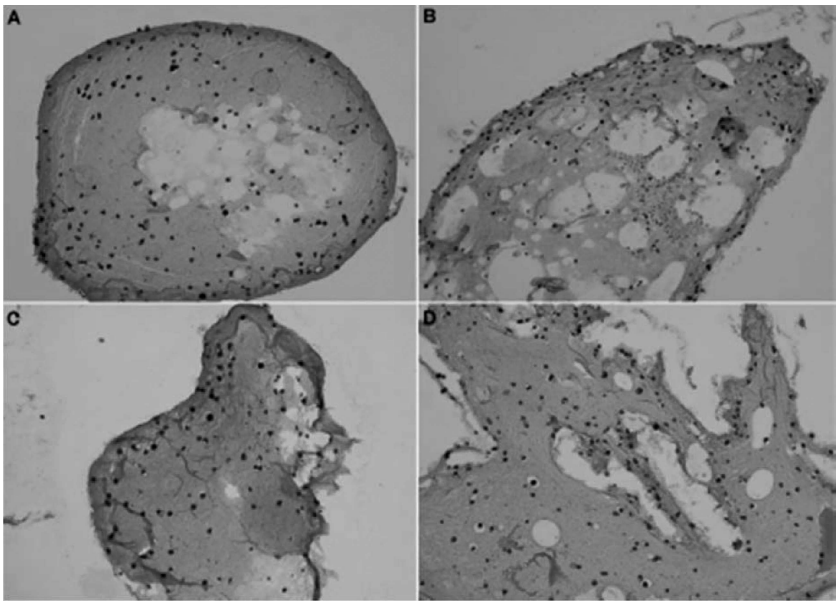

Figure 5. Safranin O staining for both groups at the time points of 48 hours and 144 hours: (**A**) control group at 48 hours; (**B**) HBO group at 48 hours; (**C**) control group at 144 hours; (**D**) HBO group at 144 hours). A more cellular appearance with abundant matrix formation was apparent in the HBO group.

decreases nutrient supply or waste removal.[25] These structural changes also change the microenvironment, especially with regard to the oxygen content.[5,6] Meanwhile, with respect to the biochemical basis, Kang *et al*[8,26] have reported that the cells of the intervertebral disc are biologically responsive and increase their production of matrix metalloproteinases, nitric oxide, IL-6, and PG-E2 when stimulated by IL-1β. Liu *et al*[27] also reported that human herniated lumbar disc cultures spontaneously produce NO which inhibits proteoglycan synthesis in the intervertebral disc. Their study revealed that NO plays an important role in the regulation of disc cell metabolism under mechanical stress and in the pathophysiology of intervertebral disc degeneration. These studies have shown that disc degeneration is related to inhibition of nuclear synthesis and enhanced matrix degradation caused by some mechanical mediators such as IL-1, IL-6, NO, PG-E2, and matrix metalloproteinases.[8–10]

Hyperbaric oxygenation is a useful modality for treating many diseases. By increasing the local oxygen concentration under pressurization, it is well known that HBO can promote neovascularization,[11] enhance wound healing by activating fibroblasts,[22] combat infection by increasing phagocytosis of leukocytes,[28] and inhibit toxin formation.[29] Recently, with regard to the biochemical aspect, several reports have disclosed a relationship between HBO ad proinflammatory cytokines, PG, and NO synthesis. Weisz *et al*[14] reported that HBO treatment in Crohn's disease decreased tumor necrosis factor-α, IL-1, and IL-6 secretion by monocytes. Yin *et al*[30] reported that the neuroprotective effect of HBO might lead to an inhibition of cyclooxygenase-2 overexpression. Kurata *et al*[31] demonstrated that hyperbaric oxygenation reduces the cytostatic activity and transcription of the nitric oxide synthetase gene of mouse peritoneal macrophages. On the basis of the results of these studies, it is reasonable to presume that HBO can halt the process of inflammation or its consequences, although the mechanisms

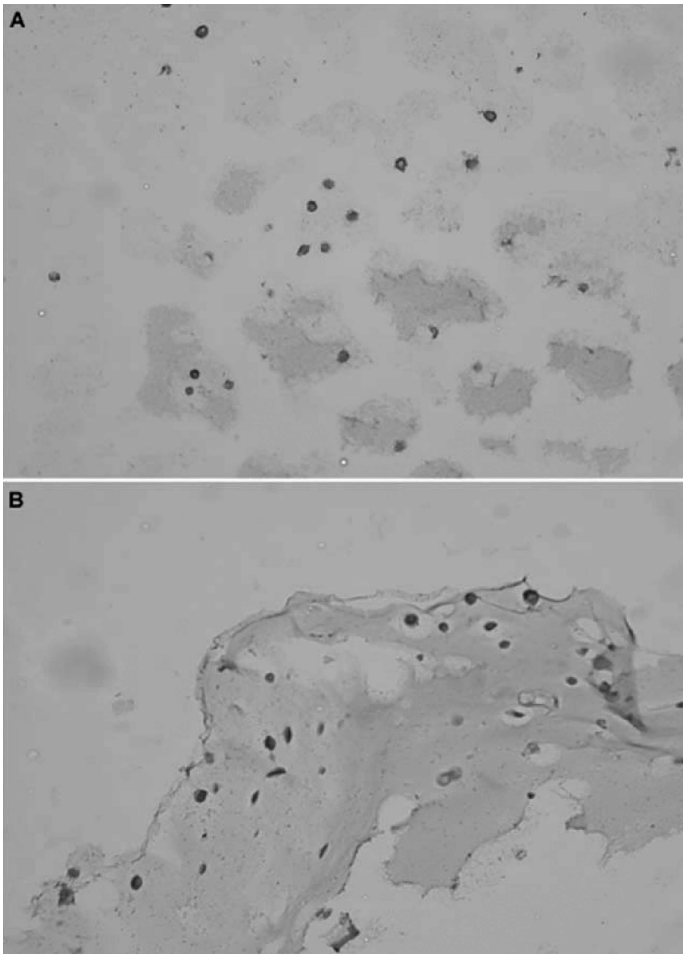

Figure 6. TUNEL staining for both groups at the time point of 144 hours: (**A**) control group, (**B**) HBO group. Reduced expression of TUNEL-labeled cells is seen in the HBO group.

TABLE 5. TUNEL Staining Cell Count (n = 4)*

Apoptotic Cell	Positive	Negative
Control group	17	2
HBO group	2	25

*The Fisher exact test, $P < 0.01$.

underlying the relationships between HBO and these inflammatory cytokines or chemical mediators are not clear.

Possibly, because of the relatively avascular character of the intervertebral disc, reports in the literature regarding HBO and intervertebral disc cells are rare. Only a few reports have focused on measuring oxygen concentration change in the disc cell under normal atmospheric pressure. Holm *et al*[32] demonstrated that the oxygen tension in the nucleus pulposus has a rapid response time as the oxygen tension in the arterial blood is changed by regulating the oxygen concentration in the inspired air. Hence, it is reasonable to presume that HBO can increase the oxygen concentration in the intervertebral disc. From the point of view of embryology, Kim *et al*[33] have reported that the nucleus pulposus originates from the cartilage of the intervertebral endplate and NPCs are similar in character chondrocytes. It is interesting that the nucleus pulposus not only has a similar avascular environment but the NPCs also share the same embryological origin as chondrocytes of cartilage. In a study of chondrocytes in cartilage, Yuan *et al*[17] demonstrated that 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.

On the basis of the earlier description, we hypothesized that HBO may have an influence on disc degeneration. In this *in vitro* study, HBO treatment increased cell growth (Figure 4) and matrix synthesis (Figure 5), but suppressed apoptosis of the NPCs (Figure 6). We also demonstrated that HBO treatment can be anti-inflammatory by suppressing the production of inflammatory cytokines and chemical mediators including IL-1β (Figure 1), PG-E2 (Figure 2), and NO (Figure 3) in cultured NPCs. Although the mechanisms that explain these results are not clear, the benefit of HBO therapy on cultured NPCs may offer a potential therapy for treating disc degeneration or even chronic back pain in 3 possible ways. First, the ability of HBO to decrease NPC apoptosis and promote matrix synthesis can be applied in treatment of mechanical back pain by halting the structural change or aging process. Second, the demonstration that HBO has an anti-inflammatory effect can be applied in treatment of chemical back pain, which is caused by nerve irritation with inflammatory cytokines. Third, as in the development of tissue engineering or gene therapy for treating degenerative disc disease, HBO may offer as an adjuvant therapy or even precondition the stem cell and scaffold before and after implantation.[34,35] However, before being applied clinically, further animal studies are necessary to verify the benefit of HBO on disc degeneration.

➢ Key Points

- ❑ Hyperbaric oxygenation increases cell growth and matrix synthesis but inhibits apoptosis of the human NPC *in vitro.*
- ❑ Hyperbaric oxygenation inhibits IL-1β, PG-E2, and NO production in the human NPC *in vitro.*
- ❑ Further animal studies are necessary to verify the influence of hyperbaric oxygenation on intervertebral disc degeneration.

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