Beneficial Effects of Hyperbaric Oxygen on Human Degenerated Intervertebral Disk Cells Via Suppression of IL-1β and p38 MAPK Signal*

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ABSTRACT: Nucleus pulposus cells (NPCs) from degenerating disks produce catabolic and inflammatory factors, including interleukin (IL)-1, nitric oxide (NO), prostaglandin E2 (PGE-2), and matrix metalloproteinaes (MMPs). An imbalance between MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs) has been proposed to exist in the degenerating disk. This study evaluates the effects of hyperbaric oxygen (HBO) on the human degenerated NPCs. NPCs were maintained in alginate bead culture. All hyperoxic cells were exposed to 100% O_2 at 2.5 atmospheres absolute (ATA) in a hyperbaric chamber. p38 MAPK phosphorylation of the NPCs was detected using the phosphor-kinase array kit. RNA was isolated for real-time quantitative polymerase chain reaction (Q-PCR) analysis of aggrecan and type II collagen gene expression. The amounts of IL-1β, NO, PGE-2, MMP-3, and TIMP-1 in the conditioned media were quantified by enzyme-linked immunosorbent assay (ELISA). Our data showed that HBO treatment decreased expression of IL-1β, increased the gene expression of aggrecan and type II collagen, suppressed the phosphorylation of p38 MAPK, decreased NO, PGE-2, and MMP-3, and increased TIMP-1 expression in NPCs as compared with the atmospheric treatment. These results support the hypothesis that IL-1β and the p38 MAPK signal may be responsible for many of the inflammatory and catabolic changes seen in the human disk degeneration, and support our proposal that HBO treatment-induced increase of the anabolic factor (TIMP-1)/catabolic factor (MMP-3) ratio may provide a therapeutic approach to slow the course of intervertebral disk degeneration. J Orthop Res 29: 14–19, 2011

Keywords: disk degeneration; hyperbaric oxygen; p38 MAPK; MMP-3; TIMP-1

The process of intervertebral disk degeneration (IDD) is believed to have a biochemical basis, with inhibition of nuclear proteoglycan synthesis and enhanced matrix degradation caused by chemical mediators that may include interleukin (IL)-1, nitric oxide (NO), prostaglandin E2 (PGE-2), and matrix metalloproteinases (MMPs).[1,2] Altered biomechanical forces in the human cervical spine can up-regulate IL-1 and TNF-α.[3] LeMaitre et al.[4] showed increasing IL-1 in human lumbar disk cells with increasing grades of degeneration. A common characteristic of IDD is the imbalanced synthesis and catabolism of extracellular matrix (ECM) proteins. MMPs are increased in degenerated disks,[5] and elevated MMPs are correlated with progressive disk degeneration in humans[6] and in animal models of IDD.[7] Loss of the anti-catabolic tissue inhibitor of metalloproteinase-1 (TIMP-1), as reported in disks of the annular stab rabbit model of degeneration[8] could contribute to the anabolic/catabolic imbalance.

p38 mitogen-activated protein kinase (p38 MAPK) is involved in both the induction and actions of inflammatory cytokines, and this kinase is implicated in MMP production, NO synthesis, and PGE-2 synthesis.[9] Blocking the p38 MAPK signal decreases IL-1 and TNF-α induction of MMP-3, IL-6, and PGE-2 in NPC cells[10,11] and articular chondrocytes.[12] These data are consistent with the hypothesis that blockade of p38 MAPK could help restore the normal homoeostatic mechanism in cytokine-activated disk cell.

Close associations have been reported among hyperbaric oxygen (HBO), p38 MAPK, IL-1β, NO, and MMPs. HBO treatment increases the tissue/microvascular pO_2,[13] decreases the IL-1β secretion of monocytes,[14] blocks postischemic plasma MMP-9 up-regulation in rats,[15,16] increases TIMP-1 expression in vitro[17] and in vivo,[18] and induces neuroprotection against forebrain ischemia via suppression of p38 MAPK.[19] We previously reported that HBO suppressed the iNOs expression and apoptosis of chondrocytes in rabbit cartilage defects.[20] Because p38 MAPK is implicated in NO synthesis[9] and hypoxia activates MAPK activity in rat nucleus pulposus cells (NPCs)[21]. The specific point of this study was to investigate the interactions among HBO, p38 MAPK, IL-1β, NO, and MMPs in human degenerated NPCs. We proposed that suppression of p38 MAPK expression in the degenerated NPCs by HBO treatment may provide a novel therapeutic strategy for IDD.

The present study examined the effects of HBO on the gene expression of ECM components (aggrecan and type II collagen), activation of p38 MAPK, protein expression of catabolic and inflammatory factors (IL-1β, PGE-2,

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NO, and MMP-3), and an anti-catabolic factor (TIMP-1) in the cell culture of a human degenerated NPCs.

MATERIALS AND METHODS

The experimental protocol was approved by the human subjects Institutional Review Board at the Chang Gung Memorial Hospital.

Nucleus Pulposus Cell Isolation and Culture

Fresh disk tissue was harvested from the degenerated lumbar intervertebral disk (IVD) of patients undergoing spinal surgery. NPCs were separated from the nucleus tissue by performing sequential enzymatic digestion, first with 0.4% pronase (Sigma, St. Louis, MO) for 1 h 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 and were seeded in three fresh flasks at a density of 5,000 cells/cm^2 and incubated in a humidified atmosphere of 5% CO_2 and 95% air until the cells attained confluence. 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 min of incubation at 37°C to facilitate further polymerization, the newly formed beads were washed three 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, NY) and cultured them 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.

Exposure to Intermittent HBO

Control cells were maintained in 5% CO_2/95% air at 1 atmosphere (atm) through 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 min per 48 h. A series of three times of HBO treatment was used. At 24 h after each treatment, conditioned media (CM) were collected and centrifuged at 1,200*g* for 5 min to remove debris, and then stored at −70°C until analysis.

IL-1β ELISA 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, Minneapolis, MN). At 24 h 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 microgram DNA.

RNA Extraction and Real-Time Q-PCR Analysis

At 24 h after each treatment, the alginate beads from each well were solubilized by incubation at 4°C for 20 min 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 two times with the dissolving buffer, collected by centrifugation, resuspended in PBS, and collected by centrifugation. Cellular RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA) and reverse-transcribed into cDNA with the ImProm-II reverse transcription system (Promega Corporation, Madison, WI) according to the manufacturer's instructions. For real-time Q-PCR detection of aggrecan and type II collagen RNA transcripts, cDNA was analyzed on an ABI PRISM 7900 sequence detection system using the TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA). The cycle threshold (C_t) values were obtained, and data normalized to GAPDH expression using the $\Delta\Delta C_t$ method to calculate relative mRNA levels of each target gene.

p38 MAPK Phosphorylation Assay

p38 MAPK phosphorylation was measured after hyperbaric or normobaric treatments. At 24 h after three times of HBO treatment, the alginate beads from each well were solubilized as described above. After mild centrifugation, the cell pellet was washed two times with dissolving buffer, collected by centrifugation, and resuspended in PBS. After mild centrifugation, the cell pellet was resuspended in lysis buffer. The cell lysates were stored at −80°C until assayed for phospho-p38 using the protocol and reagents from Human phosphor-kinase array kit (R&D Systems). Lysate protein concentration was determined using the Bradford method (Bio-Rad Protein Reagent, Hercules, CA). Membranes were washed and rinsed with ECL detection reagents (Amersham Pharmacia Biotech, Little Chalfont, UK). Dot images were photographed using ECL Hyperfilm (Amersham). Stain intensity was quantified with an image-analysis system (Image-Pro plus 5.0).

PGE-2 ELISA Assay

The levels of PGE-2 in the CM after hyperbaric or normobaric treatments were determined using a commercial immunoassay kit (Quantikine Human PGE-2; R&D System). At 24 h 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 microgram DNA.

Nitric Oxide Assay

Cell production of NO was assessed as nitrite content of CM. The levels of NO in the CM after hyperbaric or normobaric treatments were determined using the Griess reaction. At 24 h after each treatment, 200 μl of CM was sampled for analysis. The measurements were performed in duplicate. The results were normalized to microgram DNA. DNA content was determined using a DNAzol reagent (DNAzol; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

MMP3 and TIMP1 ELISA Assay

The levels of MMP3 and TIMP1 in CM after hyperbaric or normobaric treatments were determined using a commercial immunoassay kit (Quantikine Human MMP3 and Quantikine Human TIMP1; R&D System). At 24 h 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 microgram DNA.

Statistical Analysis

Data are given as mean ± standard error of the n noted in the results. p-value for the Student's t-test was calculated and a p-value of <0.05 defined statistically significant differences.

RESULTS

Effect of HBO on IL-1β Secretion

Figure 1 presents the effect of HBO on IL-1β secretion following analysis by ELISA (data are average ± SD, HBO group vs. control group: 0.16 ± 0.02 vs. 0.25 ± 0.04 pg/ml/μg DNA, $p < 0.05$; 0.19 ± 0.03 vs.

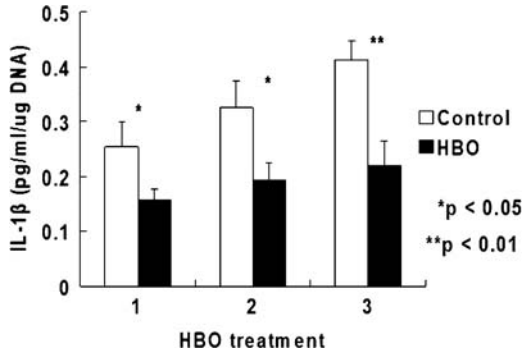

Figure 1. Effect of HBO on IL-1β secretion. HBO treatment significantly inhibited secretion of IL-1β by NPC.

0.32 ± 0.04 pg/ml/μg DNA, $p < 0.05$; 0.22 ± 0.04 vs. 0.42 ± 0.03 pg/ml/μg DNA, $p < 0.01$; $n = 4$). HBO treatment significantly inhibited secretion of IL-1β by NPC.

Effect of HBO on Gene Expression of Aggrecan and Type II Collagen

Figure 2 presents the effect of HBO treatment on transcription of aggrecan (data are average ± SD, HBO group vs. control group: 2.41 ± 0.99-fold, $p < 0.05$; 2.90 ± 1.04-fold, $p < 0.01$; 1.89 ± 0.85-fold, $p < 0.05$; $n = 6$) and type II collagen (data are average ± SD, HBO group vs. control group: 2.09 ± 0.8-fold, $p < 0.05$; 7.23 ± 3.44-fold, $p < 0.01$; 3.32 ± 1.65-fold, $p < 0.05$; $n = 6$) in NPC. HBO treatment significantly increased gene expression of aggrecan and type II collagen as compared with the control cells.

Effect of HBO on p38 MAPK Phosphorylation

The p-p38 MAPK/Internal control dot density ratio in the control group was $69.2 \pm 3.2\%$. Fifteen minutes after the third HBO treatment, the p-p38 MAPK/Internal control dot density ratio was $54.9 \pm 2.4\%$ (vs. control, $p < 0.01$, $n = 4$), at 30 min, the ratio was $56.4 \pm 0.9\%$ (vs. control, $p < 0.01$, $n = 4$), at 60 min, the ratio was $52.0 \pm 2.0\%$ (vs. control, $p < 0.01$, $n = 4$). p38 MAPK phosphorylation of NPC was significantly suppressed at

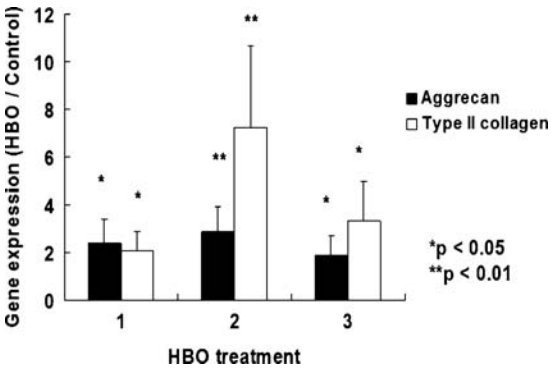

Figure 2. Effect of HBO on gene expression of aggrecan and type II collagen. HBO treatment significantly increased gene expression of aggrecan and type II collagen as compared with the control cells.

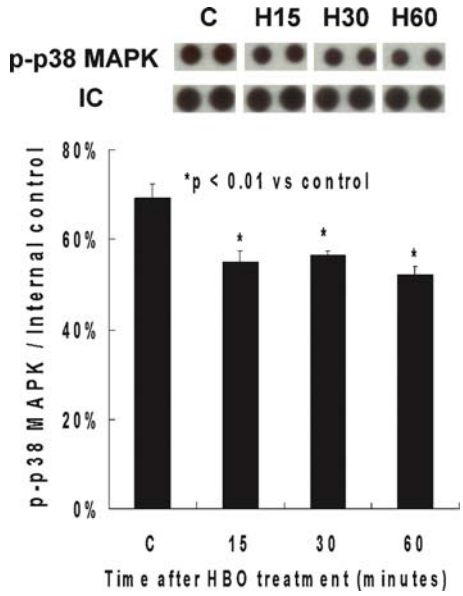

Figure 3. Effect of HBO on p38 MAPK phosphorylation. p38 MAPK phosphorylation in NPC was significantly suppressed at 15 ($p < 0.01$), 30 ($p < 0.01$), and 60 ($p < 0.01$) min after three times of HBO treatment.

15, 30, and 60 min after three times of HBO treatment (Fig. 3).

Effect of HBO on PGE-2 Secretion

Figure 4 presents the effect of HBO on PGE-2 secretion following analysis by ELISA (data are average ± SD, HBO group vs. control group: 263.4 ± 16.0 vs. 317.7 ± 24.7 pg/ml/μg DNA, $p < 0.05$; 258.8 ± 14.7 vs. 340.4 ± 16.5 pg/ml/μg DNA, $p < 0.05$; 257.5 ± 17.7 vs. 309.6 ± 24.5 pg/ml/μg DNA, $p < 0.05$; $n = 4$). HBO treatment significantly inhibited secretion of PGE-2 by NPC at each time of treatment.

Effect of HBO on NO Accumulation

Figure 5 presents the effect of HBO on nitrite accumulation after HBO treatment (data are average ± SD, HBO group vs. control group: 12.9 ± 1.53 vs. 12.9 ± 1.89 μM/μg DNA, $p > 0.05$; 10.1 ± 1.61

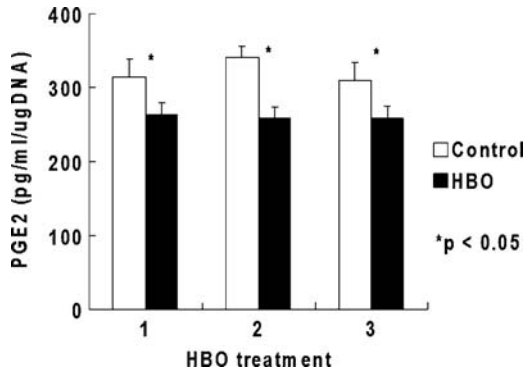

Figure 4. Effect of HBO on PGE-2 secretion. HBO treatment significantly inhibited secretion of PGE-2 by NPC at each time of treatment.

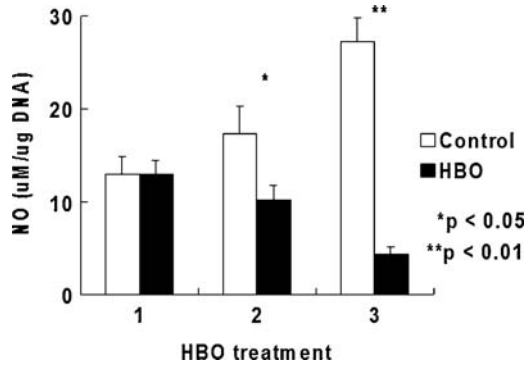

Figure 5. Effect of HBO on NO accumulation. HBO treatment significantly inhibited nitrite accumulation by NPC after two ($p < 0.05$) and three ($p < 0.01$) times of treatment.

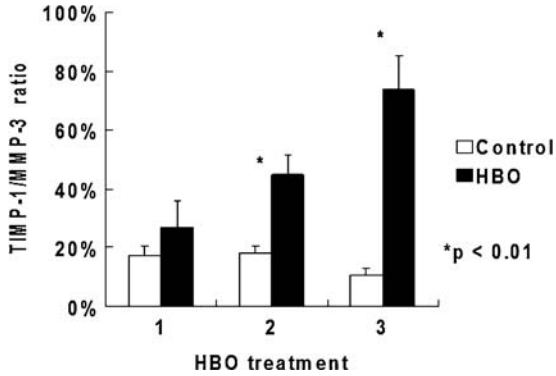

Figure 6. Effect of HBO on the TIMP-1/MMP-3 ratio. HBO treatment significantly increased the ratio of TIMP-1 to MMP-3 after two ($p < 0.01$) and three ($p < 0.01$) times of treatment.

Table 1. Effect of HBO Treatment on MMP-3 Secretion (pg/mL/μg DNA, $n = 4$)

	Control Group	HBO Group	p-Value
1× treatment	164.1 ± 32.3	143.7 ± 20.7	>0.05
2× treatments	237.7 ± 29.7	215.1 ± 33.8	>0.05
3× treatments	340.3 ± 84.1	191.5 ± 35.4	<0.05

Table 2. Effect of HBO Treatment on TIMP-1 Secretion (pg/mL/μg DNA, $n = 4$)

	Control Group	HBO Group	p-Value
1× treatment	27.6 ± 4.1	29.8 ± 8.2	>0.05
2× treatments	43.0 ± 3.0	95.4 ± 9.7	<0.05
3× treatments	35.6 ± 2.6	138.5 ± 7.8	<0.01

vs. 17.3 ± 3.05 μM/μg DNA, $p < 0.05$; 4.4 ± 0.75 vs. 27.2 ± 2.64 μM/μg DNA, $p < 0.01$; $n = 4$). HBO treatment significantly inhibited nitrite accumulation by NPC after two and three times of treatment.

Effect of HBO on MMP-3 and TIMP-1 Secretion

Table 1 presents the effect of HBO on MMP-3 and TIMP-1 secretion following analysis by ELISA. HBO treatment significantly inhibited secretion of MMP-3 by NPC after three times of treatment. Table 2 presents the effect of HBO on TIMP-1 secretion following analysis by ELISA. HBO treatment significantly increased secretion of TIMP-1 by NPC after two and three treatments. Figure 6 presents the ratio of TIMP-1 to MMP-3 after HBO treatment (data are average ± SD, HBO group vs. control group: $27.1 \pm 9.0\%$ vs. $17.1 \pm 3.5\%$, $p > 0.05$; $44.9 \pm 6.9\%$ vs. $18.2 \pm 2.1\%$, $p < 0.01$; $73.6 \pm 11.6\%$ vs. $10.8 \pm 2.3\%$, $p < 0.01$; $n = 4$). HBO treatment significantly increased the ratio of TIMP-1 to MMP-3 after two and three times of treatment.

DISCUSSION

NPCs from degenerating disks produce catabolic and inflammatory factor, including IL-1β, NO, PGE-2, and MMPs.[1,2] IL-1 inhibits the expression of types II collagen and for aggrecan in human degenerated IVD cells.[4] IL-1 treatment also causes a decrease in proteoglycan and collagen II production in animal cells.[22,23] Our data suggest that HBO treatment significantly inhibits secretion of IL-1β by NPCs (Fig. 1). In addition, mRNA expression of aggrecan and types II collagen in NPCs was up-regulated after HBO treatment (Fig. 2) which in consistent with similar observations in fibroblasts.[24,25] Inhibiting IL-1β secretion by HBO treatment may reverse disk degeneration.

Activation of the p38 pathway (phosphorylation of p38) has been characterized as pathological changes in inflammatory or apoptotic processes.[26] Recent studies have suggested that inhibition of p38 MAPK in IL-1-activated disk cells blunts production of factors associated with inflammation, pain, and disk matrix catabolism.[10,11] Suppression of p38 phosphorylation plays a key role in HBO-induced neuroprotection and that pretreatment with a p38 MAPK inhibitor could provide similar neuroprotection.[19] The present study demonstrated that p38 MAPK phosphorylation of NPC was significantly suppressed at 15, 30, and 60 min after HBO treatment (Fig. 3). Inhibition of p38 MAPK significantly increases the message for aggrecan and type II collagen transcription in NPCs (Fig. 2). The p38 MAPK inhibition-mediated increase in aggrecan and type II collagen messages may translate into changes in matrix composition, which in turn may be beneficial to disk function.

IDD is caused by IL-1, NO, PGE-2, and MMPs.[1,2] HBO treatment suppressed IL-1,[14] NO,[20] COX-2,[27] and PGE-2[27,28] expression which resulted in anti-inflammatory effects. We have demonstrated that HBO treatment significantly inhibits secretion of inflammatory factors such as IL-1β (Fig. 1), PGE-2 (Fig. 4), and NO (Fig. 5) by NPC. The regulation of PGE-2 is complex and can be modulated by arachidonic acid availability, cell enzyme content (COX-2 and PGE-2 synthases), and the activity of these enzymes.[29] HBO treatment inhibits PGE-2 production which raises the possibility of blunting the production of a factor frequently associated with painful disk degeneration. NO has been suggested to

be related to the pathophysiology of radicular pain.[30] The NO production was reduced after HBO treatment (Fig. 5). It is speculated that HBO treatment may lead to moderate radicular pain in a herniated disk.

IDD is caused by a disturbance in the equilibrium of MMP-3 and TIMP-1, and that MMP-3 contributes to degeneration of the cartilaginous endplate.[6] MMP-3 production is enhanced by IL-1 in cultured IVDs.[31] The phosphorylated p38 in NPCs under low-oxygen tension (2% O_2) was higher than cells in normoxia (20% O_2).[21] In addition, the combination of hypoxia and IL-1β induces MMP-3 but inhibits TIMP-3 mRNA expression in temporomandibular joint (TMJ) disk cells.[32] The present study demonstrated phosphorylated p38 in NPCs was down-regulated after HBO treatment (Fig. 3). HBO treatment increases the oxygen tension in cell culture medium and suppressed IL-1β secretion, which may cause a significant decrease of MMP-3 (Table 1) and an increase of TIMP-1 (Table 2) expression in NPCs. The increase of anabolic (TIMP-1)/catabolic (MMP-3) ratio contributes to the restoration of normal homoeostasis (Fig. 6). The ratio of TIMP-1 to MMP-3 is increased by p38 MAPK inhibition after HBO treatment, suggesting it might provide similar protection against the catabolic effects normally seen in cells exposed to p 38 MAPK inhibitors.[10]

Long-termed and repeated HBO treatment may increase oxidative stress. Because exposure to hyperoxia in clinical HBO protocols is rather brief (typically <2 h/day), studies show that antioxidant defenses are sufficient to reverse the biochemical stresses of ROS exposure.[33] This study demonstrated that HBO treatment of NPCs increases ECM gene expression, suppressed p38 MAPK phosphorylation, decreases protein expression of catabolic and inflammatory factors, and increases anti-catabolic factor in NPCs. From the results of this study, HBO therapy may be offer a potential method for clinical IVD repair in two ways and need the animal model be tested to verify this: (1) HBO therapy may suppress the expression of inflammatory mediators and increase the expression of Type II collagen and aggrecan in the early stage of degenerated IVD or moderate radicular pain in a herniated disk in vivo. HBO therapy may delay or reverse the IVD degeneration for the patients who are not suite for surgery. (2) For an ideal method for disk cell transplantation, we remove the degenerated cells first and then we use HBO to treat the inflammatory disk space. After suppress the expression of inflammatory cytokines, we implant the healthy disk cells, stem cells, or HBO-treated cells into the healthy and native disk space (nearly anaerobic and without inflammatory mediators). After cell transplantation, we may use HBO to improve the outcome if it is necessary.

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