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Hyperbaric oxygen inhibits the HMGB1/RAGE signaling pathway by upregulating Mir-107 expression in human osteoarthritic chondrocytes

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SUMMARY

Objective: MicroRNA (miRNA)107 expression is downregulated but high mobility group box 1 (HMGB-1), Toll-like receptors (TLRs), and receptor for advanced glycation end products (RAGE) are upregulated in osteoarthritic (OA) cartilage. We investigated mir-107/HMGB-1 signaling in OA after hyperbaric oxygen (HBO) treatment.

Design: MiR-107 mimic was transfected and the HMGB-1 was analyzed in OA chondrocytes. MiRNA targets were identified using bioinformatics and a luciferase reporter assay. After HBO treatment, the mRNA or protein levels of HMGB-1, RAGE, TLR2, TLR4, and inducible nitric oxide (NO) synthase (iNOS) and phosphorylation of mitogen-activated protein kinase (MAPK) were evaluated. The secreted HMGB-1 and matrix metalloproteases (MMPs) levels were quantified. Finally, we detected the HMGB-1 and iNOS expression in rabbit cartilage defects.

Results: Overexpression of miR-107 suppressed HMGB-1 expression in OA chondrocytes. The 3′UTR of HMGB-1 mRNA contained a 'seed-matched-sequence' for miR-107. MiR-107 was induced by HBO and a marked suppression of HMGB-1 was observed simultaneously in OA chondrocytes. Knockdown of miR-107 upregulated HMGB-1 expression in hyperoxic cells. HBO downregulated the mRNA and protein expression of HMGB-1, RAGE, TLR2, TLR4, and iNOS, and the secretion of HMGB-1. HBO decreased the nuclear translocation of nuclear factor (NF)-κB, downregulated the phosphorylation of MAPK, and significantly decreased the secretion of MMPs. Morphological and immunohistochemical observation demonstrated that HBO markedly enhanced cartilage repair and the area stained positive for HMGB-1 and iNOS tended to be lower in the HBO group.

Conclusions: HBO inhibits HMGB-1/RAGE signaling related pathways by upregulating miR-107 expression in human OA chondrocytes.

Introduction

Osteoarthritis (OA) is a widely prevalent degenerative joint disease, which is characterized by articular cartilage degradation and joint inflammation[1]. Chondrocytes are the cells in the articular cartilage and play an important role in the homeostasis of cartilage metabolism: There is no cartilage metabolism without chondrocytes. Decreased cell viability and number of chondrocytes, as well as inflammation, are associated with the onset and progression of OA[1,2]. MicroRNAs (miRNAs) have emerged as an important target for many diseases, including OA[3]. MiRNAs are endogenous

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non-coding small RNAs containing 20–25 nucleotides, which can regulate gene expression by pairing with the 3′-untranslated region (3′-UTR) of their target messenger RNAs (mRNAs). Therefore, miRNAs regulate diverse cellular processes, including cell proliferation, cell apoptosis, and cell differentiation[4]. Various miRNAs are dysregulated and therefore functionally implicated in the pathogenesis of OA[5–7]. MiR-27b was shown to regulate the expression of MMP-13 in human OA chondrocytes[8]. Overexpression of miR-9 was reported to promote interleukin (IL)-6 expression upon IL-1β-stimulation in human chondrocytes[9]. MiR-107 was reported to be downregulated in OA cartilage[10,11], however, the function of miR-107 in OA chondrocytes is not clear.

High mobility group box 1 (HMGB-1) is a ubiquitous non-histone DNA-binding protein, which is an important modulator of inflammation[12]. The dysregulation of HMGB-1 contributes to many inflammatory diseases[12]. The expression of HMGB-1, receptor for advanced glycation end products (RAGE), Toll-like receptor (TLR) 2, and TLR four are up-regulated within OA cartilage[1,13,14]. Chondrocyte derived HMGB-1 is a potential ligand for RAGE[1], TLR 2, and TLR 4[15]. Extracellular HMGB-1 interacts with RAGE, TLR 2, and TLR four present on the membranes of nearby cells, to activate NF-κB or mitogen-activated protein kinase (MAPK), which are key factors in the inflammatory response[1,15,16]. In humans, genes expression is altered by three major groups of distinctly regulated MAPK cascades; extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK. Phosphorylation of ERK, JNK, and p38 MAPK induces in the expression of genes encoding inflammatory response factors such as nitric oxide (NO)[17,18]. Previous studies have identified certain matrix metalloproteases (MMPs), such as MMP-1, MMP-3, MMP-9, and MMP-13 in human OA cartilage[19,20]. The production of MMPs was significantly increased after chondrocytes were treated with the RAGE ligands such as HMGB-1[1] or S100A4[2]. HMGB-1 binding with RAGE could contribute to cartilage degradation through the upregulation of MMP-13 expression[1].

Although the articular cartilage is an avascular tissue, which functions under low oxygen tension, a further decrease in the oxygen tension of synovial fluid and environmental changes may result in traumatic injury or degenerative diseases of the articular cartilage[21]. Hypoxia has been suggested to play a central role in the induction of tissue damage in OA[4]. Hypoxia is a potent inducer of extracellular HMGB-1, which may in turn play an important role in the development of arthritis[22]. MiR-107 expression was increased or unchanged in the presence of hypoxia[23] and increased in the hyperoxic conditions[24] in diverse cell types. Hyperbaric oxygen (HBO) treatment increases the tissue/microvascular O_2 levels to improve the hypoxia[9]. MiR-107 expression was downregulated in OA cartilage[10,11]. To analyze the biological impact of this down-regulation, we overexpressed the MiR-107 and found the reduced HMGB-1 expression in OA chondrocytes. HBO increased mir-107 expression in OA chondrocytes. Bioinformatics were used to predict putative target sequences for miR-107 in human HMGB-1 mRNA and this was confirmed by a luciferase reporter assay. The effects of HBO treatment on the miR-107/HMGB-1 signaling-mediated catabolic pathway were also investigated in human OA chondrocytes. Finally, we examined the effects of HBO on HMGB-1 and inducible nitric oxide synthase (iNOS) expression in rabbit cartilage defects.

Method

The experimental protocols used in the present study were approved by the Human Subjects Institutional Review Board at the Chang Gung Memorial Hospital. All animals were cared for in accordance with regulations of the National Institutes of Health of the Republic of China, under the supervision of a licensed veterinarian.

Surgical procedures and cells isolation and cultivation

Articular cartilage specimens (tibial plateaus and femoral condyles) were obtained from 28 Ahlbäck grade IV or Kellgren and Lawrence grade IV OA patients who receive TKA surgery. OA chondrocytes were released from the OA cartilage by digestion with 1 mg/ml collagenase (Sigma–Aldrich, MI, USA) in Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 2% fetal bovine serum (FBS) and incubated at 37°C. The isolated cells were seeded in T-75 flasks (Falcon, Amsterdam, The Netherlands) containing 15 ml of medium supplemented with 10% FBS. The cultures were incubated in a humidified atmosphere of 5% CO2 and 95% air. Cells were used at passage one for each experiment.

Effect of miR-107 overexpression on HMGB-1 expression

OA chondrocytes were transfected with miR-107 mimics and negative control (NC) by Lipofectamine 3,000 according to manufacturer's protocols and the HMGB-1 mRNA expression was analyzed (see Appendix).

Exposure to intermittent HBO intervention

Control cells were maintained in 5% CO_2/95% air at 1 atm (atm) throughout the experimental period. The hyperoxic cells were exposed to 100% O_2 for 25 min and then to 5% CO_2/95% air for 5 min at 2.5 atm absolute (ATA) in a hyperbaric chamber (Perry Baromedical Corporation, FL) for a total treatment time of 90 min every 48 h. HBO treatment was administered three times. At 24 h after each treatment, the conditioned media (CM) were collected by contributive at 1000 g for 10 min, filter the medium through 0.22 μM filter, and stored at −70°C prior to further analysis.

MiRNA target prediction and dual-luciferase reporter assay

The Target Scan 7.1 and miRnalyze online software were used to analyze the putative target genes of miR-107. The 3′-UTR of HMGB-1 (50–56 bases) containing the potential miR-107 binding site was cloned into pmir GLO dual-luciferase miRNA reporter vectors (Promega, WI, USA). A mutated 3′-UTR of HMGB-1 was created using the New England Biolabs Q5 Site-Directed Mutagenesis Kit (#E0554S) and introduced into the potential miR-107 binding site. A luciferase reporter vector clone containing the wild type (WT) or mutant (Mut) HMGB-1 3′-UTR were was obtained commercially from Gene Labs (Taipei, Taiwan). The reporter vectors containing the WT or mutant (Mut) HMGB-1 3′-UTR were transfected into OA chondrocytes using Lipofectamine 2000 (Invitrogen) (see Appendix). After incubation with or without HBO the transfected cells were lysed. Firefly and renilla luciferase activities were detected using the dual-luciferase assay system (Promega, WI, USA).

Transfection of OA chondrocytes with miRNA inhibitor and analysis after HBO treatment

OA chondrocytes were transfected with miR-107 inhibitor by Lipofectamine RNAiMAX (Invitrogen) and the HMGB-1 expression was analyzed after HBO treatment. Cellular RNA was isolated using an RNeasy mini kit (Qiagen, CA, USA) and reverse-transcribed into cDNA using the ImProm-II reverse transcription system (Promega). For real-time qPCR detection, the cDNA was analyzed on an ABI PRISM 7,900 sequence detection system using the TaqMan PCR

Master Mix (Thermo Fisher). Cellular protein was extracted using M-PER mammalian protein extraction reagent (Thermo Fisher) (see Appendix).

RNA extraction and real-time qPCR detection of HMGB-1, TLR2, TLR4, RAGE, and iNOS

Cells were plated at a density of 3×10^5 cells per 100-mm culture dish in 10 ml of DMEM/F-12 containing 5% FBS. At 12 h after the third HBO treatment, RNA extraction was performed as described above, for real-time qPCR detection of HMGB-1 (Assay ID: Hs01923466), RAGE (Assay ID: Hs00542584), TLR2 (Assay ID: Hs00610101), TLR4 (Assay ID: Hs00152939), and iNOS (Assay ID: Hs00167257) RNA transcripts.

Protein extraction and Western blot analysis of HMGB-1, TLR2, TLR4, RAGE, and iNOS

At 24 h after the third HBO treatment, protein extraction and Western blot analysis of HMGB-1, TLR2, TLR4, RAGE, and iNOS were performed (see Appendix).

HMGB-1 ELISA assay

The post-treatment levels of HMGB-1 in the CM were determined using an ELISA assay with a Human HMGB-1 ELISA kit (LifeSpan Biosciences, WA, USA). At 24 h after each treatment, 200 μl of CM was sampled and analyzed to detect the HMGB-1 levels.

MAPK phosphorylation assay

At 30 and 60 min after the third HBO treatments, the phosphorylation levels of ERK, JNK, and p38 MAPK were measured using the Human phosphor-kinase array kit according to the manufacturer's protocol (R&D Systems, MN, USA). Dot images were obtained using an ECL Hyper film and the staining intensity was quantified using the image analysis system.

Preparation of cytosolic and nuclear fractions for IκBα and NF-κB p65 detection

At 60 min after the third HBO treatment, the cells were rinsed with PBS, treated with 0.05% trypsin, and then collected by centrifugation. NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher) were used to isolate cytoplasmic and nuclear extracts from the cells and the protein was separated by 10% SDS-PAGE. After blocking with 10% nonfat milk, the membranes were incubated overnight at 4°C with 1:1,000 diluted mouse antibodies to detect IκBα (Cell Signaling Technology, MA, USA) and β-actin (Abcam) in the cytoplasmic extracts and NF-κB p65 (Cell Signaling Technology), TATA binding protein (TBP; Abcam) in the nuclear extracts. The membranes were then washed and rinsed with ECL detection reagents and the band images were photographed using the Hyperfilm. The staining intensity of IκBα, β-actin, NF-κB p65, and TATA was quantified using the image analysis system.

MMP-9 and MMP-13 ELISA assay

The levels of MMP-9 and MMP-13 in the CM after treatments were determined using Quantikine® Human MMP-9 and MMP-13 ELISA kits, respectively (R&D Systems). At 24 h after each treatment, 200 μl of CM was sampled and analyzed to detect the levels of MMPs. The results were normalized to 10^6 cells.

Effect of HBO on rabbit cartilage defects

A rabbit cartilage defect model was created and the effect of HBO on HMGB-1 and iNOS expression in repaired cartilage was performed by immunohistochemically analysis (see Appendix). The staining intensity ratios of HMGB-1 and iNOS were quantified by using an image-analysis system (see Appendix).

Statistical analysis

Articular cartilage specimens were obtained from 28 OA patients who receive TKA surgery. Each human knee yield one sample. Three or four sample were used in each experimental item (n = three or 4). The control and HBO samples separated from the same (Control group: without HBO treatment) so we used paired t-test to analyze the control/HBO ratio in this study. Data were represented as mean ± 95% confidence interval (CI). The P-values for the paired Student's t-test or repeated measures ANOVA were performed using the SPSS software package (Version 12.0, Chicago, IL). A P-value of <0.05 was considered statistically significant.

Results

MiR-107 overexpression results in decreased expression of HMGB-1

Transient transfection assays were used to directly confirm the effect of overexpression of miR-107 on HMGB-1 expression in OA chondrocytes (see Appendix and Supplementary Figs. S1(a) and S1(b)).

HBO treatment increases miR-107 expression in OA chondrocytes

MiR-107 was downregulated in OA cartilage. HBO treatment significantly increased miR-107 expression in OA chondrocytes (HBO/control ratio = 1.71 ± 0.201 fold, $^{**}P = 0.002$, $n = 4$). These results indicated that miR-107 might play an important role in the inhibition of OA progression after HBO treatment.

HMGB-1 is a direct target of miR-107

To investigate the potential molecular mechanism of miR-107, we screened the putative target genes of miR-107 were screened using Target Scan 7.1 and miRnalyze online software. It was found that HMGB-1, an important regulator of inflammation, was a direct target of miR-107. The 3′ UTR of HMGB-1 contains a potential binding element for miR-107 with a 7-nucleotide match to the miR-107 seed region [Fig. 1(a) and (b)]. Cross-species conservation of the miR-107 seed sequence in the 3′UTR of HMGB-1 mRNA was identified by the Target Scan algorithm [Fig. 1(c)]. These findings suggest that the hsa-miR-107 may target the HMGB-1 mRNA by directly recognizing its seed-matched sequence present in the 3′UTR.

To validate the direct targeting of HMGB-1 by miR-107, the wild-type (WT) HMGB-1 3′-UTR containing the target sequences, or a mutant variant (Mut) thereof was cloned into a dual-luciferase reporter vector [Fig. 2(a)]. Overexpression of miR-107 after HBO treatment significantly inhibited luciferase activity of the WTHMGB-1 3′-UTR (0.55 ± 0.135 fold; $^{**}P = 0.003$, $n = 4$; Fig. 2(b)), whereas mutation of the miR-107 binding sites (MT) abolished this inhibitory effect of miR-107 in human OA chondrocytes (1.01 ± 0.184 fold, $P = 0.903$, $n = 4$; Fig. 2(b)). These results identified HMGB-1 as a target gene of miR-107 after HBO treatment.

The expression of HMGB-1 in OA chondrocytes transfected with anti-miR-107 was also examined. As shown in Fig. 2(c), HBO treatment significantly decreased the mRNA expression of HMGB-1

(a) Targetscan

	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 50-56 of HMGB1 3' UTR	5' ...UGAUAUUUUGGAUAUUGCUGCAU...
	||||||
hsa-miR-107	3' ACUAUCGGGACAUGUUACGACGA

(b) miRnalyze

Transcript ENST00000399489.1

Gene: high mobility group box 1 (HMGB1)

Prevalent number: 1

Gene (Transcript)	Position in the UTR	Seed match	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	Pct
high mobility group box 1 (ENST00000399489.1)	50-56	7mer-1A	-0.20	> 90	-0.20	5.284	N/A

(c)

-------40----------50----------60----------bases-

Human 5'-------AUUUUGgauauugcugcauaucgagcuaaa------3'

Chimp 5'--------AUUUUGgauauugcugcauaucgagcuaaa------3'

Rhesus5'--------AUUUUGgauauugcugcauaucgagcuaaa------3'

Rabbit 5'--------AUUUCCgauauugcugcauaucgagcuaaa------3'

Pig 5'-------AUUUUAgauauugcugcauaucgagcuaaa------3'

Cat 5'-------AUUUUCgauauugcugcauaucgagcuaaa------3'

Elephant 5' ------CUUG---gauauugcugcauaucgagcuaaa------3'

Opossum 5'----- UU---AU--auauugcugcauaucgagcuaaa------3'

Lizard 5'---UAG----------gauauugcugcauaucgagcuaaa------3'

Seed sequence

Fig. 1. Seed sequence of miR-107 in the 3′UTR of HMGB-1 mRNA. (a) TargetScan predicted duplex of miR-107 with the seed sequence in the 3′UTR of human HMGB-1 mRNA. The sequences in white are the locations of the potential seed-matched sequence for the miRNAs studied. (b) miRnalyze predicted duplex of miR-107 with the seed sequence in the 3′UTR of human HMGB-1 mRNA. (c) Cross-species conservation of the miR-107 seed sequence in the 3′UTR of human HMGB-1 mRNA as identified by the TargetScan algorithm (sequences in red).

(0.63 ± 0.214 fold; $^{*}P = 0.012$, $n = 4$), whereas transfection with miR-107 inhibitors increased the mRNA level of HMGB-1 in OA chondrocytes after HBO treatment (0.92 ± 0.095 fold; $P = 0.065$, $n = 4$). Western blot analysis [Fig. 2(d)] indicated that HBO treatment led to a significant decrease in the protein expression of HMGB-1 (0.78 ± 0.126 fold; $^{*}P = 0.002$, $n = 4$), whereas knockdown of miR-107 reversed HMGB-1 protein expression in HBO-treated OA chondrocytes (0.96 ± 0.065 fold; $P = 0.118$, $n = 4$). These data indicated that HMGB-1 was negatively mediated by miR-107 at the post-transcriptional level in OA chondrocytes after HBO treatment. Overexpression of miR-107 after HBO treatment significantly inhibited the mRNA [Fig. 2(c)] and protein [Fig. 2(d)] expression of HMGB-1 in OA chondrocytes.

Effect of HBO on the mRNA and protein expression of HMGB-1, RAGE, TLR2, TLR4, and iNOS

Fig. 3(a) shows the effect of HBO intervention on the transcription of HMGB-1, RAGE, TLR2, TLR4, and iNOS. HBO treatment significantly suppressed the mRNA expressions of HMGB1 (0.63 ± 0.214 fold; $^{*}P = 0.012$, $n = 4$), RAGE (0.64 ± 0.221 fold; $^{*}P = 0.014$, $n = 4$), TLR2 (0.68 ± 0.243 fold; $^{*}P = 0.025$, $n = 4$), TLR4 (0.58 ± 0.199 fold; $^{**}P = 0.007$, $n = 4$), and iNOS (0.57 ± 0.248 fold; $^{*}P = 0.012$, $n = 4$) in OA chondrocytes. Fig. 3(b) showed the protein expression of HMGB-1 (0.52 ± 0.23 fold; $^{*}P = 0.012$, $n = 3$), RAGE (0.66 ± 0.161 fold; $^{*}P = 0.012$, $n = 3$), TLR2 (0.42 ± 0.182 fold; $^{**}P = 0.005$, $n = 4$), TLR4 (0.51 ± 0.211 fold; $^{**}P = 0.009$, $n = 4$), and iNOS (0.48 ± 0.254 fold; $^{*}P = 0.013$, $n = 3$), which were down-regulated after culturing for three rounds of HBO treatment. Fig. 3(c) shows the quantification of the relative protein expression levels.

Effect of HBO on the secretion of HMGB-1

HBO treatment significantly inhibited the extracellular release of HMGB-1 by OA chondrocytes in association with the protein level (Control group vs HBO group: 114.9 ± 43.1 vs 97.4 ± 33.7 after 1st treatment; 125.9 ± 49.4 vs 89.6 ± 29.9 after 2nd treatment, $n = 4$; 135.8 ± 44.9 vs 86.1 ± 45.5 after 3rd treatment, $^{**}P < 0.001$, $n = 4$; Fig. 4).

Effect of HBO on MAPK phosphorylation

Extracellular HMGB-1 interacts with RAGE present on the membrane of nearby cells to activate MAPKs. To assess the molecular mechanisms of the catabolic pathways mediated by HMGB-1/RAGE in OA chondrocytes after HBO treatment, the effects of HBO on MAPK activity were evaluated (Fig. 5). The phosphorylated dot

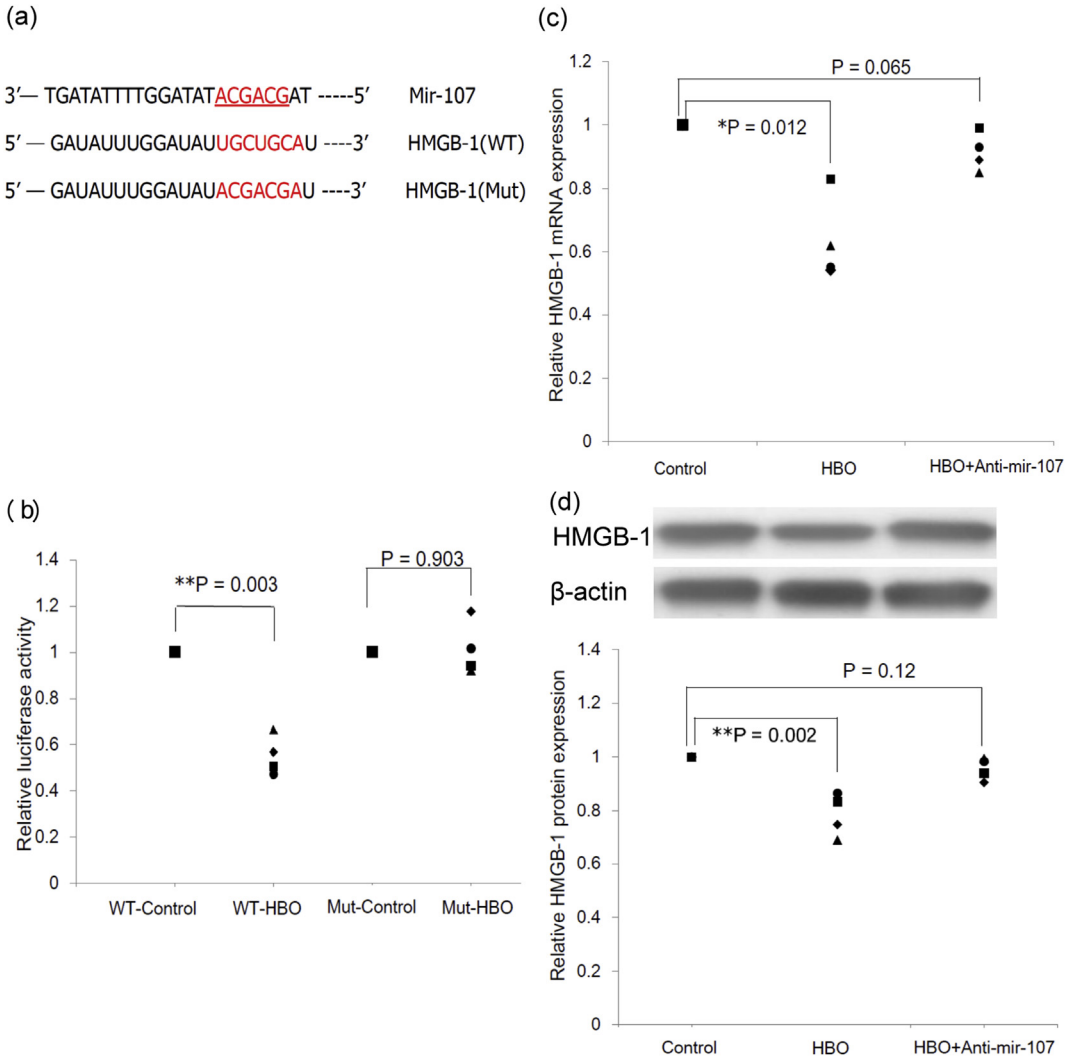

Fig. 2. HMGB-1 is a direct target of miR-107. (a) Diagram of the binding site between miR-107 and the HMGB-1 3′-UTR. The reporter vectors contain the wild type (WT) or mutant of HMGB-1 3′-UTR. (b) Dual-luciferase reporter assay of HMGB-1 3′-UTR. The reporter vectors containing the WT or mutant of HMGB-1 3′-UTR were transfected into OA chondrocytes. Luciferase activity was significantly downregulated after hyperbaric oxygen (HBO) treatment (**$P = 0.003$; $n = 4$) in the WT but not in the mutant type ($P = 0.903$, $n = 4$). (c) Real-time PCR analysis of HMGB-1 mRNA expression in OA chondrocytes transfected with miR-107 inhibitors. HMGB-1 mRNA expression was down-regulated after HBO treatment (*$P = 0.012$; $n = 4$). MiR-107 inhibitors reversed the suppressive effects of HBO ($P = 0.065$; $n = 4$). (d) Western blot analysis of HMGB-1 protein expression in chondrocytes transfected with miR-107 inhibitors. Values were normalized against β-actin. HMGB-1 protein expression was significantly downregulated after HBO treatment (**$P = 0.002$; $n = 4$). MiR-107 inhibitors reversed the suppressive effects of HBO ($P = 0.12$; $n = 4$).

density ratios for HBO and control groups at 30 and 60 min after administering the third HBO treatment. At 30 min, the ratios were 74.9% ± 15.1% for p38 MAPK phosphorylation (*$P = 0.013$, $n = 4$), 85.1% ± 8.5% for ERK phosphorylation (*$P = 0.012$, $n = 4$), and 94.9% ± 16.3% for JNK phosphorylation ($P = 0.391$, $n = 4$). At 60 min, the ratios were 34.8% ± 4.4% for p38 MAPK phosphorylation (***$P < 0.0001$, $n = 4$), 38.7% ± 3.8% for ERK phosphorylation (***$P < 0.0001$, $n = 4$), and 43.3% ± 9.0% for JNK phosphorylation (**$P = 0.0002$, $n = 4$). HBO treatment significantly suppressed MAPK phosphorylation in OA chondrocytes.

Effect of HBO on the protein expression of IκBα and NF-κB p65

HMGB-1 has been previously reported as an inducer of the NF-kB inflammatory signaling pathway. HBO treatment significantly suppressed the mRNA expressions of iNOS [Fig. 3(a)]. As NF-κB is a central transcription factor that regulates the expression of iNOS, the effects of HBO on the nuclear translocation of NF-κB p65 subunits were examined (Fig. 6). The protein expression of IκBα was significantly upregulated at 60 min after administering the third HBO treatment (1.92 ± 0.436 fold; *$P = 0.019$, $n = 3$). In addition, the levels of NF-κB p65 in the nucleus (0.28 ± 0.143 fold; *$P = 0.029$, $n = 3$) were significantly decreased after HBO treatment [Fig. 6(a)]. Fig. 6(b) shows the quantification of relative protein expression levels. IκBα resides in the cytoplasm to prevent NF-κB translocation to the nucleus. HBO treatment decreased the NF-kB inflammatory signaling.

Effect of HBO on MMP-9 and MMP-13 secretion

As MAPK signaling and NF-κB activation may contribute to cartilage degradation through the upregulation of MMP expression, ELISA analysis was performed to detect MMP-9 and MMP-13, which are released by HBO-treated primary OA chondrocytes (Fig. 7). Fig. 7(a) shows the effect of three rounds of HBO treatment on MMP-9 secretion (**$P < 0.001$; Table I). Fig. 7(b) shows the effect of

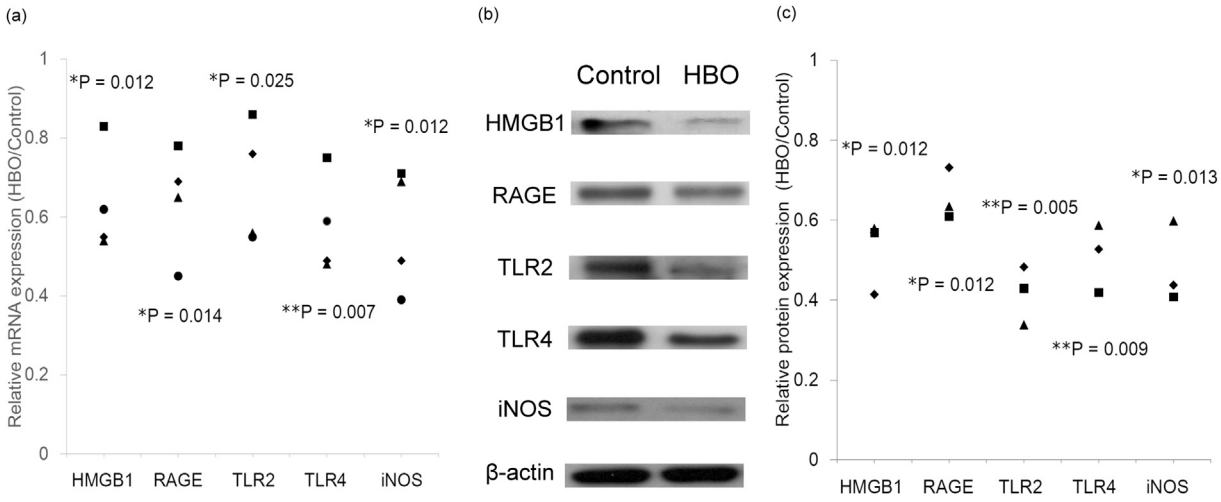

Fig. 3. Effect of HBO on the mRNA and protein expression of HMGB-1, receptor for advanced glycation end products (RAGE), and inducible nitric oxide synthase (iNOS). (a) HBO treatment significantly suppressed the mRNA expressions of HMGB1 (*$P = 0.012$; $n = 4$), RAGE (*$P = 0.014$; $n = 4$), and iNOS (*$P = 0.012$; $n = 4$) in OA chondrocytes. (b) Western blot analysis was performed to examine the protein expression of HMGB-1, RAGE, and iNOS. The protein expression of HMGB-1 (*$P = 0.012$; $n = 3$), RAGE (*$P = 0.045$; $n = 3$), and iNOS (*$P = 0.012$; $n = 3$) were significantly downregulated after culturing for three rounds of HBO treatment. (c) The quantification of relative protein expression levels.

three rounds of HBO treatment on MMP-13 secretion (**$P < 0.001$; Table I). HBO treatment significantly inhibited MMP-9 and MMP-13 secretion by OA chondrocytes.

Effect of HBO treatment on cartilage defect repair

The gross appearance of the defects and repaired cartilage were examined. Morphological observations showed HBO markedly enhanced cartilage repair (see Appendix and Supplementary Figs. S2(a)–2(d)).

Effect of HBO treatment on HMGB-1 and iNOS expression

HBO treatment markedly suppressed HMGB-1 and iNOS expression in repaired cartilage (see Appendix and Supplementary Figs. S2(e)–2(h)).

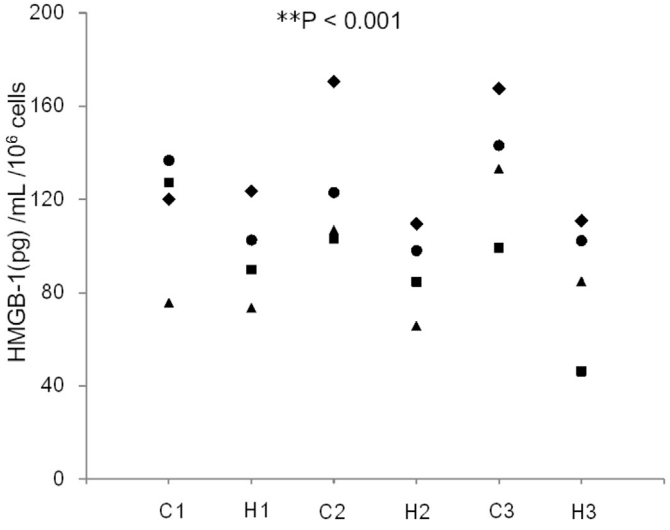

Fig. 4. Effect of HBO on the secretion of HMGB-1. HBO treatment significantly inhibited the extracellular release of HMGB-1 by OA chondrocytes (Control group vs HBO group: 114.9 ± 43.1 vs 97.4 ± 33.7 after 1st treatment; 125.9 ± 49.4 vs 89.6 ± 29.9 after 2nd treatment, $n = 4$; 135.8 ± 44.9 vs 86.1 ± 45.5 after 3rd treatment, **$P < 0.001$, repeated measures ANOVA, $n = 4$).

Discussion

MiR-107 is increasingly thought to serve key functions in humans[25,26]. It regulates gene expression involved in cell division[27], hypoxic stress response[28], and angiogenesis[29] in different tissues and cells. MiR-107 has also been implicated in human colon carcinomas[29] and neurodegenerative disease[30]. MiR-107 expression was increased in the retina following hyperoxia[24]. MiR-107 decreases hypoxia signaling by suppressing the expression of hypoxia inducible factor-1β (HIF-1β) in human colon cancer cells[29]. MiR-107 is significantly downregulated in inflamed colons[3]. MiR-107 is downregulated in OA[10,11]. After verifying the re-expression levels of miR-107 by real-time qPCR (Fig. S1(a)), we assessed the ability of the miR-107 to diminish HMGB-1 levels. As shown in Fig. S1(b), overexpression of miR-107 significantly decreased the levels of HMGB-1.

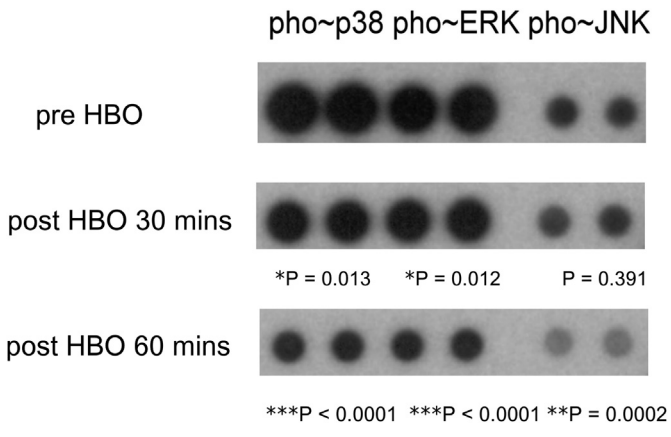

Fig. 5. Effect of HBO on MAPK phosphorylation. HBO treatment significantly suppressed ERK, JNK, and p38 MAPK phosphorylation in OA chondrocytes. Phosphorylation dot density ratios for the HBO and control groups were determined at 30 and 60 min after the third round of HBO treatment. At 30 min, the ratio for p38 MAPK (*$P = 0.013$; $n = 4$) and ERK (*$P = 0.011$; $n = 4$) was significantly decreased. At 60 min, the ratio for p38 MAPK (***$P < 0.0001$, $n = 4$), ERK (***$P < 0.0001$, $n = 4$), and JNK (**$P = 0.0002$, $n = 4$) were significantly decreased. HBO treatment significantly suppressed MAPK phosphorylation in OA chondrocytes.

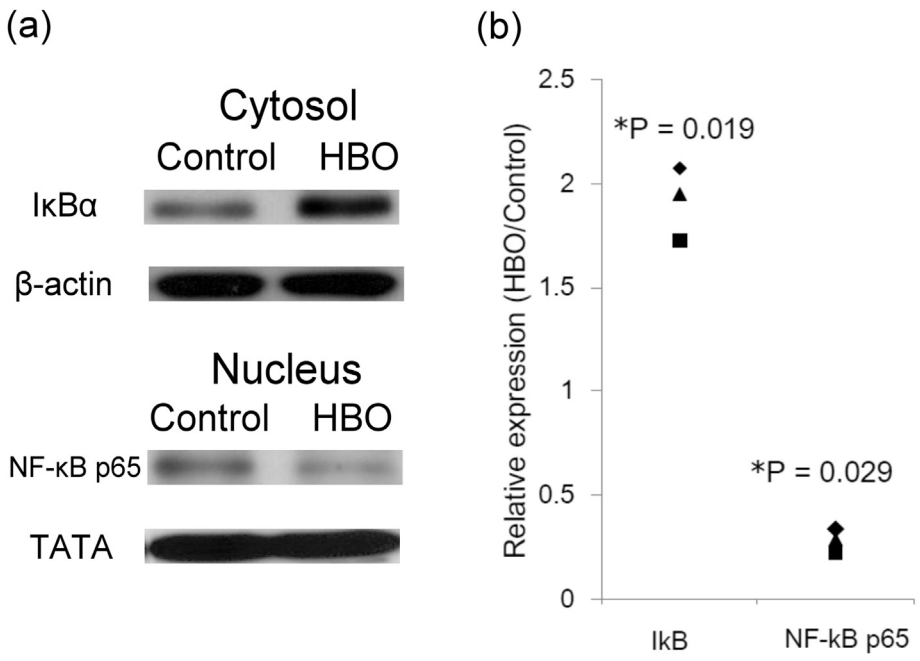

Fig. 6. Effect of HBO on the protein expression of IκBα and NF-κB p65. (a) The cytosolic protein level of IκBα was significantly upregulated at 60 min after the administration of the third HBO treatment (*$P = 0.019$, $n = 3$). In addition, the levels of NF-κB p65 in the nucleus was significantly decreased after HBO treatment (*$P = 0.029$, $n = 3$). IκBα resides in the cytoplasm to prevent NF-κB translocation to the nucleus. HBO treatment decreased the NF-kB inflammatory signaling. (b) Quantification of the relative protein expression levels.

In this study, for the first time, we reported that miR-107 played an important role in OA chondrocytes after HBO treatment. To investigate the potential role of miR-107, we detected miR-107 expression in OA chondrocytes after HBO treatment. Hypoxia is a potent inducer of extracellular HMGB-1[6] and miR-107 can modulate the cellular response to hypoxic in tumors[29]. A decreased expression of miR-107 was shown in OA chondrocytes[10,11]. The results of the present study showed that HBO treatment increased miR-107 expression to suppress inflammation in OA chondrocytes (Fig. 1). HMGB-1 was identified as a target gene of miR-107 after HBO treatment. MiR-107 may regulate inflammatory signaling by targeting HMGB-1 (Figs. 2 and 3). HBO treatment significantly increased miR-107 expression and therefore decreased the HMGB-1 production from OA chondrocytes (Figs. 1–3).

HMGB-1 is released from the cells after translocation from the nucleus to the cytoplasm or the external of the cell[31], which indicates that secretion of HMGB-1 may be associated with activation of the inflammasome complex[32]. RAGEs expression increased with progression of OA[1]. Treatment with HMGB-1 resulted in phosphorylation of the MAPK and the p65 subunit of NF-κB in cultured chondrocytes[1]or mouse knee joint[33], both of which are well-characterized mediators of RAGE signaling[1,34]. Hypoxia induces MAPK activity in rat nucleus pulposus cells (NPCs)[35]. Suppression of MAPK phosphorylation plays a key role in the protection of NPCs after HBO treatment[36]. Consistent with these previous reports, the present study suggested that the reduced HMGB-1 secretion (Fig. 5) and RAGE expression (Fig. 4) after HBO treatment suppressed p38 MAPK, ERK, and JNK phosphorylation in OA chondrocytes (Fig. 6). These results suggest that HBO plays a role in treating OA chondrocytes by down-regulating HMGB-1/RAGE/MAPK signaling expression. The inhibition of HMGB-1 and RAGE by HBO treatment showed effective anti-inflammatory action in OA chondrocytes. In addition, reduced expression of HMGB-1 and iNOS in repaired cartilage by HBO treatment was also shown in our animal model (Fig. S2(e), (f), (g), (h)).

In most cell types, NF-κB is composed of a p65/p50 heterodimer. IκBα, IκBβ, IκBγ and IκBε reside primarily in the cytoplasm and function to prevent NF-κB translocation to the nucleus[10]. Under normal conditions, NF-κB is present in an inactive state and mainly located in the cytoplasm. However, once activated, the p65 subunit dissociates from its inhibitor IκBα and translocate from the cytoplasm to the nucleus to activate the transcription of its target genes[10]. Hypoxia induces inflammatory responses by activating of NF-κB, which is a major mediator of inflammation and controls the transcriptional programs, which execute and regulate the inflammatory responses[11]. HBO treatment reduces inflammatory responses in patients with acute pancreatitis by upregulating of IκB activation and downregulating of NF-κB levels in granulocytes[25]. The present study further investigated the HMGB-1/RAGE/NF-κB signaling pathway in OA chondrocytes, and the results demonstrated that IκB expression was upregulated in response to HBO treatment, which decreased the translocation of NF-κB from the cytosol into the nucleus (Fig. 7). NF-κB mediated iNOS mRNA [Fig. 4(a)] and protein [Fig. 4(b)] expressions were subsequently downregulated after HBO intervention. Because the presence of NF−κB binding sites in the RAGE promoter creates a positive feedback loop through increasing RAGE expression[34], NF-κB mediated RAGE mRNA [Fig. 4(a)] and protein [Fig. 4(b)] expressions were downregulated after HBO intervention. These results suggest that HBO plays a role in treating OA chondrocytes by down-regulating HMGB-1/RAGE/NF-κB/iNOS signaling expression. These findings supported our previous study that HBO treatment suppressed the iNOS expression in OA chondrocytes[37] or OA animal model[38].

Upregulation of MMP-9 results in destruction of articular cartilage in patients with OA[19]. NF-kB p65-specific short interfering RNA (siRNA) inhibits the expression of iNOS and MMP-9 in IL-1β- and TNF-α-induced chondrocytes[20]. MMPs production is significantly increased in chondrocytes treated with RAGE ligands[38,39]. Binding of HMGB-1 to RAGE may contribute to cartilage degradation by upregulating MMP-13 expression[38]. Overexpression of HMGB-1 antagonists inhibited the upregulation of iNOS, MMP-1, MMP-3, and MMP-9 induced by IL-1β in human chondrocytes[40]. HBO treatment significantly inhibits the secretion of MMP-13[12] and

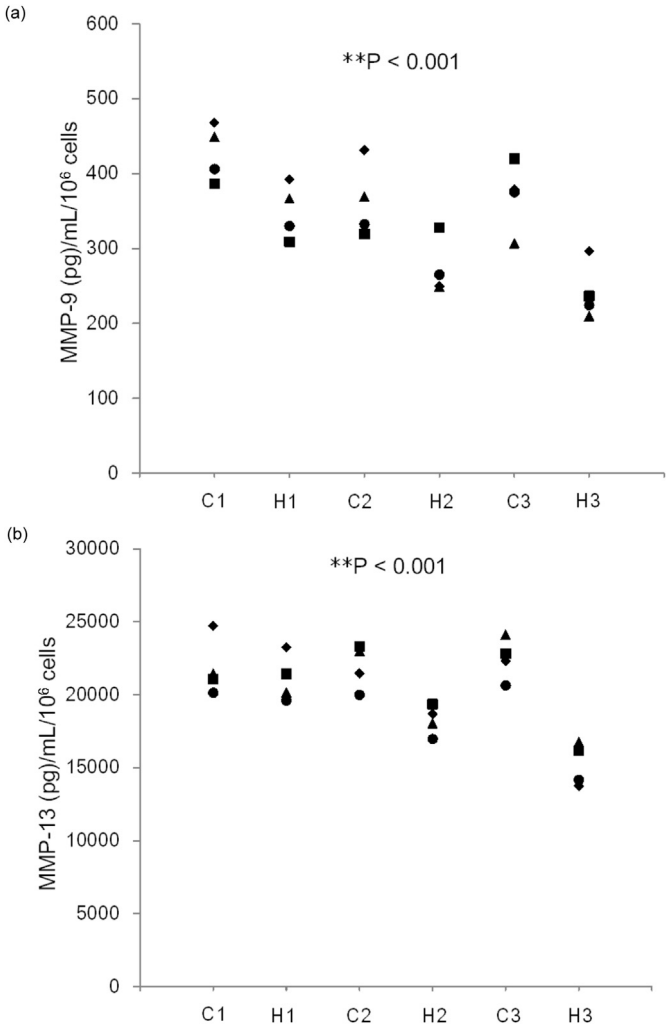

Fig. 7. Effect of HBO on MMP-9 and MMP-13 secretion. (a) HBO treatment significantly inhibited MMP-9 secretion (**$P < 0.001$, repeated measures ANOVA; $n = 4$) by OA chondrocytes. (b) HBO treatment significantly inhibited MMP-13 secretion (**$P < 0.001$, repeated measures ANOVA; $n = 4$) by OA chondrocytes. HBO treatment decreased the MAPK and NF-kB signaling, which contribute to the regulation of matrix metalloproteases (MMPs) regulation.

Table I
Effect of hyperbaric oxygen (HBO) on the extracellular release of matrix metalloproteases (MMPs) by OA chondrocytes

	Control		HBO		*P*-value
MMP-9	Mean	95% CI	Mean	95% CI	
1st treatment	427.4	59.8	349.6	59.4	**$P < 0.001$
2nd treatment	363.2	79.7	272.9	59.2	
3rd treatment	370.3	74.5	241.8	60.5	
	Control		HBO		*P*-value
MMP-13	Mean	95% CI	Mean	95% CI	
1st treatment	21,847.2	2001.3	21,108.6	1,615.9	**$P < 0.001$
2nd treatment	21,947.9	1,531.8	18,258.7	1,017.5	
3rd treatment	22,477.7	1,430.9	15,220.6	1,481.9	

Differences between control and HBO groups were analyzed (using repeated measures ANOVA; $n = 4$). A *P*-value of <0.05 was considered statistically significant.

MMP-9[7] in different cells. In the present study, the expression of HMGB-1 and RAGE was reduced in response to HBO treatment (Fig. 4), which may lead to the downregulation of MMP-9 [Fig. 7(a)] and MMP-13 [Fig. 7(b)]. In addition, HBO increases the protein expression of IκBα, which leads to the downregulation of NF-κB signaling (Fig. 6), iNOS (Fig. 3), and MMP-9 secretion [Fig. 7(a)]. HBO intervention can reduce MMPs associated extracellular matrix damage and promote the repair of OA cartilages (Fig. S2).

HBO serves as primary or adjunctive therapy for a diverse range of medical conditions (*ex.* crush injury, traumatic ischemia, radiation necrosis of soft tissue and bone)[41,42]. One of the mechanisms is by increasing the oxygen diffusion to the tissues by raising dissolved oxygen level in plasma or body fluid. Because oxygen is a gas, the effectiveness of the HBO treatments is that the gases are pushed into the tissue not just delivered through the blood stream[41,42,17]. The basis of modulating pressure and oxygen concentration is to regulate cellular metabolism or tissue microenvironment. Hypoxia has been suggested to play a central role in the induction of tissue damage in OA[4]. Therefore, the HBO treatment maybe a good choice to study avascular tissue like cartilage. In our previous animal studies, HBO increased the oxygen tension in synovial fluid and suppressed iNOS expression and apoptosis of cells in rabbit OA model[38]. In the present study, we further found HBO treatment markedly suppressed HMGB-1 and iNOS expression in repaired cartilage (Figs. S2(e), (f), (g), (h)). Recently, HBO therapy downregulated HMGB-1 and NF-κB expression in clinical patients with acute spinal cord injury also has been reported[43]. The effects of HBO on HMGB1/RAGE, TLR2, 4/NF-κB signaling in clinical OA patients are needed to be further investigation.

In post-natal growth cartilage, nutrition is supplied by the metaphysical subchondral circulation to the hypertrophic layer while the germinal and proliferative layers are supplied from epiphysial subchondral vessels[44]. It follows that the nutrition of chondrocytes is carried out by diffusion of tissue fluid from subchondral vessels. The effects of HBO on cells/explants maintained under hypoxic conditions also has been reported. Calderwood showed the beneficial effect of HBO on the transplantation of epiphysial growth cartilage in rabbits[45]. HBO increasing the amount of oxygen in tissue fluid should be of great benefit to chondrocytes when the normal subchondral vasculature has been grossly disturbed after transplantation[45]. In addition, our previous study also showed the beneficial effects of HBO in chondrocyte transplantation via upregulating of platelet-derived growth factor-BB (PDGF-BB) receptors expression[46].

The results of the present study indicate that HBO treatment of OA chondrocytes exerts a protective effect by mitigating inflammation and its subsequent activation. This effect is induced through inhibition of the miR-107/HMGB-1/RAGE, TLR2, 4/MAPK, NF-κB signaling pathway and subsequent suppression of pro-inflammatory cytokines and MMPs levels.

Author's contributions

Conception and design: YLJ, LSS, and USWN.

Acquisition, analysis and interpretation of data: YLJ, LSS, NCC, Tu YK and YCY.

Drafting the article: YLJ, LSS, and USWN.

Final approval of the article: YLJ, LSS, NCC, Tu YK, YCY, and USWN.

Conflicts of interest

None.

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Ethical approval

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

Supplementary data

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