Up-regulation of proteoglycan 4 in temporomandibular osteoarthritic synovial cells by hyaluronic acid

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BACKGROUND: Hyaluronic acid (HA) injection is widely used in the treatment of temporomandibular joint (TMJ) osteoarthritis (OA). Proteoglycan 4 (PRG4) is another joint lubricant that protects surface of articular cartilage. But few studies had explored the role of HA in regulation of PRG4 expression in TMJ OA. In this study, the effects of HA on the expression of PRG4 in osteoarthritic TMJ synovial cells were investigated in hypoxia, which was similar to the TMJ physiologically.

METHODS: Synovial cells were isolated from the TMJ OA patients and were treated with or without HA under normoxia or hypoxia for indicated time periods. The proliferation of synovial cells was measured using Cell Counting Kit-8 (CCK-8). The gene expression of HAS2, VEGF, and PRG4 was detected by quantitative real-time PCR, and the secretion of PRG4 and VEGF was assayed by enzyme-linked immunosorbent assay (ELISA). Immunofluorescence was used to examine the protein expression of hypoxia-induced factor-1α (HIF-1α).

RESULTS: Hyaluronic acid markedly increased the proliferation of osteoarthritic synovial cells in hypoxia. The expression of HAS2 and PRG4 mRNA of osteoarthritic synovial cells under hypoxia was enhanced by HA treatment. However, HA had no effect on reducing the VEGF and HIF-1α expression in synovial cells in hypoxia.

CONCLUSIONS: Hyaluronic acid could promote the expression of HAS2 and PRG4, but could not modulate HIF-1α and VEGF expression of TMJ osteoarthritic synovial cells in hypoxia.
protocol was approved by the Human Research Ethics Committee, School & Hospital of Stomatology, Wuhan University. Synovial membrane specimens were cut into small (1 mm³) pieces and placed as explants in 25-cm² tissue culture flasks (NEST, China) as described previously (8). Synovial cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), penicillin (100 units/ml; Hyclone), and streptomycin (100 μg/ml; Hyclone) in a humidified atmosphere containing 5% CO². After the third passage, morphologically homogeneous fibroblast-like cells were obtained, which were used for experiments until passage 5. In all the experiments, the synovial cells were plated in 6-well, 24-well, or 96-well plates (Coster, Badhoevedorp, the Netherlands). When the synovial cells were grown to confluence, they were starved for 16 h in serum-free DMEM to synchronize in a non-proliferating and non-activating phase firstly. Then, the synovial cells were transferred to 10% FBS medium and divided into four groups (normoxia, normoxia + HA, hypoxia, and hypoxia + HA group) with or without 100 μg/ml HA (1.63 × 10⁴ Da, product number: 53747; Sigma-Aldrich) dissolved in serum-free DMEM. Then, the four groups were incubated for indicated time periods to start each experiment.

Hypoxia conditions
Hypoxia was induced using an anaerobic Incubator Chamber (Galaxy 170R; Eppendorf, Hamburg, Germany), which can provide the environment of 1% O₂, 5% CO₂, and 94% N₂.

Cell proliferation assay
Cell proliferation assay of synovial cells was assessed by Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Synovial Cells were seeded in 96-well plates at 10⁴ cells per well and pre-incubated for 24 h. Then, synovial cells were treated as described above for 6, 12, 24, or 48 h under normoxia or hypoxia. Then, 10 μl CCK-8 solutions were added to each well and the synovial cells were incubated for 2 h. The absorbance at 450 nm was measured on a microplate reader (Nanodrop; Thermo, Rockford, IL, USA) in five samples of each group.

Enzyme-Linked Immunosorbent Assay (ELISA)
Synovial cells were plated at 5 × 10⁴ cells per well in 24-well plates. When the synovial cells were grown to confluence, the medium was replaced with 8% FBS medium for 24 h; then, the cells were changed to the medium containing 4% FBS to start the experiment. After 6 and 24 h under normoxia or hypoxia, the supernatants were collected and secretion of PRG4 and vascular endothelial growth factor (VEGF) was analyzed using ELISA kits (PRG4; Elabscience, Wuhan, China; VEGF; Neobioscience Technology Co., Ltd, Shenzhen, China) according to the manufacturers’ protocols, respectively. Absorbance was measured at 450 nm using a micro-plate reader (Nanodrop; Thermo) in three samples of each group.

Quantitative real-time PCR for the detection of hyaluronan synthase 2 (HAS2), PRG4, and VEGF
Cells were seeded at a density of 2 × 10⁵ cells/well in 6-well culture dishes and pre-treated as described above. After various times, total RNA was extracted from cultured cells using RNAiso Plus (TakaRa, Dalian, China) according to the manufacturer’s instructions. Total RNA was reverse-transcribed into cDNA using First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA). For the measurement of mRNA, the primer sequences of HAS2, PRG4, and VEGF were showed as follows: β-actin: Fwd 5’ GTC CAC CGC AAA TGC TTC TA, Rev 5’ TGC TGT CAC CTT CAC CGT TC; HAS2: Fwd 5’ CTC TTT TGG ACT GTA TGG TGC C, Rev 5’ AGG GTA GG TAG CCT TTT CAC A; PRG4: Fwd 5’ ACC CAA TCA AGG CAT TAT CAT C Rev 5’ CAT CCA GAA ATA ATG ACC TCG G VEGFA: Fwd 5’ CAG CTA CTG CCA TCC AAT CG Rev 5’ TTG TTG TGC TGT AGG AAG CTC A. Real-time PCRs were carried out using a TaKaRa SYBR Premix ExTaq kit (TaKaRa) under the instruction of the manufacturer. The amount of targets was obtained by dividing the level of HAS2, PRG4, and VEGF mRNA expression by the level of β-actin mRNA expression.

Immunofluorescence for hypoxia-induced factor-1α (HIF-1α)
After incubated for 6 and 24 h, human synovial cells were fixed in freshly prepared 4% formaldehyde in PBS for 10 min and permeated with the 0.1% Triton-X for 5 min. Following blockade of non-specific binding sites by incubation with 2.5% BSA in PBS for 30 min, the cells were incubated with a monoclonal anti-HIF-1α antibody diluted at 1:300 (Santa Cruz Biotech, Santa Cruz, CA, USA) at 4°C overnight. HIF-1α antibody binding was detected by incubating with a TRITC-conjugated goat anti-mouse IgG (Pierce, Rockford, IL, USA) diluted at 1:100 for 1 h at room temperature. Then, the cells’ nuclei were stained with 4’6-Diamidino-2-phenyl-indole (DAPI) (Beyotime Institute of Biotechnology, Shanghai, China). Finally, all the samples were washed three times with PBS, mounted, and examined by fluorescence microscopy (Olympus, Tokyo, Japan).

Statistical analysis
Statistical analyses were carried out with the SPSS 20 statistical software program. The data were presented in the form of mean ± SD. Gaussian distribution and homogeneous variance were tested in all original data. Statistical
analysis was analyzed by one-way analysis of variance (ANOVA) followed by least significant difference test to compare differences between group means. P-values $< 0.05$ were considered significant.

### Results

**Effect of HA on osteoarthritic synovial cell proliferation**

With the addition of HA, the proliferation level of synovial cells of each group was even at 6 h, while from 12 to 48 h, the proliferation of synovial cells was inhibited by hypoxia. Interestingly, HA could increase synovial cell proliferation in normoxia or hypoxia (Fig. 1).

**Effect of HA on the expression of HAS2, PRG4, and VEGF genes under hypoxia**

The gene expression of HAS2 and PRG4 was decreased at most of the time points under hypoxia (Fig. 2A, B). However, HA significantly increased the expression of HAS2 and PRG4 genes. On the other hand, the expression of VEGF gene was markedly enhanced under hypoxia, but the incubation of HA did not affect the expression level of VEGF gene at 6 and 24 h (Fig. 2C).

**Effect of HA on hypoxia-induced PRG4 and VEGF secretion**

Hyaluronic acid dramatically enhanced the PRG4 secretion of synovial cells at 24 h under hypoxia (Fig. 3A). In addition, the secretion of VEGF was all significantly enhanced in all groups from 6 to 24 h; for example, the treatment with HA did not suppress the expression of VEGF under hypoxia (Fig. 3B).

**Effect of HA on HIF-1α nuclear translocation protein by hypoxia**

As HIF-1α is an important regulator for VEGF, the effect of HA on the expression of HIF-1α was studied by immunofluorescence. At 6 and 24 h, the nuclear translocation of HIF-1α could be all visualized in synovial cells in the experimental groups. Furthermore, there was obviously accumulation with HA treatment at 24 h (Fig. 4).

### Discussion

Intra-articular injection of HA has been demonstrated as an effective treatment of OA, which could release the OA pain and lubricate the knee joint (9, 10). The same result was found in TMJ; Long et al. (1) reported inferior joint space
injection with HA was a valid method of treating disc displacement without reduction of TMJ. Many studies tried to explain the mechanism of the effects of HA on OA. For example, HA could down-regulate gene expression of TNF-α, IL-8 in synovial cells (11) and inhibit IL-1β-stimulated production of MMP-1, MMP-3, and MMP-13 in articular cartilage (12). Furthermore, exogenous HA could enhance secretion of HA from synovial cells and mediate nutrition to the avascular surface of the joint (13). However, exogenous HA is quickly degraded by hyaluronidase in 12 h once it is injected into the joint space (14–16). So we detected the short-term effect of HA on the expression of PRG4 within 24 h. In this study, we found that HA also could up-regulate PRG4 expression of osteoarthritic TMJ synovial cells within 24 h in vitro.

PRG4 has been identified to act synergistically with HA (17) via adsorbing to various types of substrates and forming complexes with itself and HA. The protein could form a discontinuous nano-film that exerted repulsive forces, which was the basis for its lubricating and anti-adhesive properties when bound to the surface of cartilage (18). Intra-articular supplementation with PRG4 reduced cartilage damage as indicated by histology and radiography (7). PRG4 may be degraded, and synthesis appeared to be reduced in arthritic joints, due to loss of synovial cell or inflammatory cytokines (19). PRG4 concentrations found a significant decline in the group with OA (6). This study also showed that the expression of PRG4 in osteoarthritic TMJ synovial cells was dramatically suppressed by hypoxia, which may relate to various inflammatory cytokines secreted in that situation. HA, however, enhanced the protein release of PRG4, especially at 24 h indicated by ELISA assay. This phenomenon suggested that the lubricating effect of exogenous HA might be associated with the up-regulation of PRG4. Although PRG4 gene expression was up-regulated by HA, PRG4 secretion in normoxia group was suppressed. The possible reason might be related to the complex regulation of PRG4 secretion. PRG4 secretion is determined by a combination of mechanical and chemical stimuli (20). For example, TGF-β1 and FGF-2 could up-regulate the expression of PRG4 (21, 22). On the other hand, proinflammatory cytokines such as TNF-α and IL-1α/β were shown to suppress secreted PRG4 levels in chondrocytes cultures (23, 24).

For another, HASs are responsible for the synthesis and regulation of different molecular weight HA. High molecular weight hyaluronic acid (HMWHA, molecular mass>1 × 10⁶ Da), which is synthesized by HAS2, appears to be anti-angiogenic, whereas oligosaccharide degradation products of HA induce angiogenesis in vivo (24, 25). In this study, HAS2 expression was significantly decreased in hypoxia, but HA could enhance HAS2 mRNA expression, which may be a therapeutic mechanism of HA. The exogenous HA may increase synovial cell HA secretion by up-regulating HAS2 expression although the detailed pathway is unknown.

Most studies about the effect of HA on osteoarthritic synovial cells were performed under normoxic situation (5, 26), while the TMJ joint space is in hypoxia. In TMJ OA, oxygen tension was even lower in osteoarthritic joint compared with healthy joint (27). It is believed that hypoxia inhibits proliferation of synovial cells as well as leads to angiogenesis which contributes to the progression of OA (28, 29). So this study focused on the role of HA in osteoarthritic synovial cells under hypoxia. The synovial cell proliferation was inhibited in hypoxia, but fortunately, HA could promote the proliferation of synovial cells in a time-dependent manner. However, from our quantitative real-time PCR results, we could not get obvious time-dependent manner in PRG4, VEGF, and HAS2 gene expression from 6 to 24 h. Furthermore, as mentioned above, the half-time of exogenous HA is 12–24 h, so the effect of exogenous HA on the protein secretion of PRG4 and VEGF was detected at initial and final time points.

Our previous study reported that hypoxia could mainly induce the expression of VEGF121 and VEGF165 in synovial cells to promote inflamed angiogenesis of TMJ (30). Hypoxia is a pivotal regulator of angiogenesis, and VEGF was expressed increasing in OA synovium, articular cartilage, and synovial fluid (31, 32). The increased

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**Figure 4** Immunoﬂuorescence staining of HIF-1α in osteoarthritic synovial cells incubated with or without HA under normoxia or hypoxia for 6 and 24 h. Bar = 10 μm.
VEGF production in OA has also been associated with the stimulation of HIF-1α in hypoxic situation. It has been reported that expression of HIF-1α was up-regulated in osteoarthritic synovial cells (33). But the opinion about the role of HA on HIF-1α and VEGF expression is in conflict. As mentioned above, HMWHA is anti-angiogenic, but, degradation products of HA actively stimulate endothelial cell proliferation and induce angiogenesis (34). HMWHA is more likely to degrade into oligosaccharide HA in in vitro and in vivo. As mentioned above, HMWHA is anti-angiogenic, but, degradation products of HA actively stimulate endothelial cell proliferation and induce angiogenesis (34). HMWHA is more likely to degrade into oligosaccharide HA in in vitro and in vivo.

Moreland LW. Intra-articular hyaluronan (hyaluronic acid) and VEGF production in OA has also been associated with the stimulation of HIF-1α in hypoxic situation. It has been reported that expression of HIF-1α was up-regulated in osteoarthritic synovial cells (33). But the opinion about the role of HA on HIF-1α and VEGF expression is in conflict. As mentioned above, HMWHA is anti-angiogenic, but, degradation products of HA actively stimulate endothelial cell proliferation and induce angiogenesis (34). HMWHA is more likely to degrade into oligosaccharide HA in in vitro and in vivo. As mentioned above, HMWHA is anti-angiogenic, but, degradation products of HA actively stimulate endothelial cell proliferation and induce angiogenesis (34). HMWHA is more likely to degrade into oligosaccharide HA in in vitro and in vivo.

By this study, HA could promote the expression of HAS2 and PRG4, but not modulate HIF-1α and VEGF expression of TMJ osteoarthritic synovial cells in hypoxic condition. The detailed role of HA on PRG4 expression needs to be investigated.

References


**Acknowledgements**

This research was supported by Grant No. 81071266 and No. 30901684 of National Science Foundation of China.

**Conflict of interest**

The authors declare that they have no competing interests.