Effects of Chemically Induced Hypoxia on In Vitro Expression of Hypoxia Inducible Factor-lα, Vascular Endothelial Growth Factor, Aggrecanase-1, and Tissue Inhibitor of Metalloproteinase-3 in Rat Mandibular Condylar Chondrocytes

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Aims: To examine the expression of hypoxia inducible factor-lα (HIF-1α), vascular endothelial growth factor (VEGF), aggrecanase-1 (ADAMTS-4), and tissue inhibitor of metalloproteinase-3 (TIMP-3) in rat mandibular condylar chondrocytes under hypoxic conditions and examine the relationship of these expressed factors in early condylar cartilage growth. **Methods:** $CoCl_2$ was used to mimic a hypoxic microenvironment by chemically inducing hypoxia. Chondrocytes, which were obtained from the mandibular condyles of 3-week-old female Sprague-Dawley rats, were treated with 125 μmol/L $CoCl_2$ for 48 hours. The HIF-1α, VEGF, ADAMTS-4, and TIMP-3 mRNA levels in chondrocytes were detected using a semiquantitative polymerase chain reaction (PCR) at 12, 24, and 48 hours after the initiation of hypoxia and normoxia conditions. A univariate variance analysis (pairwise least significant difference *t* test) using a SPSS 13.0 software package was performed to test for differences between different groups. **Results:** PCR analysis of the chondrocytes showed that the expression of HIF-1α and VEGF mRNA increased at 12, 24, and 48 hours after induction of hypoxia. HIF-1α expression gradually increased throughout the study duration, while VEGF expression peaked at 12 hours. Compared to normoxia conditions, hypoxia resulted in constantly elevated expression levels of both. On the other hand, there was no significant difference in the ADAMTS-4 and TIMP-3 mRNA expression between hypoxic and normoxic conditions throughout the study ($P > .05$). **Conclusion:** An upregulated HIF-1α and VEGF mRNA expression indicates their important roles in cartilage vascularization and development in newly hypoxic microenvironments. Additionally, chemically induced hypoxia has little effect on the expressions of ADAMTS-4 and TIMP-3.
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Key words: *HIF-1α, VEGF, aggrecanase-1 (ADAMTS-4), TIMP-3, hypoxia, $CoCl_2$, TMJ cartilage*

The temporomandibular joint (TMJ) is a major growth center of the mandible and the weight-bearing joint in the functional movements of the masticatory system. Adequate stress can promote the metabolism of the chondrocytes, while abnormal stresses may lead to degenerative changes or even growth arrest in chondrocytes. However, the exact mechanism of the transduction of signals that allows biomechanical stress to affect chondrocyte responses is not yet clear.

Under physiologic conditions, condylar chondrocytes are maintained in a hypoxic microenvironment, as there is no direct blood supply to the proliferative and hypertrophic zones in condylar cartilage.[1,2] Hypoxia inducible factor 1α (HIF-1α), one of the major mediators of the hypoxic response, appears to play an essential role in the arrest of chondrocyte growth and thereby survival in vitro.[3] Studies have shown that condylar chondrocytes express both HIF-1α and vascular endothelial growth factor (VEGF) in condylar cartilage,[3,4] while hypoxia is a major inducer of VEGF, an angiogenesis and vascular permeability factor, and HIF-1α, a transcriptional activator of VEGF.

Additionally, one of the suggested causes of cartilage destruction in degenerative joint diseases is the loss of aggrecan.[5] This is carried out by cartilage aggrecanases and is considered a critical early event in the

process, followed by the degradation of collagens by matrix metalloproteinases (MMPs).[5] Using a well-established rat model of dietary loading during early development, previous studies showed that VEGF expression was higher in the hard-food group[6]; similarly, aggrecanase-1 (ADAMTS-4) expression was higher, but tissue inhibitor of metalloproteinase-3 (TIMP-3) expression was correspondingly lower in the group eating hard food in the early stage after altered loading.[6,7] Furthermore, a previous study showed that mechanical overloading could induce VEGF expression in cartilage discs via HIF-1α.[8] All these results indicate that HIF-1α and VEGF expression, when induced by an increased food load, might be related to the expression of ADAMTS-4 and TIMP-3. Thus, it is reasoned that there might be some correlative mechanisms between mechanical loading (dietary loading) and hypoxia, which lead to the expression of HIF-1α, VEGF, ADAMTS-4, and TIMP-3 in chondrocytes. Since $CoCl_2$ is a commonly used hypoxia-mimetic agent,[9] it was utilized in the present study, the aim of which was to examine the expression of HIF-1α, VEGF, ADAMTS-4, and TIMP-3 in rat mandibular condylar chondrocytes under hypoxic conditions and examine the relationship of these expressed factors in early condylar cartilage growth.

Materials and Methods

A total of 60 3-week-old female Sprague-Dawley rats (obtained from the Research Ethics Boards at the Experimental Animal Center of Guangxi Medical University) were sacrificed by sedation. After their mandibles were exposed, the soft tissue on the surface of the condyles was removed. The mandibular condyles were bilaterally excised, and the fibers and perichondrium on the surface of the condylar cartilage were removed. The condylar cartilage was cut into small pieces that were about 1 mm^3 in volume. The individual pieces were then mixed with 0.02% ethylene diaminetetraacetic acid (EDTA) + 0.25% trypsin complex digestive juice in about 10 times the volume of each piece, and thereafter incubated at 37°C in 5% CO_2 for approximately 20 minutes. Digestion was terminated when the tissue pieces were loose and soft. Next, the samples were centrifuged for 5 minutes at 1,000 rpm, the supernatant decanted, and the pellet resuspended in 5 mL 0.2% type II collagenase. The samples were incubated at 37°C for about 3 hours while being gently agitated to mix. The cartilage tissue blocks became inflated and flocculent macroscopically. Digestion was stopped when microscopy revealed that large numbers of cells were isolated. The digested products were then filtered through a 200-mesh stainless steel sieve, and the resultant filtrate containing the individual cells was centrifuged for 5 minutes at 1,000 rpm. Pelleted cells were resuspended in medium. The cell number was determined using a hemocytometer, and cell viability was examined using the trypan blue dye exclusion method. The cell counting was done by an investigator (HH) and was repeated three times. The total number of cells was represented as the mean (± standard deviation) of these three measurements.

Chondrocytes (3×10^4 cells/mL) were subpackaged into culture bottles and incubated in a 37°C, 5% CO_2 humidified incubator. Cell attachment was measured 12 hours later using an inverted phase contrast microscope. Images of the attached cells were captured and cell morphologies recorded. The medium was refreshed on the third day after culture, and replaced every 2 days thereafter. Primary cells were subcultured when they reached 80% to 90% confluency. The same method was used to subculture chondrocytes. Cells were cryogenically frozen for further use and thawed when required. The growth curves of the cultured cells were obtained by using the well-established 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method.

Cell Identification

Condylar chondrocytes were identified using two different methods: toluidine blue staining and type II collagen detection.

Toluidine Blue Staining. This was performed according to Terry's method.[10] The chondrocytes were fixed in 4% formaldehyde for 30 minutes and then stained for 4 hours with 10 g/L toluidine blue at normal temperature. Cultures were then washed twice with 95% alcohol. This technique stains the nuclei dark blue and the cytoplasm and extracellular matrix purple under a light microscope.

Type II Collagen Detection. A two-step immunohistochemical staining kit protocol was used. The chondrocyte sections were fixed in 4% formaldehyde for 20 to 30 minutes, then they were incubated with 0.1% Triton X-100 for 10 minutes at room temperature. Endogenous peroxidase activity was quenched by treatment with 0.2% H_2O_2 for 3 hours. After washing with phosphate buffer solution (PBS), the sections were incubated at 4°C with diluted monoclonal rabbit anti-rat type II collagen primary antibody (0.001 mg/mL, Boster Biological Technology) overnight. For negative and positive controls, the sections were incubated in 0.01 mol/L PBS without any primary antibody. Immunostaining was visualized with streptavidin peroxidase (SP) kits (Maixin) by using a peroxidase and diaminobenzine substrate. The sections were counterstained with Mayer's hematoxylin and subsequently examined with an image analyzer

under a light microscope (Leica Leitz). Type II collagen was seen as tan particles in the cytoplasm under microscope. The presence of type II collagen and morphologic characteristics indicated that the cultured cells were condylar chondrocytes.

$CoCl_2$-Induced Hypoxia Model for Condylar Chondrocytes

In order to prevent dedifferentiation and phenotypic instability,[11] the second-generation chondrocytes were chosen from the first-generation chondrocytes that had been digested with trypsin, randomly divided into four experimental groups, and seeded on 96-well plates.[12] When the cells reached 50% confluency, high glucose Dulbecco's modified eagle's medium (DMEM) containing different concentrations of $CoCl_2$ (group A, 75 μmol/L; group B, 125 μmol/L; group C, 175 μmol/L; and normoxic group, 0 μmol/L) was added. After 48 hours, the culture medium and cells were assayed for lactic dehydrogenase (LDH) activity, according to the instructions of the enzyme-linked immunosorbent assay (ELISA) kit, and absorbance was measured at a wavelength of 440 nm. Percentage LDH release rate, which represents the degree of cell damage, was calculated according to the following formula: LDH activity in culture medium / (LDH activity in culture medium + LDH activity in cells) × 100%.

Chondrocyte Culture in 125 μmol/L $CoCl_2$

At 50% confluency, 125 μmol/L $CoCl_2$ (Anpel) was added to the second-generation cultures to induce hypoxia. For the normoxic control subjects, cultures were not treated with $CoCl_2$. At 12, 24, and 48 hours after treatment, mRNA levels of cytokines were measured with a semiquantitative polymerase chain reaction (PCR).

Detection of HIF-1α, VEGF, ADAMTS-4, and TIMP-3 mRNA Expression

The total RNA was extracted from each experimental group, in accordance with the RNA extraction kit instructions. RNA was then converted to cDNA by reverse transcriptase, following kit instructions from the manufacturer (Promega). For conventional PCR reactions, 2 μL of cDNA was used, while β-actin mRNa levels were used concurrently as an internal reference (Table 1).

The PCR conditions for HIF-1α were as follows: pre-denature at 94°C for 2 minutes; then 30 cycles of 95°C for 45 seconds, 64°C for 1 minute, and 72°C for 1 minute, with final extension at 72°C for 15 minutes.

VEGF PCR conditions were as follows: pre-denature at 95°C for 5 minutes; then 35 cycles of 95°C for 45 seconds, 61°C for 30 seconds, and 72°C for 1 minute, with final extension at 72°C for 10 minutes.

Table 1 PCR Primer Sequences and Their Amplified Product Sizes

Primer	Sequence (5′–3′)	Size (bp)
HIF-1α	Forward 5′-AAGTCTAGGGATGCAGCAC-3′ Reverse 5′-CCAGATCACCAGCATCTAG-3′	130
VEGF	Forward 5′-GGTGAGAGGTCTAGTTCCGAAC-3′ Reverse 5′-CCATGAACTTACTGCTCTTCTT-3′	115
ADAMTS-4	Forward 5′-CGCTGACCGCCAATGCCAACTG-3′ Reverse 5′-GCCCAAGGTGAGTGCTTCGTCTG-3′	385
TIMP-3	Forward 5′-GGCAAGAAGCTGGGTAAAGGAG-3′ Reverse 5′-GTCGGATGCAGGCGTAGTGTT-3′	121
β-actin	Forward 5′-TCGCTACGCCAACACAGTGC-3′ Reverse 5′-CATCTCCTGCTTGCTGATCC-3′	274

Amplicon size and abundance were analyzed by agarose gel electrophoresis using 2% agarose. The Discovery Series Quantity One (Version 4.4) analysis software (Bio-rad) was used to quantify band intensities of HIF-1α, VEGF, ADAMTS-4, and TIMP-3 amplicons, and these were then compared to the internal β-actin control. Gene expression (from each sample) was defined as the intensity of each target gene/intensity of the β-actin band. Gene expression experiments were repeated three times and then subject to statistical analysis.

Statistical Analysis

Experimental data were represented as means ± standard deviation. A univariate variance analysis (pairwise least significant difference *t* test) using a SPSS 13.0 software package was performed to test for differences between groups. $P < .05$ was considered to reflect a statistically significant difference.

Results

Observation of Cell Growth and Morphologies

Primary chondrocytes obtained from rat condylar tissue had a survival rate of 95% ($P > .05$), as detected by trypan blue staining. They are characterized by their spherical shape, suspension in medium, and strong refractive index during the early stages of culturing. During the first 24 hours of incubation, some cells gradually adhered to the bottom of the plate, completely attached, and formed protrusions (Fig 1a). At 3 to 5 days, these cells rapidly proliferated, extended their protrusions, and obtained a triangular morphology. Moreover, they formed a monolayer with weak cell-to-cell contacts. Their cell nuclei were clear and either round or oval shaped, with one to two nucleoli. There were many mitotic cells, and some cells engulfed matrix particles into the cytoplasm (Figs 1b and 1c). The cells were completely confluent after 7 to

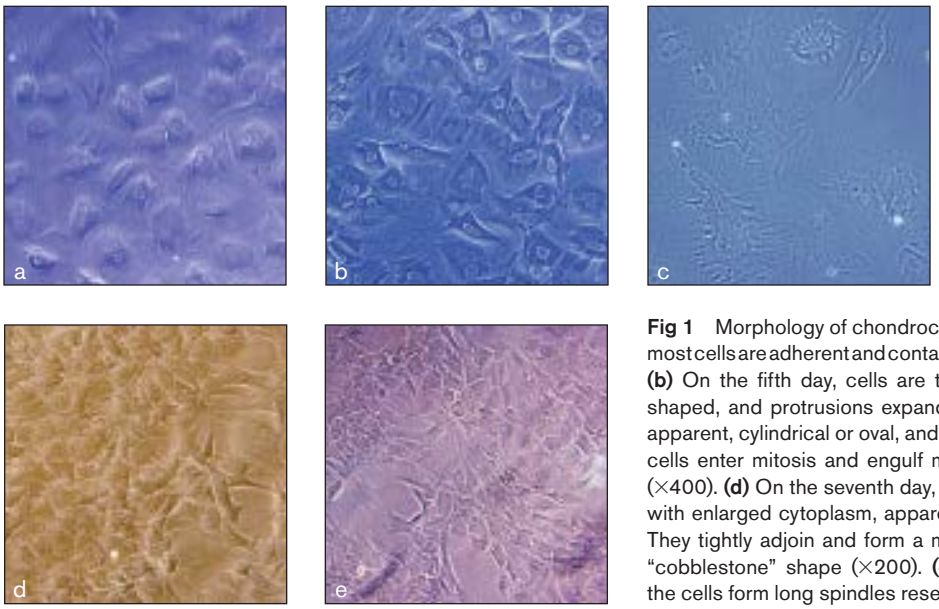

Fig 1 Morphology of chondrocytes. **(a)** At 24 hours after seeding, most cells are adherent and contain stretched-out processes (×200). **(b)** On the fifth day, cells are triangular-, spindle-, or polygonal-shaped, and protrusions expand gradually (×200). **(c)** Nuclei are apparent, cylindrical or oval, and contain one or two nucleoli. Some cells enter mitosis and engulf matrix granules into the cytoplasm (×400). **(d)** On the seventh day, cells are mostly polygonal-shaped with enlarged cytoplasm, apparent nucleoli, and strong refraction. They tightly adjoin and form a monolayer, reminiscent of a typical "cobblestone" shape (×200). **(e)** Fifth-generation chondrocytes; the cells form long spindles resembling fibroblasts (×200).

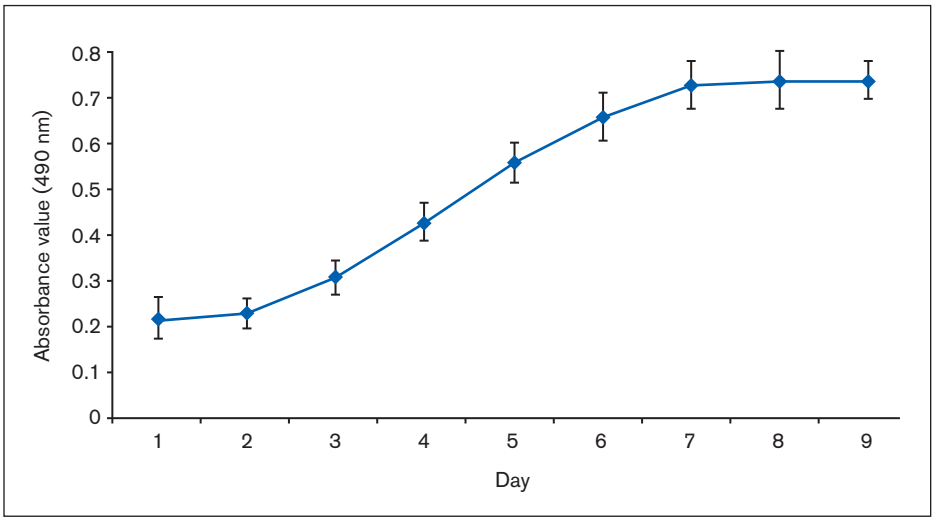

Fig 2 The growth curve of second-generation chondrocytes shown as mean ± SD of absorbance value.

8 days of growth. These cells were mostly polygonal, with abundant cytoplasm, clear nucleoli, and a strong refractive index. Their outer membranes were continuous and formed a tight monolayer (Fig 1d).

The primary chondrocytes were subcultured about 7 or 8 days after their initial seeding. First- or second-generation cells thrived and looked like primary cells, which were mostly polygonal and spindle-shaped. Fourth-generation cells gradually stretched and became more spindle-shaped cells, which had a weak refractive index. After the fifth generation, most cells were spindle-shaped and resembled fibroblasts (Fig 1e). These spindle-like cells spread and flattened out as the subculture continued. They were relatively long and large with a weak refractive index.

Growth Curve of Chondrocytes

The growth of second-generation condylar chondrocytes was characterized by a slow phase, which lasted for 1 to 2 days, followed by a logarithmic phase in the next 3 to 7 days. Finally on the eighth day, the cells reached confluency, grew slowly, and presumably entered the plateau phase (Fig 2).

Identification of Condylar Chondrocytes

Toluidine blue staining was used; this stains the chondrocyte nuclei a dark blue color, while the cytoplasm and extracellular stains purple (Fig 3a). Small metachromatic granules surrounding the cells were also observed using this technique. Chondrocytes were detected by the presence of type II collagen, as

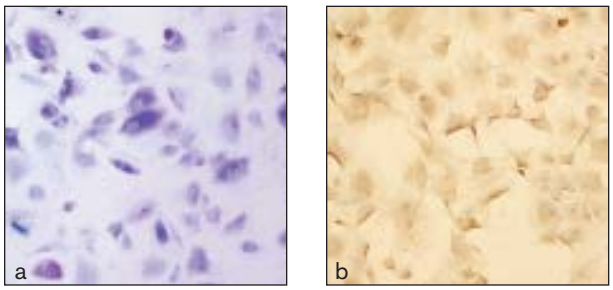

Fig 3 **(a)** Nuclei of chondrocytes stained dark blue. Cytoplasm and extracellular matrix appear purple (toluidine blue staining, ×200). **(b)** Cytoplasm and extracellular matrix express brown particles, while the nuclei are not stained (IHC, ×200).

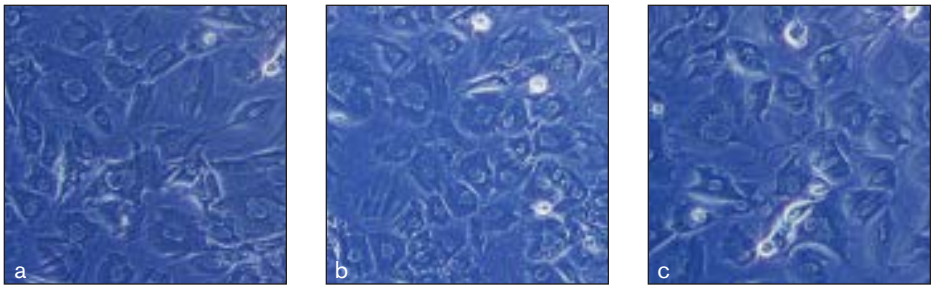

Fig 4 Cartilage cells treated for 48 hours. **(a)** With 75 μmol/L $CoCl_2$, the cells grow well and maintain typical morphology. **(b)** With 125 μmol/L $CoCl_2$, cell membrane boundaries are ambiguous. Some cells shrink and appear rounded. **(c)** With 175 μmol/L $CoCl_2$, a large number of cells are reduced in size and have compromised nuclear architecture.

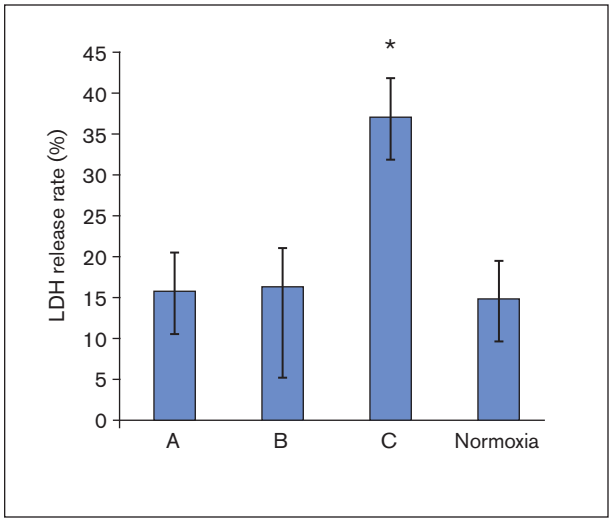

Fig 5 Comparison of lactic dehydrogenase (LDH) release rates in different concentrations of $CoCl_2$: 75 μmol/L (group A), 125 μmol/L (group B), and 175 μmol/L (group C), compared with that of the normoxic group. * $P < .01$.

tan granules in the cytoplasm and extracellular matrix, on immunohistochemical staining. In fact, type II collagen is specifically expressed in the cytoplasm and extracellular matrix of chondrocytes (Fig 3b).

Morphologic Changes of Chondrocytes Under Different Concentrations of $CoCl_2$

Cells treated with 75 μmol/L of $CoCl_2$ (group A) were similar to the normoxic control group; they grew well and had typical morphologic structures (Fig 4a). However, cells treated with 125 μmol/L $CoCl_2$ (group B) did not have obvious cell boundaries, and some cells were rounded and reduced in size (Fig 4b). Cytotoxic effects were observed in cells treated with 175 μmol/L of $CoCl_2$ (group C). These cells were largely reduced in size, rounded, had compromised nuclear membrane structure, and were suspended in the medium (Fig 4c).

LDH Release Rate Under Different Concentrations of $CoCl_2$

The LDH release rate positively correlated with the $CoCl_2$ concentration; however, while this value was similar in groups A and B to values from the normoxic group, it dramatically doubled in group C ($P < .01$) (Fig 5).

HIF-1α, VEGF, ADAMTS-4, and TIMP-3 mRNA Expression Levels

HIF-1α, VEGF, ADAMTS-4, and TIMP-3 mRNA expression were analyzed in response to treatment, and

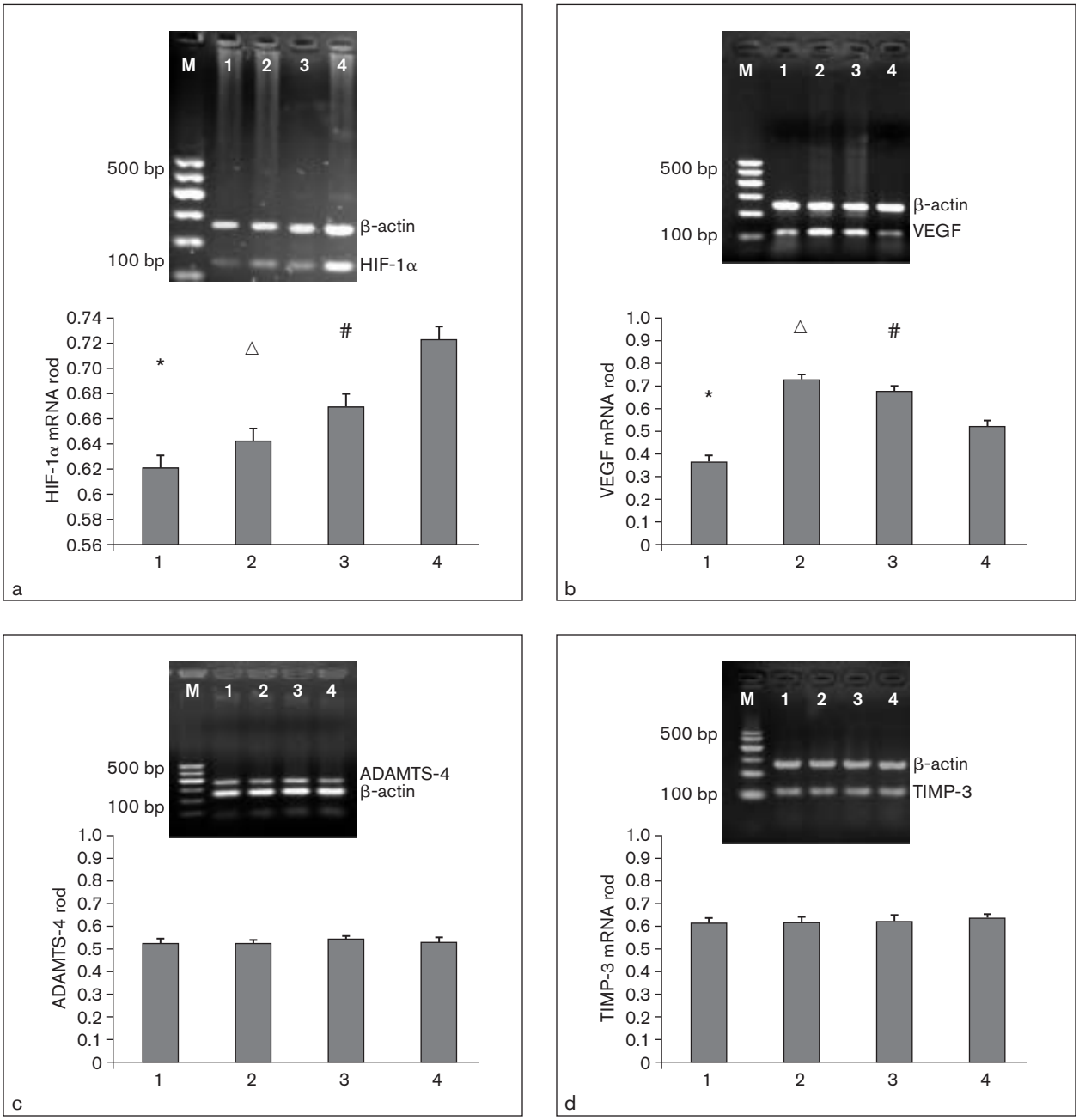

Fig 6 **(a)** HIF-1α, **(b)** VEGF, **(c)** ADAMTS-4, and **(d)** TIMP-3 mRNA expression in condylar cartilage cells. M = marker; 1 = normoxic group; 2 = hypoxia for 12 hours; 3 = hypoxia for 24 hours; 4 = hypoxia for 48 hours. * Compared with 2, 3 or 4, $P < .05$; △ compared with 3 or 4, $P < .05$; # compared with 4, $P <. 05$.

their levels were compared to those of the expression of β-actin (Fig 6). HIF-1α mRNA expression was low under normoxic conditions. However, its levels were significantly higher after treatment with $CoCl_2$ for 12, 24, and 48 hours ($P < .05$). HIF-1α mRNA expression was found to be time dependent, as statistically significant increases were detected when comparing expression levels from 12 to 24 hours, 12 to 48 hours, and 24 to 48 hours ($P < .05$).

Like HIF-1α, VEGF expression levels were also elevated throughout the entire duration of the experiment. However, VEGF expression levels did not increase in a time-dependent fashion. Instead, a peak was observed 12 hours after treatment, which was then followed by a steady drop in levels at subsequent time points.

PCR results showed that ADAMTS-4 and TIMP-3 mRNA were expressed under normoxic conditions. Their expression levels in the hypoxic cell culture model did not significantly change after treatment with $CoCl_2$, and minor changes did not reach statistical significance ($P > .05$) (Fig 6).

Discussion

The establishment of a simple yet effective chondrocytic culture model under hypoxia is necessary to investigate the development of the TMJ and its associated diseases.[13] There are two ways to create such a hypoxic model. One method is to use special devices to control the partial pressure of oxygen, and the other is to sequester oxygen by adding chelators such as $CoCl_2$ into the medium. The former requires special equipment and is difficult to control for exact conditions. Oxygenation of cells in the $CoCl_2$ medium has been widely used to establish hypoxic culture models for different cells, such as endothelial cells, kidney epithelial cells, retinal pigment epithelial cells, etc.[14,15] In the present study, $CoCl_2$, which is a well-characterized and highly established hypoxia mimetic,[16,17] was used. Moreover, because there is a strong correlation between the $CoCl_2$ concentration and deoxygenation, the use of this reagent allows for precise control over the extent and timing of the onset of hypoxia. The concentration range of $CoCl_2$ commonly used is 100 to 250 μmol/L.[18,19] However, the effective range needs to be determined empirically for each cell line. For example, studies have shown that only 100 μmol/L of $CoCl_2$ could effectively simulate the effects of hypoxia in accessory nerve cells.[20] In the present study, the LDH release rate was used as an index to assess the effects of the different concentrations of $CoCl_2$ on chondrocyte growth, survival, and damage. According to the results of the LDH release rate, 175 μmol/L $CoCl_2$ was toxic to cells and not suitable for the establishment of a hypoxic model, so a concentration of 125 μmol/L was employed to establish subsequent hypoxic models in the present study.

Previous studies have confirmed that HIF-1α and VEGF are necessary cytokines for chondrocyte survival and the normal development of long bones.[21] The study showed that HIF-1α and VEGF are expressed, albeit weakly, in chondrocytes cultured under normoxic conditions (Fig 6), suggesting that they might be under autocrine control and regulated by subsequent physiologic processes.

Lin et al have confirmed that HIF-1α upregulation is mediated by the extent and length of hypoxia.[22] They showed that HIF-1α upregulation was detected at 30 minutes when cells were incubated with 0.2% oxygen. This effect lasted for at least 48 hours. Gene expression was examined in the first 48 hours from the onset of hypoxia, since chondrocytes are very sensitive to loading changes and readily initiate adaptive mechanisms following this early observational period.[7] The studies found that HIF-1α and VEGF expression levels were higher in hypoxic cells after 12, 24, and 48 hours of treatment.

The relationship between VEGF and HIF-1α was further explored in the hypoxia cell culture model. VEGF and HIF-1α are closely associated with hypoxia. It is generally thought that HIF-1α is an important upstream regulatory factor of VEGF and a core regulatory element in hypoxia-mediated angiogenesis.[23] HIF-1α binds to the promoter or enhancer regions of VEGF and other target genes, enforces their transcription, and increases their mRNA stability.[24] Some studies have found that VEGF mRNA levels increase commensurately with HIF-1α levels in hypoxic smooth muscle cells or tumor cells.[25,26] However, in the hypoxic model of chondrocyte cells, HIF-1α expression levels continued to rise throughout the entire 48-hour duration of the study, while VEGF expression levels were elevated initially but peaked at 12 hours and finally declined. These results are consistent with previous studies conducted on hypoxic osteoblast culture models.[27] According to these studies, significant variations in the hypoxic responses occurred among different cell types correlating to the varying concentrations of the hypoxia regulators to which they were subject.[28]

As described earlier, the degradation of extracellular matrix occurs because of a loss of aggrecan, which is a critical early event that is mediated by aggrecanases.[5] Studies have shown that a change in food hardness could bring a loading change in the TMJ. Meanwhile, stress might first affect VEGF expression levels in early stages, thereby generating changes in aggrecans, MMPs, and TIMPs expression levels. In this way, the development and degradation of condylar cartilage matrix are tightly regulated.[5,6] Pufe et al found that mechanical overloading increased the expression of HIF-1α, VEGF, and MMP, while decreasing that of TIMP.[8]

In this study, the results repeatedly showed that VEGF and HIF-1α are closely associated with $CoCl_2$-induced hypoxia, but ADAMTS-4 and TIMP-3 expression levels did not change under $CoCl_2$-induced hypoxic conditions. Therefore, the results are quite different from those of the aforementioned studies, suggesting that there may be different mechanisms acting on the expression of ADAMTS-4 and TIMP-3 than those on HIF-1α and VEGF when mechanical overloading of the TMJ occurs in vivo and chemically induced hypoxia conditions occur in vitro.

Inflammatory cytokines, such as interleukin-1β (IL-1β), were also found to be strongly associated with hyperalgesia over the TMJ.[29] Mechanical loading and inflammatory cytokines can initiate a matrix destructive pathway in tendon.[30] Moreover, the combination of hypoxia and IL-1β may also contribute to the degradation or remodeling of the extracellular matrix, via an increase in MMPs and decrease in TIMP-3 in the TMJ.[13] These data taken together suggest that

the chemically induced hypoxic chondrocytic model should be combined with inflammatory cytokines to fully investigate the pathogenesis of TMJ disorders.

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