Study on the effects of mechanical pressure to the ultrastructure and secretion ability of mandibular condylar chondrocytes

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ABSTRACT

During mandibular movement, condyle is subjected to repetitive compression and the mandibular condylar chondrocytes (MCCs) can detect and respond to this biomechanical environment by altering their metabolism. The present study was undertaken to investigate the effects of pressure to the ultrastructure, aggrecan synthesis, nitric oxide (NO) and prostaglandin F$_{1\alpha}$ (PGF$_{1\alpha}$) secretion in MCCs.

In vitro cultured rabbit MCCs were incubated and pressed under continuous pressure of 90 kPa for 60 min and 360 min by hydraulic pressure controlled cellular strain unit. The ultrastructure, aggrecan mRNA expression, activity of nitric oxide synthase (NOS) and PGF$_{1\alpha}$ secretion were investigated. Besides, nitric oxide inhibitor was used together with pressure to investigate the role of NO in mechanical effects.

The appearance of MCC on TEM showed that after been pressed under 90 kPa for 60 min, the cellular processes became elongated and voluminous, together with aggrecan mRNA increasing. Under 90 kPa for 360 min, some of the cells showed distinct sign of apoptosis and the aggrecan mRNA decreased. Pressure of 90 kPa could cause increase of NOS activity and decrease of PGF$_{1\alpha}$ composition. Inhibitor experiments indicated that pressure-induced upregulation of aggrecan mRNA and inhibition of PGF$_{1\alpha}$ synthesis was partly mediated by NO.

Continuous pressure could cause changes on the ultrastructure and function of MCC, as well as up-regulation of aggrecan synthesis, increase of NO secretion and decrease of PGF$_{1\alpha}$ composition. NO was the upstream molecule, which mediated the response of aggrecan and PGF$_{1\alpha}$ to mechanical pressure.

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1. Introduction

Articular cartilage of the synovial joint experiences loading conditions which can produce tissue stresses exceeding hundreds of atmospheres, requiring chondrocytes and their extracellular matrix to withstand and biologically respond to these physical forces in order to maintain and remodel articular cartilage to meet functional requirements. This process is termed mechanotransduction and can be resolved into extracellular and intracellular events. Temporomandibular joint (TMJ) is the only joint that keeps relatively active reconstruction ability for all one’s life. During the normal mandibular movement, it is subjected to repetitive compressive loading and the mandibular condylar chondrocytes (MCCs) within the tissue can detect and respond to this biomechanical environment by altering their metabolism.
Studies relating to biological and physical stimuli at the cellular level to the load-induced biological response of chondrocytes, such as altered ultrastructure and secretion of matrix macromolecules, could provide key information in understanding cartilage remodelling processes.

Articular cartilage covers the end of bones and protects the underlying bone against shearing and compressive forces. Cartilage is composed of a proteoglycan and collagen-rich extracellular matrix containing chondrocytes. Collagen forms a meshwork that imparts tensile strength, and proteoglycans form large aggregates that provide resistance to compression.\(^1\) The maintenance of cartilage matrix integrity is critically dependent on mechanical stimulation and cartilage thickness reflects the total load transmitted by the joint. Among all the matrix molecules secreted by chondrocytes, aggrecan, the large aggregating extracellular proteoglycan, is the main proteoglycan component of the cartilage matrix. It was reported that load-induced flow stimulated the aggrecan production during dynamic loading of cartilage\(^8\) and IL-4 involved membrane hyperpolarization under mechanical stimulation was also related with upregulation of aggregan mRNA level.\(^9\)

Studies in osteoblasts, endothelial cells and primary chondrocytes have demonstrated the varied production of soluble factors, such as prostaglandins (PG) and nitric oxide (NO), in response to mechanical stimulation.\(^10\)–\(^13\) NO is a diatomic free radical that acts as an inter- and intracellular messenger molecule in many cell types, including articular chondrocytes. NO is synthesized from L-arginine by enzymes known as nitric oxide synthases (NOS) of which three isoforms have been identified. Two isoforms, collectively known as cNOS, are constitutively expressed while the third isoform, iNOS, is expressed following the induced activation by endotoxins, LPS or cytokines. cNOS and iNOS activity can be inhibited by structural analogues of L-arginine such as L-(1-iminoethy)-ornithine (L-NIO).\(^14\) PG is one of the most important paracrine signalling factors in the transferring process of mechanical stimulation in the cells,\(^15\) which has been shown to mediate adaptive bone formation in vivo.\(^16\)–\(^17\)

Several studies have demonstrated that physical forces can influence NO production by articular chondrocytes in vitro, with conflicting results. Under shear force, it was reported that fluid-induced shear stimulated the release of NO and PG by isolated bovine chondrocytes.\(^16\)–\(^19\) Under tissue force, cyclic tensile strain was shown to inhibit both NO and PG synthesis by lapine articular chondrocytes and temporomandibular fibrochondrocytes.\(^20\)–\(^21\) Under pressure, some reported static and intermittent compression increases NO production by pig cartilage explants,\(^22\) whereas, some others reported the inhibition of NO and PG by dynamic compression in the agarose/chondrocyte cylinders.\(^23\)–\(^24\) Furthermore, several authors have reported an induction of PGE2 via COX in parallel with an upregulation of NO.\(^25\)–\(^26\) However, contradictory evidence exits, with further studies reporting that the suppression of NO synthesis augmented PGE2 synthesis. Still further, Chowdhury et al.\(^27\) demonstrated that dynamic compression-induced inhibition of PGE2 synthesis and stimulation of proteoglycan synthesis were NO mediated in the primary chondrocytes.

Our study aimed to characterize the changes of form and function of MCCs at different pressure conditions and to test the hypothesis that static pressure condition alters the ultrastructure and synthesis of aggrecan, NO and PGE\(_2\), which also differs with different pressure level. In order to gain insight into molecular mechanism which could be responsible for altered aggrecan and PGE\(_2\) synthesis, we use a NO inhibitor to further testify the role of NO in regulation of its downstream molecules.

2. Materials and methods

2.1. Source of cartilage and basic culture procedure

For this study, three New Zealand white rabbits, 1 or 2 weeks old, were killed. Bilateral mandibular condyles with part of the ramus were removed aseptically from the temporomandibular joints and stored in sterile Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grang Island, NY). The mandibular condylar cartilages were dissected under a microscope and MCCs were cultured according to the method of Engel et al.\(^27\) After being digested with 0.25% trypsin for 1 h and 0.1% collagenase for 2 h, the cells were separated from the debris by filtration through a 40-\(\mu\)m-mesh nylon sieve, suspended in DMEM, and identified by type II collagen immunocytotoxic reaction. After being passed for one to two times since the initial plating, MCC got stable growth character. Because the primary MCC could only keep their chondrocytes phenotype for about sixth to seventh passages, 5–10 \(d\) cultures, nonconfluent, of the third to fourth passages were used in all experiments in an attempt to limit changes in gene expression (dedifferentiation). Morphologically, the cells studied were typically flattened with a polygonal cell shape and did not show the fibroblastic appearance of dedifferentiated chondrocytes.

2.2. Mechanical pressure on the cell culture

Hydrostatic pressurization has been a very frequently used modality for compression of cells, tissues, or explant cultures. It included negative (vacuum) and positive pressurization. Hydrostatic compression holds several substantial attractions: simplicity of the equipment, spatial homogeneity of the stimulus, ease of configuring multiple loading replicates (via manifolding), and ease of delivering and transducing either static or transient loading inputs. There is no physical impediment of metabolite transport processes between the culture layer and the nutrient medium. Moreover, the loading delivered is not dependent on the state of adhesion between the culture and its substrate. To simulate compressive stress to the cultured MCC, a self-designed hydraulic pressure-controlled cellular strain unit was applied, which followed the model developed by Yousefian and Firouzian.\(^28\) The pressure was generated by continuously compressing the gas phase (2% CO\(_2\) in air) in a closed culture chamber (humidity 98%), which was placed in a 37 °C incubator. It can exert accurate, adjustable and identical hydraulic pressure upon cells and can fairly well imitate mechanical circumstance of the MCC in vivo.

2.3. Mechanical pressure condition selection

To select the proper pressure condition combined by different pressure time and pressure value, the alkaline phosphatase
(ALP) activity and cell proliferation were adopted for investigation. ALP was determined in cell lysates of cell culture. Cell cultures were washed twice with PBS, harvested in 0.5 ml H2O by scraping, and sonicated for 10 min at 1250 × g (3000 rpm) and the supernatant was transferred to eppendorf tubes and stored at −20 °C until assay for ALP activity, which was determined using p-nitrophenyl phosphate (Merck) as a substrate at pH 10.3 and the absorbance was determined at 410 nm using a microplate reader. ALP activity was expressed as nmol p-nitrophenol released per hour and per mg protein (cells). The cell proliferation was performed in 96-well microtiter plates and tested by methabenzthiazuron (MTT) method. The absorbance was determined by enzyme-linked immunosorbtent assay (Bio-Rad, USA). Data were analysed using one-way analysis of variance, and the significance among means was determined by Dunnett t method.

2.4. Transmission electron microscope of MCCs

Nonconfluent MCC cells from the third to fourth passages plated in 55-mm plastic Petri dishes (Nunc) were digested with 0.25% trypsin for 5 min and centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the cell deposit block was fixed immediately after being removed from sacrificed animals by immersion in glutaraldehyde (2.5% glutaraldehyde in 0.1 M sodium cacodylate with 2 mM CaCl2, pH 7.3). Cell specimens fixed in glutaraldehyde can be kept for up to a couple of days without degradation. Then, they were washed three times using TEM rinsing buffer (0.13 M NaH2PO4, 0.05% MgCl2, pH 7.4), with 10 min for each time. Following dehydration with acetone and post-fixing with 0.1 M cacodylate buffered 1% osmium tetroxide for about 1 h, tissue specimens were stained using 1% uranyl acetate and lead citrate overnight in dark. They were then dehydrated using increasing concentrations of ethanol alcohol, from 25 to 100% ethanol. Fully dehydrated tissues were then embedded in resin, which was cured in oven at a temperature of 60 °C for 1 or 2 days. TEM specimens were prepared by cutting 50 nm thick foils from the resins using Ultracut UCT Microtome (Leica). The thin slices were then transferred onto copper TEM grids. After further staining in 1% osmium tetroxide for about 3 min, the samples were ready for TEM observation. The TEM analysis was carried out in a high resolution TEM (JEM-2000EX, Japan).

2.5. In situ hybridization of aggrecan mRNA

MCC cells from the third to fourth passages were plated at a density of 1 × 10^5 ml^-1 in six-well microtiter plates placed with coverglass beforehand and incubated at 37 °C under 5% CO2 in air in DMEM supplemented with 10% FBS for 24 h. The subcultures were divided into five groups (n = 6 per group) with different treatments: 60 min continuous pressure of 90 kPa; 360 min continuous pressure of 90 kPa; pretreatment with 1 mM 1-N-(1-iminoethyl)-ornithine (l-NIO) for 12 h plus continuous pressure of 90 kPa for 60 min or for 360 min. The subcultures without l-NIO or pressure were used as the control.

For the detection of aggrecan mRNA, oligonucleotides with the following sequence were synthesized: Aggrecan 5’-CTC TGC GAA GCA GTA CAC GTC ATA G-3’. Digoxigenin UTP-labelled single-stranded RNA probes were prepared using a DIG RNA Labelling Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions.

At the end of culture, cover slips with cells from each group were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 10 min at 4 °C. Following this, cells were digested with proteinase K, post-fixed with 4% paraformaldehyde (PFA), rinsed with water, acetylated for 10 min with acetic anhydride, and rinsed with PBS. Cover slips were prehybridized in 50% formamide, 4 × SSC (0.6 M NaCl, 60 mM sodium citrate), 10% dextran sulphate, 2 mM EDTA, and 1 × Denhardt’s. The RNA probe was applied to cover the tissue (400 ng/ml) and an RNase-free parafilm slide cover placed over the cells. Hybridization was performed at 50 °C for 18–36 h, and cover slips were carefully washed with 2 × SSC at room temperature, then at 37 °C, and finally with 50% formamide, 1 × SSC at 37 °C. Visualization using DIG-antibody was done following blocking and rinsing as described in the DIG kit. Cover slips were incubated in 4% PFA in PBS at 4 °C for 8 h and then washed in TBS. After that, a 1:1000 dilution of NBT/BCIP substrate was added with 100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl2 for 2 min in a Coplin jar. Staining was stopped by incubating cells for 15 min in 250 ml of TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) at room temperature and then rinsed in water. Cover slips with cells were overturned and mounted with one to two drops of Aquamounti (preheated to 50 °C) onto slides, which were viewed and photographed on a Nikon Labophot light microscope with Nikon CF60 objectives (200×).

2.6. Biochemical analysis of NO

The Greiss reaction assay was used to indirectly measure NO production by quantifying the production of the reactive nitrogen intermediate, nitrite (NO3−). This assay is a colorimetric assay, which quantifies the accumulation of nitrite in the culture medium. Nitrite is stable in culture medium even in the presence of cells. The amount of nitrite present is determined by comparison with a standard curve using solutions of sodium nitrite (Sigma). MCCs (10^5 cells/ml) were seeded into round bottom 96-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ). An aliquot of 100 ml of conditioned culture media was removed from each well and placed into a flat-bottom 96-well tissue culture plate. An equal volume of the Greiss reagent (1% sulphanilamide/0.1% naphthylenediamine/2.5% phosphoric acid) was added, plates were incubated 10 min at room temperature and the absorbance at 550 nm was recorded. Results are expressed as NOS activity (U/ml), which was calculated according to molar amounts of nitrite per 1 ml serum per minute.

2.7. Radioimmuology assay of 6-keto-PGF1α

Concentrations of PGF alpha was determined in 25–100 ml of incubation medium by using 6-keto-PGF1αRIA Kit from the Institute of Isotopes. PGF was assayed according to Chang et al. with sensitivities of 25 pg/ml, respectively. The cross-reactivity of the PGF antiserum was <0.1% to other metabolizing productions of prostaglandin. Inter- and intra-assay
coefficients of variation did not exceed 3.5 and 10%, respectively.

3. Statistical analysis

High power image analysis system (HPIAS) 1000 (Tongji University, China) was used for measuring the ISH staining results, which was corrected by the same area of the same slide as the blank. The positive ratio of the cells was calculated as following. Five ISH slides were chosen randomly from each group for light microscope and 10 different visual fields under high power lens (magnification, 400×) were chosen randomly from each slide for observation. From each visual field, the numbers of the positive cells and the total number of the cells were accounted, respectively. The ratio of them was just the positive frequency. The average data was calculated based on five random visual fields of each group. The semi-quantitative analysis of mRNA content was determined by the absorbency of all the positive cells under one high power visual field, which conformed the same randomization principle as mentioned above. The data from image analysis of aggrecan ISH staining, biochemical analysis of NO and radioimmunology assay of PGF1α were all expressed as mean ± S.E.M. Statistical evaluations were conducted by Student’s t-test for paired data. For multiple comparisons, ANOVA followed by Turkey analysis was used. A P-value smaller than 0.05 was considered statistically significant.

4. Results

4.1. Morphology and identification of the cells

Phase-contrast microscope showed that cells grown in primary culture underwent distinct morphological changes with respect to shape, size, and density of the cells. At 12 h after initial plating, the cells attached to the plate were round and refractile, varying in their size. After 3 days, most of the cells became dark, enlarged, and spread out. Fusiform and spindle-shaped cells were present in low density areas, and polygonal cells in high-density areas. Small round cells were scattered throughout the cultures. By the seventh day, the culture had reached confluence. The cells enlarged evidently with one or two large and round nuclei. There were plentiful granule in the cytoplasm (Fig. 1). The cells from third passage plated for type II collagen staining showed positive in cytoplasm (Fig. 2).

4.2. Mechanical pressure condition selecting

The ALP activity and proliferation of the control cells showed increasing gradually with the culture time prolonged. Under 30 kPa pressure, ALP activity showed no variation and the cell proliferation decreased when the pressure prolonged to 720 min (P < 0.05, Table 1). Under 60 kPa pressure, ALP activity increased (P < 0.05, Table 1) but cell proliferation decreased (P < 0.01, Table 1) when the cells being pressed for more than 360 min. Under 90 kPa pressure, ALP activity increased from time point of 60 min (P < 0.05, Table 1) but cell proliferation decreased till the cells being pressed for more than 360 min (P < 0.01, Table 1). According to the principle that the shorter is the cells being processed in vitro, the better it is for their growth, the short-period pressure condition was fixed on 90 kPa for 60 min, under which ALP activity of the cells could be promoted without affecting the cell proliferation and the long-period pressure condition was fixed on 90 kPa for 360 min, under which the cell proliferation was prohibited. Based on that results, the succeedent pressure experiments were all grouped as 90 kPa/60 min pressure, 90 kPa/360 min pressure and control.

4.3. Ultrastructure of MCCs

The appearance of MCC under TEM showed that the cells of the control developed well with short cellular microvillus around them without any sign of apoptosis. In the cells, there were plenty of rough endoplasmic reticulum, normal mitochondria with distinct ridge, small quantity of lysosomes and lipid, abundant viscid cytoplasm and big nucleus with one to two notch (Fig. 3a). After been pressed under 90 kPa for 60 min,
the cells showed elongated and voluminous cellular processes. And the rough endoplasmic reticulum were dilated and narrowly arranged (Fig. 3b). When the pressure prolonged to 360 min, some of the cells showed distinct sign of apoptosis such as chromatin condensation, chromosomal clumping and margination. There were expansive mitochondria without ridge, a great amount of lipid globules and matrix vesicles, as well as the typical apoptotic body (Fig. 3c).

![Ultrastructure of MCC under TEM (N = 6).](image1)

**Fig. 3 – Ultrastructure of MCC under TEM (N = 6).** (a) Cells of the control developed well without any sign of apoptosis and surrounded by a thin pericellular zone containing short cellular microvillus. In the cells, there were plenty of rough endoplasmic reticulum, normal mitochondria with distinct ridge, small quantity of lysosomes and lipid, abundant viscid cytoplasm and big nucleus with one to two notch (magnification, 4000×; bar = 2 μm). (b) After been pressed under 90 kPa for 60 min, the cells showed elongated and voluminous cellular processes. Rough endoplasmic reticulum of the cells showed dilated and narrowly arranged (magnification, 10 000×; bar = 500 nm). (c) When the pressure prolonged to 360 min, some cells showed distinct sign of apoptosis with the typical apoptotic body (magnification, 4000×; bar = 2 μm).

![Expression of aggrecan mRNA in MCCs by ISH (light microscope, N = 6; magnification, 200×; bar = 10 μm).](image2)

**Fig. 4 – Expression of aggrecan mRNA in MCCs by ISH (light microscope, N = 6; magnification, 200×; bar = 10 μm).** (a) There was small amount of aggrecan mRNA in the cells of control observed as the pearl blue granules dotted in the cytoplasm. (b) After been pressed under 90 kPa for 60 min, the cells showed the strong positive expression of target mRNA, large indigo granules in the cytoplasm. (c) When the pressure prolonged to 360 min, some small pearl blue granules could still be observed.

### Table 1 – ALP activity and cell proliferation under different pressure (X ± s)

<table>
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<tr>
<th>Time (min)</th>
<th>0 kPa</th>
<th>30 kPa</th>
<th>60 kPa</th>
<th>90 kPa</th>
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<tr>
<td>0</td>
<td>0.102 ± 0.003</td>
<td>0.098 ± 0.006</td>
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<tr>
<td>60</td>
<td>0.104 ± 0.006</td>
<td>0.101 ± 0.004</td>
<td>0.107 ± 0.003</td>
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<tr>
<td>360</td>
<td>0.142 ± 0.006</td>
<td>0.134 ± 0.005</td>
<td>0.132 ± 0.004</td>
<td>0.203 ± 0.015*</td>
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<tr>
<td>720</td>
<td>0.183 ± 0.005</td>
<td>0.176 ± 0.002</td>
<td>0.212 ± 0.004*</td>
<td>0.198 ± 0.009*</td>
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<tr>
<td>Proliferation (n = 6)</td>
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<tr>
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</tr>
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<td>360</td>
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<td>0.39 ± 0.02**</td>
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<tr>
<td>720</td>
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<td>0.57 ± 0.03a</td>
<td>0.42 ± 0.02**</td>
<td>0.38 ± 0.03**</td>
</tr>
</tbody>
</table>

* vs. 0 kPa P < 0.05.
** vs. 0 kPa P < 0.01.
4.4. Expression of aggrecan mRNA in MCCs (Table 2)

The results of ISH showed that there was small amount of aggrecan mRNA in the cells of control (151.3 ± 7.29), which was observed as the pearl blue granules dotted in the cytoplasm (Fig. 4a). After been pressed under 90 kPa for 60 min, the cells showed the strong positive expression of target mRNA, which could be observed as large indigo granules in the cytoplasm (Fig. 4b). When the pressure prolonged to 360 min, the mRNA signal expressed weaker than that of the cells under 90 kPa for 60 min. Still some small pearl blue granules could be observed (Fig. 4c). The positive frequency in the 90 kPa/60 min pressure group was 49.23 ± 3.67% higher and that of 90 kPa/360 min pressure group was 18.35 ± 1.75% higher than that of control (P < 0.05). With L-NIO treatment, expression of aggrecan mRNA of 90 kPa/60 min group (201.1 ± 9.32) was still stronger than that of control (141.43 ± 8.53) (P < 0.05) but weaker than that of 90 kPa/60 min group without L-NIO intervention. Whereas, that of 90 kPa/360 min group (153.5 ± 9.07) showed no difference with that of the control (P > 0.05). The increase of positive frequency of the two pressure groups were 22.79 ± 2.43% (P < 0.05) and 2.36 ± 1.38% (P > 0.05), respectively.

4.5. NOS activity and 6-keto-PGF$_1\alpha$ secretion of MCCs under pressure

Under the pressure, the NOS activity of MCCs was prominently promoted through 1–12 h (Fig. 5). The differences between stimulated and unstimulated cells were showed as Fig. 6, from which we could see that the stimulation effects of pressure to NOS activity in 1 h group was the highest and then it decreased with the pressure time prolonged. Pressure of 90 kPa significantly inhibited the 6-keto-PGF$_1\alpha$ secretion of MCCs from 4 h on (Fig. 7). The inhibition effects increased with the pressure continued (Fig. 8). While the pressure-induced PGF$_1\alpha$ inhibition could be partly weakened in the presence of 1mM L-NIO (Fig. 7).

5. Discussion

5.1. Selection of the pressure condition

It is not possible for us to know the real pressure value that the condylar cartilage endured in vivo. So the values in the simulation experiment in vitro varied a lot according to different mechanical instruments, and different researchers. But the intra-articular pressure is always used as the practical standard. In TMJ, the range of intra-articular pressure with normal occlusion in ICP is from 0.5 to 1.5 kPa. For the intra-articular cavity of TMJ is very small and the relative function parts of fossa, disc and condyle are closely contact with each other so the real pressure that the cartilage endured should be much higher than that of intra-articular pressure. Besides, the results of our finite element analysis showed that when a normal occlusion was in ICP, the stress in the condylar cartilage was about 0.3 MPa. So at first we confined the range of

| Table 2 – Expression of aggrecan mRNA in MCCs (X ± s) |
|-----------------|-----------------|-----------------|
|                | 0 kPa           | 90 kPa/60 min   | 90 kPa/360 min |
| Untreated       | 151.3 ± 7.29    | 232.5 ± 8.26$^*$| 194.7 ± 8.21$^*$|
| 1 mM L-NIO      | 141.43 ± 8.53   | 201.1 ± 9.32$^*$| 153.5 ± 9.07$^*$|

* vs. 0 kPa P < 0.05.
$^*$ vs. 0 kPa P < 0.01.
* vs. untreated P < 0.01.
observed that although most of MCCs could keep the normal form and function, there were still some cells showed elongated and voluminous cellular processes, which was probably related to the improvement of secretion and anchoring function of chondrocytes optimized by mechanical pressure. The exact significance of that phenomenon need further investigation.

5.2. Effects of pressure to the ultrastructure of MCC

It was reported that occlusal trauma could result in the regressive changes in tempromandibular condylar cartilage and synovial membrane and presumed that it was related with the changes of biomechanical environment of TMJ secondary to the occlusal trauma. The results of this study manifested that after been pressed under 90 kPa for 360 min, some of MCCs showed distinct sign of apoptosis. It confirmed that excessive pressure could really induce the regressive remodelling of mandibular condylar cartilage. It was also observed that although most of MCCs could keep the normal form and function, there were still some cells showed elongated and voluminous cellular processes, which was probably related to the improvement of secretion and anchoring function of chondrocytes optimized by mechanical pressure. The exact significance of that phenomenon need further investigation.

5.3. Effects of pressure to the proteoglycan secretion of MCC

Under normal circumstances, the articular cartilage deformed when enduring the mechanical stress and resumed to original state when it was unloaded. Whereas, the excessive load which surpassed the adaptability of cartilage or the insufficient mechanical stimulation could result in the damage of chondrocytes, proteinase release and proteoglycan loss in the matrix.

Aggrecan is the main proteoglycan component of the cartilage matrix, which is very important for the maintaining of normal physiological function of TMJ cartilage. Valhmu et al. reported that aggrecan mRNA synthesis of primary chondrocytes increased under the pressure of 0.1 MPa for 1 h and decreased back to the basic level at 4 h. Our results confirmed that short-period (60 min) low pressure condition (90 kPa) upregulated the synthesis of aggrecan mRNA, which was probably related to the improvement of remodelling process of mandibualr condylar cartilage under pressure. With the pressure time prolonged, the aggrecan mRNA level returned back gradually but still higher than that of the control at 360 min. It suggested that at that time the MCCs still kept the relatively higher ability for matrix synthesis while the potential reservoir of that ability already decreased.

5.4. Effects of pressure to NO and PGF synthesis of MCC

There are only small amount of NO synthesized by the normal MCCs. Mechanical compression clearly influences NO production by chondrocytes. The current findings are consistent with previous studies which demonstrated that shear stress, applied to isolated chondrocytes and static or intermittent compression, applied to cartilage explants induced an increased NO synthesis. However, cyclic tensile strain and dynamic pressure acts to suppress IL1-induced induction of NO or PGE2 synthesis in isolated lapine chondrocytes cultured in monolayer or human chondrocytes cultured in agarose constructs. The differences to our study may, potentially, be attributed to several factors including different nature of mechanical stimulus, different in vitro culture model systems, and various mechanical conditions combined by different stimulus time, value and frequency.

It was reported that mechanical stimulation of shear stress, uniaxial strain and micro-gravity could all stimulate the PG synthesis by osteoblasts. An increased PGE synthesis is one of the important factors for cartilage degenerative disorders in osteoarthritis. The increased expression of PGE was also observed in the articular fluid of patients with temporomandibular disorders. The current results showed that 90 kPa static pressure could suppress the PGF synthesis, which is consistent with the previous study.

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**Fig. 7** – 6-Keto-PGF$_{1\alpha}$ secretion of MCCs cultured under 90 kPa static pressure through 12 h. Values are estimated from six replicates. The results of ANOVA followed by Turkey analysis. For multiple comparisons indicate differences among the three groups as follows: vs. control $P < 0.05$, vs. control $P < 0.01$, and vs. pressure $P < 0.01$.

**Fig. 8** – The differences between the cells with or without pressure stimulation for 6-keto-PGF$_{1\alpha}$ secretion of MCCs. Bars represent mean and standard error of six replicates. Paired Student’s t-test results indicate differences between the odds of two adjacent pressure time point. Values as follows: ***$P < 0.001$.**
demonstrating that strain induced while pressure reduced the PG synthesis for in vitro cultured cartilage tissue.\textsuperscript{29} Chowdhury et al.\textsuperscript{2} had also proved that IL-1\beta-induced PGE\textsubscript{2} production could be inhibited apparently by dynamic compression in human chondrocytes. All of these results confirmed that feasible mechanical stimulus is very important for TMJ cartilage to keep its normal structure and function.

5.5. Action of NO in mechanotransduction process of MCC

As a kind of small gas signal molecule, NO acts directly to the intracellular target molecules without the help of any specific membrane receptor. It is even more important for the cartilage which is a kind of tissue short of blood supply. The current findings showed that aggrecan synthesis was augmented by the mechanical pressure and the promotion effects could be partly impaired by \textsuperscript{1}-NO\textsubscript{O} confirming that synthesis of NO by nitric oxide synthase enzymes is required to mediate this mechanotransduction process. The results suggest, therefore, that the regulation of proteoglycan synthesis by mechanical compression is partly mediated by NO but not completely achieved through common pathways. Furthermore, pressure inhibited PGF\textsubscript{1\alpha} production was partially reversed by \textsuperscript{1}-NO\textsubscript{O}, suggesting that NO is an upstream mediator of PGF\textsubscript{1\alpha} production in the compression-induced signalling cascade and participates in the PGF regulation under pressure. On the other hand, we can see from Figs. 4 and 6 that the pressure-induced NO increase descended with the pressure time prolonged. By the contrast, the pressure-inhibited PGF increased by degrees with the time. The different variation trend between the two molecules suggested us that there are still other upstream signal molecules participating in the regulation of NO, PGF and proteoglycan under pressure.

6. Conclusions

Continuous pressure could cause changes on the ultrastructure and function of MCC, as well as up-regulation of aggrecan synthesis, increase of NO secretion and decrease of PGF\textsubscript{1\alpha} composition. NO was the upstream molecule, which mediated the response of aggrecan and PGF\textsubscript{1\alpha} to mechanical pressure.

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