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Investigation for Effects of Cyclical Dynamic Compression on Matrix Metabolite and Mechanical Properties of Chondrocytes Cultured in Alginate

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Abstract: Articular cartilage is suffered from high mechanical loads under normal physiological conditions and articular chondrocytes regulate the composition of peri-cellular matrix (PCM), in response to mechanical signals. This study aims to investigate influence of cyclical dynamic compression on matrix metabolite and mechanical properties of chondrocytes cultured in alginate. Two months old New Zealand white rabbits were sacrificed by air embolism. Chondrocytes were taken from knee joints under aseptic conditions and cultured in alginate. The suspension was gelled in a shape of disc in CaCl₂ solution and processed for intermittent compression by using Flexcell-5000 compression system kept under an uncompressed condition as a control. The gene regulation of aggrecan (Agg), collagen α1 (Col-2), matrix metallo-proteinases 13 (MMP-13) and collagen X (Col-10) were determined by reverse transcription polymerase chain reaction (RT-PCR) at 7th, 14th and 21st day post loading. The alteration in mechanical properties of chondrocytes induced by compression was evaluated using micropipette aspiration technique combined with a half-space model. A significant up-regulation was observed in gene expression of Agg and Col-2 on 7th day or MMP-13 and Col-10 on 14th day respectively after loading of compression. In response to compression, chondrocytes exhibited viscoelastic solid creep behavior. Young’s modulus almost approximated to control group (0.65±0.42kPa). However, viscoelastic properties decreased gradually along with intermittent compression. Viscoelastic properties in experimental 21st day, including equilibrium modulus (Eₑ), the instantaneous modulus (E₀) and apparent viscosity (μ) were significantly lower than experimental 7th day and 14th day (P<0.001), no significant difference was found between experimental 7th day and 14th day (P>0.05). In conclusion, chondrocytes cultured in alginate are stable of mechanical properties and suitable to construct seed cell of tissue engineering, and can keep phenotype and mechanical properties.

Key words: Alginate, Chondrocytes, Compression, Mechanical properties, Tissue engineering

Introduction

The articular cartilage is under a variety of stress in a complex physiological environment, and the role of the compression which can cause the chondrocytes changes of biology and mechanical properties is an important factor in the joint movement. The changes of the mechanical properties will directly affect the physiological function of articular cartilage. Our previous studies demonstrated that the mechanical properties of chondrocytes cultured in alginate were more stable than the monolayer cultured in vitro. However, it is unclearly whether chondrocytes can maintain their normal biological and mechanical properties under the different mechanical stimulation in alginate.

Recently studies revealed continuous cyclic compressive loading to be beneficial in the cultivation of chondrocyte-seeded scaffolds and hydrogels. Chondrocytes cultured in alginate could induce the production of cartilage-specific extracellular matrix components and promote chondrogenesis in embryonic precursor cells. Thus, the further study on the mechanical properties of chondrocytes will expound a series process of stress condition and response in maintaining normal physiological function. To understand the biological properties of chondrocytes under dynamic compression and the mechanical properties and the metabolism responding to mechanical stimulation is vital for functional engineering cartilage.

Therefore, chondrocytes cultured in alginate were submitted to dynamic compression with FX-5000™ Compression System (Flexcell, USA) in this study. Then we measured the viscoelastic properties of chondrocytes cultured in alginate from rabbit knee articular cartilage using micropipette aspiration technique with a standard linear viscoelastic solid model.

Materials and Methods

Preparation and culture of chondrocytes

New Zealand white rabbits (n=20) were acquired from Medical
Full-thickness articular cartilage were removed from the femoral condyles and tibial plateaus of rabbit knee joints immediately following euthanasia.

The chondrocytes (mainly pick up the hyaline cartilage) were isolated by using sequential 0.4% pronase (Sigma, USA) and 0.025% collagenase-2 (Sigma) digestions in Dulbecco’s Modified Eagle Medium-F12 (DMEM-F12, HyClone, USA). After straining through a 100μm nylon cell strainer (BD, USA) and centrifugation, the chondrocytes were mixed with alginate in a cell density of 4×10⁵ cells/ml. The suspension was gelled in a shape of disc in CaCl₂ solution and each disc contains 50il suspensions. The discs of chondrocytes and alginate compound were cultured in Biopress™ compression plates (Flexcell, USA). A volume of 4 ml DMEM-F12 culture medium was added to each well. The cell/alginate constructs were maintained in culture for 6 days in 5% CO₂ at 37°C.

**Compression experiments**

In this study, the chondrocytes/alginate constructs were placed into individual wells of Biopress™ compression plates (Flexcell, USA) and processed for intermittent compression on these constructs by using the FX-5000™ Compression System (Flexcell, USA). The strain regimen consisted in cyclical compression with pulses of 20 kPa at a frequency of 0.5 Hz for 1 hour per day in 5% CO₂ at 37°C, continuous loading 7th day, 14th day and 21st day. As a control, cell/alginate constructs were maintained under uncompressed conditions.

**Reverse transcription and RT-PCR analysis for mRNA expression**

The total RNA was isolated from the chondrocytes/alginate constructs and cDNA was synthesized using PrimeScript™ RT (Takara, Japan) followed by a 20 μl reaction for real time PCR amplification using SYBR® Premix Ex Taq™ (Takara, Japan) according to the manufacturer’s protocol. Sequences of the primers used were as follows: Agg, forward: TCTACCGCTGTAGGGTG ATGC, reverse: TTCACCACTGACCTCAAGG; Col-2, forward: ACACCTGCAAAGTCCAGATG, reverse: GTGA TGTTCTGGGG AGGCCCTC; MMP-13, forward: ACACGGATCTGCAAGA GA, reverse: CGTGAGAAGTGGTATTGGGATGCA; Col-10, forward: AGCCAGGGTTGC CAGGACC, reverse: CCAGGAGCACCATACTCGT; GAPDH, forward: GGTGAA GGTCGGAGGTGAACG, reverse: AGTTAAAAGCAGCCCTGGT GA. Amplification was performed in iCycler iQ (Bio-Rad, USA) with a temperature profile of an initial de-naturation step of 2 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and an extension step of 3 min at 72°C. Levels of gene expression were determined by using the comparative Ct values with GAPDH gene as endogenous control.

**Measurement of viscoelastic properties of chondrocytes**

The viscoelastic properties of the isolated chondrocytes were tested with the micropipette aspiration technique, in a similar fashion to that described previously⁵. The micropipettes were pulled from borosilicate capillaries (Olympus, Japan) using a Pipette Puller (Sutter, USA) with a range of inner tip diameters from 4 to 5 microns, and the pipettes were soaked in 5% Acetum for at least 48 hours before use.

The chondrocytes/alginate constructs were dissolved as cell suspension. One milliliter of chondrocyte suspension (4×10⁵ cells/ml) was loaded into a small chamber. The tip of the micropipette was made to approach the surface of the spherical chondrocytes, and the cells were gripped at the tip of the micropipette under a negligible negative pressure. The negative pressure was fixed to a desired value (0.3 to 0.4 kPa), which caused a time-dependent aspiration deformation in a portion of the cells. The length of chondrocyte aspiration was determined from optical imaging recorded every 2 seconds until 200 seconds total time, which represented an instantaneous deformation. The time-dependent aspiration was measured in each graph by Olympus Measurement Software (Olympus, Japan) and the exponential curve was fitted by Origin 8.0 Software. The viscoelastic parameters, including, \( E_\infty \) (equilibrium modulus), \( E_0 \) (instantaneous modulus), and \( \mu \) (apparent viscosity), were calculated coupled with the standard linear viscoelastic solid model as an earlier theoretical study has described⁶.

**Statistical analysis**

Data were presented as mean ± standard deviation (SD) in this study. Statistical analysis between the groups was performed using one-way analysis of variance (ANOVA) in SPSS 17.0 software. A P value <0.05 was considered statistically significant.

**Results**

**Gene expression analysis following compression**

Real time PCR analysis indicated that the expression levels of Agg and Col-2 were significantly increased in response to dynamic compression at 7th day compared to all other time points (P<0.05), then returned to levels near or below the control values. A significant (P<0.05) increase in Col-10 and MMP-13 activity in response to dynamic compression at the 14th day time point was observed in comparison with the control constructs that remained uncompressed and all other time points (Fig. 1).

**Viscoelastic behaviors of the chondrocytes**

In response to an applied constant negative aspiration pressure of 0.3 to 0.4 kPa, all chondrocytes exhibited standard linear viscoelastic solid properties under dynamic compression. Compare
to the chondrocytes cultured in alginate with unloading dynamic compression, the chondrocytes showed an initial elastic response followed by a viscoelastic creep behavior, and then continued to enter into the micropipette with a monotonically decreasing rate of deformation under dynamic compression. When determined the differential pressure ($\Delta p$), the length of chondrocyte aspiration ($L$) and the inner diameter of the micropipette ($a$), the Young’s modulus ($E$) was (n=50, 0.65±0.42 kPa). The exponential curve between $\Delta p$ and standardized length ($L/a$) was fitted by Origin 8.0 Software (Fig. 2).

**Differences in viscoelastic properties of chondrocytes on different compression days**

Model predictions of the creep data have shown that the average equilibrium modulus ($E_\infty$), the instantaneous modulus ($E_i$), and the apparent viscosity ($\mu$) of the chondrocytes in the experimental 21st day was significantly lower than the experimental 7th day and 14th day ($P<0.001$). Furthermore, no significant difference was found between the experimental 7th day and 14th day ($P>0.05$, Table 1). Meanwhile, exponential fitting data have shown that the $E_\infty$ (0.36±0.10kPa), $E_i$ (0.66±0.12kPa), and $\mu$ (6.56±0.91kPa·s) of the chondrocytes in the experimental 7th day were, respectively, higher than the $E_\infty$ (0.23±0.06kPa), $E_i$ (0.50±0.07kPa), and $\mu$ (3.87±0.59kPa·s) of the chondrocytes in the experimental 21st day ($P<0.001$). The chondrocytes in the experimental 21st day were too stiff to demonstrate viscoelastic creep in this study.

The instantaneous length of the time-dependent aspiration into the micropipette was significantly increased for the chondrocytes in the experimental 21st day, but the time of the viscoelastic creep

![Figure 1](image1.png)

**Figure 1.** Changes in gene expression in response to dynamic compression on different days. A: The expression of Agg increased at the 7th day. B: Col-2 expression increased at the 7th day. C: Col-10 expression increased at the 14th day. D: MMP-13 expression increased at the 14th day.

![Figure 2](image2.png)

**Figure 2.** The standard linear elastic properties of chondrocytes at 3rd day. A: Elastic behavior of chondrocytes in response to the compression at 7th day fit with linear regression. B: The Young’s modulus ($E$) compared with control, *$P<0.05$.

<table>
<thead>
<tr>
<th>Groups</th>
<th>$E_\infty$ (kPa)</th>
<th>$E_i$ (kPa)</th>
<th>$\mu$ (kPa·s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compress 7th day</td>
<td>0.36±0.10</td>
<td>0.66±0.12</td>
<td>6.56±0.91</td>
</tr>
<tr>
<td>Compress 14th day</td>
<td>0.32±0.08</td>
<td>0.63±0.09</td>
<td>6.33±0.83</td>
</tr>
<tr>
<td>Compress 21st day</td>
<td>0.23±0.06*</td>
<td>0.50±0.07*</td>
<td>3.87±0.59*</td>
</tr>
</tbody>
</table>

* $P <0.001$, compared with the experimental 7th day and 14th day.

Table 1. Viscoelastic parameters of chondrocytes from the rabbits knee articular cartilage cultured in alginate on different compression days (n=50, Mean ± SD)
reaching equilibrium was reduced to (50±10) seconds compared with the experimental 7th day and 14th day (Fig. 3F). There was no significant difference detected in the amplitude aspirated into the micropipette or the time reaching equilibrium (110±20) seconds between the experimental 7th day and 14th day as the control groups (Fig. 3A, B, C, D and E).

Discussion

Chondrocytes as seed cell of tissue engineering are always cultured in vitro without the mechanical stimulation. These chondrocytes lacking the mechanical stimulation do not have the biomechanical properties of normal cartilage cells in cartilage tissue engineering. It has been suggested to build a better tissue-engineered cartilage by physiological mechanical stimulation⁹.

The scaffold materials of tissue engineering play an important role in growing microenvironment of chondrocytes. Previous studies have demonstrated that three-dimension culture such as alginate culture could induce the production of cartilage-specific extracellular matrix components and promote chondrogenesis in embryonic precursor cells⁹. Alginites have been used in a variety of clinical applications, because they are tolerated well when placed in contact with the body and they have a similar structure to glycosaminoglycans (GAG), one important constituent of the extracellular matrix⁹. Alginate scaffold is similar to the cartilage physiological conditions which not only can enhance the nutrient and oxygen transfer to affect the chondrocytes metabolism, but also provide the possibility of applying a proper mechanical stimulus to the cell-construct to influence the biosynthesis activity in chondrocytes¹⁰. However, it has not been tested in cartilage tissue engineering.

In this study, chondrocytes cultured in alginate were exposed to cyclical dynamic compression which could cause a series of physiological changes of chondrocytes, including the stress and strain changes of the articular cartilage. Dynamic compression can promote the secretion of extracellular matrix from chondrocytes, which is similar to the articular cartilage in vivo. Collagen I and proteoglycan are main composition characteristic of cartilage extracellular matrix, which play an important role in the biomechanical function such as resistant to compression and deformation¹⁰. In this context, recent investigations of loaded physical extracts from four-week swine ulnae showed losses by immunohistochemistry in Agg, Col-2 and Col-10 under static compressive loading¹¹. In this study, chondrocytes were isolated from the rabbits knee articular cartilage and cultured in alginate under cyclical dynamic compression with pulses of 20 kPa at a frequency of 0.5 Hz. The gene expression of Agg and Col-2 were significantly up-regulated on 7th day after dynamic compression, indicating that the proper mechanical stimulation could increase the extracellular matrix of cartilage, which would be important significance to explain the earlier study of the unconfined dynamic compression induced changes in cartilage biosynthesis. These results showed that the influence between chondrocytes and matrix related to the frequency dependence of cells mechanics properties, responded the hypothesis of chondrocytes could regulate
biosynthesis under dynamic compression.

However, chondrocytes anabolic is different under different stimulation. The study by Mauck\textsuperscript{13} using bovine chondrocytes embedded in agarose gel had shown that high dynamic compressive loading decreased Agg and Col-2 activity when measured 24 h after the end of the compression regimen. Another study using human chondrocytes were harvested from knee joints of patients having undergone total knee replacement and seeded into a collagen type I hydrogel, which were subjected to mechanical stimulation for 28 days with 10% continuous cyclic compressive loading at a frequency of 0.3 Hz. Then gene expression analyses revealed a significant increase in Agg and Col-2 under cyclical dynamic compressive loading\textsuperscript{14}. These opposite results illustrated the difference of chondrocytes biosynthesis was attributed to the different scaffold materials, the different mechanical stimulation and the cell sources. Our study found that as stimulation increased in compression days, chondrocytes tended to hypertrophy that showed gene expression of Col-10 and MMP-13 increased obviously, Col-2 was degradation and the structural integrity of chondrocytes was damaged. Although these four genes (Agg, Col-2, Col-10 and MMP-13) demonstrated changes critical for physeal structural integrity and function, it is likely that the broader effects of static compression on rabbit physes would have been forthcoming if additional genes and the counterpart secreted molecules had been assessed\textsuperscript{15}.

Studies of cell mechanics found that the mechanical properties of chondrocytes could significantly affect the physiological function of the articular cartilage. Micropipette aspiration of cells in suspension is a well-characterized method of measuring elastic and viscoelastic properties of cells\textsuperscript{16,17}. The viscoelastic properties of the isolated chondrocytes from normal articular cartilage were tested using the micropipette aspiration technique, showed that all chondrocytes exhibited standard linear viscoelastic solid properties\textsuperscript{18}. In this study, the micropipette aspiration technique coupled with a standard linear viscoelastic solid model was used to measure the biomechanical properties of isolated chondrocytes from rabbit knee cartilage under cyclical dynamic compressive loading. The results indicated that chondrocytes cultured in alginate with continuous cyclic compressive loading have defined patterns of viscoelastic behavior that are different from the compression days, which have shown that the changes in the mechanical properties of articular cartilage are due to alterations in the matrix that occur with age in previous studies\textsuperscript{19-22}. In our study, we found that the early deformation rate and the equilibrium length of time-dependent aspiration into the micropipette were increased in chondrocytes with compression 21th day under the same negative pressure, and the viscoelastic properties ($E_{\text{com}}$, $E_{r}$, and $\mu$) in chondrocytes with compression 21st day were reduced absolutely compared to compression 7th day and 14th day chondrocytes. It implies that the changes of viscoelastic properties in chondrocytes with compression and ageing may play an important role in the changes of biomechanics of articular cartilage. Therefore, we speculated that some of the signaling pathway, including Ihh signaling\textsuperscript{23}, PI-3 kinase-Akt signaling\textsuperscript{24}, p38 MAPK signaling pathway\textsuperscript{25}, may involve in the mechanisms of the chondrocyte function or maturing in mechanotransduction of chondrocytes. In the following studies, we would investigate the specific mechanism.

This study demonstrated chondrocytes cultured in alginate three-dimensional under physiological dynamic compressive loading in vitro are stable of mechanical properties by micropipette aspiration and suitable to construct the seed cell of tissue engineering. Moreover, the chondrocytes under the physiological compression cultured in alginate can keep the phenotype and the mechanical properties, but the matrix decreases and the mechanical properties decline with the increasing of compression days. Understanding the biomechanics properties of chondrocytes cultured in alginate and how the mechanical properties influences the cell response to compression stimulation will be vital to functional cartilage engineering.

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Competing Interests

The authors have declared that no COI exists

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