Effect of Mechanical Stress in Combination with Verapamil on Levels of Aggrecan and *ADAMTS-5* mRNAs and Proteins in Human Osteoarthritic Chondrocyte/Agarose Constructs

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To the Editor: Osteoarthritis (OA) has the highest prevalence and economic impact among arthritic maladies and is the most common cause of long-term disability among individuals over 65 years of age. OA is characterized by the degeneration of articular cartilage and the loss of cartilage matrix in affected joints. It is widely accepted that an imbalance between the biosynthesis and the degradation of extracellular matrix (ECM) occurs in OA cartilage, leading to degeneration and gradual cartilage loss. During the onset and progress of OA, changes occur in the composition of the ECM, which is composed primarily of type II collagen and aggrecan (AGC). The breakdown of AGC is believed to be initiated by a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS)-4 and ADAMTS-5.[1]

Mechanical stimulation has been shown to have a strong influence on chondrocyte biosynthetic activity.[2] Furthermore, multiple studies have suggested a regulatory role for intracellular Ca^{2+} in endochondral ossification, a process that includes chondrocyte proliferation, differentiation, and apoptosis.[3] Based on the above findings, we hypothesized that mechanical stress alone or in combination with the voltage-sensitive Ca^{2+} channel inhibitor verapamil would stimulate chondrocytes with respect to AGC biosynthesis.

OA chondrocytes were obtained from 10 OA patients (aged 58–75 years) who had undergone total knee replacement at the Department of Orthopedic Surgery, Huadong Hospital, Fudan University. The patients met the American College of Rheumatology classification criteria for the diagnosis of OA. Informed consent from the patients was obtained for all cartilage samples used in this study. Chondrocytes were cultured at a density of 2×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% of mixed norvancomycin hydrochloride (10 mg/L, wt/vol in normal saline [NS]), amikacin sulfate injection (10 mg/L, wt/vol in NS), and fluconazole injection (0.1 mg/L, wt/vol in NS). Chondrocytes were cultured as a monolayer in humidified atmosphere at 37°C and 5% CO_2.

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The agarose hydrogels were liquefied by heating in boiling water until they were homogeneous at temperatures below 40°C. Chondrocytes were then centrifuged and suspended in the agarose solution at a density of 2×10^6 cells/ml. The cells were counted by the trypan blue exclusion method using a hemocytometer, and 8 ml of the mixture was poured into a culture dish with a diameter of 60 mm. The chondrocyte/agarose constructs were then allowed to gel at room temperature and punched to obtain cylindrical gels (5 mm in diameter and 3 mm in height) that were placed into the wells of a Biopress™ compression plate (Flexcell International Corporation, Burlington, North Carolina, USA). The cell/agarose constructs were maintained in culture for 24 h in 5% CO_2 at 37°C.

The experimental and control group samples were subjected to mechanical load using a FX-5000C Flexcell® Compression Plus™ System (Flexcell International Corporation, Burlington, North Carolina, USA). After placing the chondrocyte/agarose constructs into the wells of the Biopress™ compression plates, the stationary platens were adjusted to the height of the samples, and the compression plates were placed on the Biopress™ baseplate. The strain regimen was programmed using the Flexcell software. Compressions of 0, 6, 12, and 24 kPa were applied to the cell/agarose constructs using a static waveform for 0.5, 1, and 2 h. An experimental group of primary OA chondrocytes was treated with verapamil (40 μmol/L). The verapamil solution (5 mg in 2 ml distilled water) was diluted with 10 ml saline, and 120 μl of the diluted solution was added directly to

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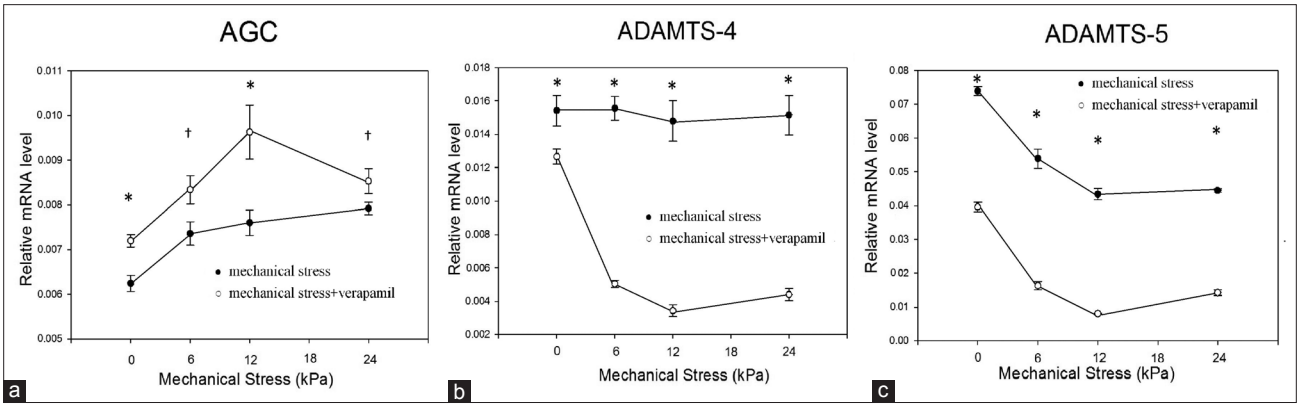

Figure 1: The expression of *AGC* (a), *ADAMTS-4* (b), and *ADAMTS-5* (c) mRNA under different mechanical loadings (0–24 kPa) in concert with 40 μmol/L verapamil treatments. These experiments were repeated three times. $n = 10$. Columns represent mean; bars represent SD. *$P < 0.01$, †$P < 0.05$. SD: Standard deviation; *AGC*: Aggrecan; *ADAMTS*: A disintegrin and metalloproteinase with thrombospondin-like motifs.

the culture medium of the wells in the Biopress™ compression plates before compression.

Total RNA and protein were extracted from chondrocytes in agarose gels in accordance with the manufacturer's protocol. The results are presented as the mean ± standard deviation. Statistical significance was assessed by analysis of variance or Student's *t*-test using SPSS Version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant for all tests.

To investigate the effect of mechanical loading on *AGC, ADAMTS-4*, and *ADAMTS-5*, mRNA expression in human chondrocytes was quantified using real-time polymerase chain reaction. As the mechanical loading time was increased from 0.5 to 2 h, the expression of AGC mRNA was significantly upregulated at 6 and 12 kPa ($P < 0.001$ and 0.002, respectively) and *ADAMTS-5* mRNA was downregulated at 6, 12, and 24 kPa ($P < 0.001$). By contrast, no significant differences in the expression of *ADAMTS-4* mRNA were observed for any of the loading time or loading strengths studied ($P = 0.640$, 0.764, and 0.988, respectively).

Then, *AGC*, *ADAMTS-4*, and *ADAMTS-5* mRNA expression under different mechanical loadings (6–24 kPa) was also compared. *AGC* expression was significantly different among three loading groups at 0.5, 1, and 2 h ($P = 0.006$, 0.005, and <0.001, respectively). ADAMTS-5 expression was different among groups with different culture strengths at 1 and 2 h (both $P < 0.001$). No significant differences in the expression of *ADAMTS-4* mRNA were observed under the range of loading strengths studied ($P = 0.561$, 0.939, and 0.807, respectively).

As shown in Figure 1a, verapamil increased levels of AGC mRNA in OA chondrocytes at all intensities of mechanical stress examined (0, 6, 12, and 24 kPa; $P = 0.004$, 0.015, 0.006, and 0.029, respectively). By contrast, as shown in Figure 1b and 1c, verapamil treatment decreased levels of *ADAMTS-4* and *ADAMTS-5* mRNA in OA chondrocytes at all intensities of mechanical stress examined (all $P < 0.01$).

Western blotting analysis of cellular extracts of OA chondrocytes cultured in control and mechanically stressed constructs revealed that 6, 12, and 24 kPa mechanical stress increased expression of AGC and decreased expression of ADAMTS-5. The effects of mechanical stress were also shown increase with time. By contrast, stress and time had no significant effect on levels of ADAMTS-4 compared to control samples. Consistent with AGC mRNA measurements, increased levels of AGC protein were also observed in cells exposed to mechanical stress and verapamil compared to mechanical stress alone.

Levels of AGC and ADAMTS proteins in OA chondrocytes were also assessed immunohistochemically. A notable increase in the intensity of AGC immunostaining was observed in chondrocyte/agarose constructs under mechanical stress. A slight increase in color intensity was detected in response to increased levels of mechanical loading. Consistent with Western blotting analyses, verapamil treatment further increased staining for AGC and decreased staining for ADAMTS-4 and ADAMTS-5.

Using these agarose constructs, we confirmed that mechanical stress, both independent and/or in combination with verapamil, can increase chondrocyte AGC biosynthesis. In the separate experiments, constructs were subjected to increasing intensities of mechanical stress (0, 6, 12, and 24 kPa). The present data showed that levels of AGC protein increased in response to higher stress intensities. Takamatsu *et al*.[4] found that verapamil (up to 50 μmol/L) inhibited Wnt/beta-catenin signaling and reduced cartilage degradation. The present study demonstrated that manipulation of intracellular Ca^{2+} concentrations in combination with mechanical loading could result in significant stimulation of AGC synthesis and inhibition of ADAMTS-4 and ADAMTS-5 production.

In conclusion, mechanical stress, both independently and in combination with verapamil, was demonstrated to increase the production of AGC and decrease the production of ADAMTS-5 protein in human OA chondrocytes cultured in three-dimensional agarose constructs. The data presented provide insight into the therapeutic importance of proper physical exercise for patients with OA and protective effects of exercise on the maintenance of articular cartilage in healthy individuals.

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Conflicts of interest

There are no conflicts of interest.

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