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Effects of Osmolarity on the Spontaneous Calcium Signaling of In Situ Juvenile and Adult Articular Chondrocytes

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Abstract

Calcium is a universal second messenger that mediates the metabolic activity of chondrocytes in articular cartilage. Spontaneous intracellular calcium ($[Ca^{2+}]_i$) oscillations, similar to those in neurons and myocytes, have recently been observed in chondrocytes. This study analyzed and compared the effects of different osmotic environments (hypertonic, hypotonic, and isotonic) on the spontaneous $[Ca^{2+}]_i$ signaling of *in situ* chondrocytes residing in juvenile and adult cartilage explants. In spite of a lower cell density, a significantly higher percentage of chondrocytes in adult cartilage under all osmotic environments demonstrated spontaneous $[Ca^{2+}]_i$ oscillations than chondrocytes in juvenile cartilage. For both juvenile and adult chondrocytes, hypotonic stress increased while hypertonic stress decreased the response rates. Furthermore, the spatiotemporal characteristics of the $[Ca^{2+}]_i$ peaks vary in an age-dependent manner. In the hypotonic environment, the $[Ca^{2+}]_i$ oscillation frequency of responsive adult cells is almost tripled whereas the juvenile cells respond with an increased duration and magnitude of each $[Ca^{2+}]_i$ peak. Both juvenile and adult chondrocytes demonstrated significantly slower $[Ca^{2+}]_i$ oscillations with longer rising and recovery time under the hypertonic condition. Taken together, these results shed new insights into the interplay between age and osmotic environment that may regulate the fundamental metabolism of chondrocytes.

Keywords

Cartilage; Chondrocyte; Age; Calcium; Osmotic; Signal Transduction

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Introduction

The strong correlation between aging and prevalence of osteoarthritis has led to a widespread concept that natural aging alters the phenotype and metabolic activity of chondrocytes, the major cell population responsible for articular cartilage homeostasis²². Tissue engineering studies showed that chondrocytes from juvenile cartilage increased the synthesis rate of proteoglycan and collagen during *in vitro* culture compared with those from adult cartilage^{33, 49}. As the physiological role of cartilage is mainly mechanical, e.g, load bearing, shock absorbing, and lubricating, physical stimuli induced by daily loading plays an essential role in regulating chondrocyte activity. Hydraulic pressure, fluid flow, mechanical compression, osmotic stress, and plasma membrane stretch can all induce biochemical responses in chondrocytes^{2, 7, 19, 20, 43, 53}. However, how chondrocytes' mechanotransductive response is orchestrated by the interplay between age and physical stimuli, for the most part, has not been investigated.

During the process of aging, one of the most striking changes in cartilage's extracellular matrix (ECM) is the loss of proteoglycans, which is associated with a loss of water content and decrease of apparent stiffness. Chondroitin and keratan sulfates on glycosaminoglycan (GAG) chains are negatively charged, which attract extra cations into the tissue increasing the osmolarity of the interstitial fluid. The resulting Donnan osmotic pressure within the ECM contributes significantly to cartilage's support of compressive loading^{37, 41}. Meanwhile, daily physiologic compression also alters the density of fixed charges causing cartilage to dynamically exude and imbibe water during loading and unloading. Both effects subject chondrocytes to fluctuating levels of hyper- and hypo-osmotic stress^{22, 41}. Therefore, oscillation of environmental osmolarity is an ordinary physical stimulus chondrocytes experience during daily functions and may be altered due to aging. A number of studies proved that osmotic stress can regulate the fate of chondrocytes, e.g, gene expression, apoptosis, and chromosomal condensation^{7, 27, 42, 46}, and further the degeneration and repair of cartilage matrix^{42, 50}.

The ability of calcium (Ca^{2+}) and phosphate ions to alter the local electrostatic fields and protein synthesis are believed to be two universal tools cells use for cellular signal transduction⁹. Intracellular calcium ($[\text{Ca}^{2+}]_i$) signaling is among one of the earliest responses of chondrocytes under various physical stimuli including mechanical loading³², fluid flow^{12, 13, 53}, electrical stimulation⁵¹, and osmotic stress^{7, 15, 16, 52}. In addition, recent studies proved that chondrocytes demonstrate spontaneous $[\text{Ca}^{2+}]_i$ oscillations, similar to those in neurons and myocytes. Without the presence of any external stimulation, chondrocytes located in hydrogels or their own ECM can release robust and repetitive $[\text{Ca}^{2+}]_i$ peaks^{18, 32, 43, 54}. While most relevant studies have focused on the $[\text{Ca}^{2+}]_i$ response immediately upon exposure of osmotic shock, chondrocytes' spontaneous $[\text{Ca}^{2+}]_i$ signaling under various osmotic environments remains elusive. Furthermore, previous $[\text{Ca}^{2+}]_i$ signaling studies have primarily investigated chondrocytes grown on monolayer cultures or in hydrogels, however, the mechanical stiffness, ultrastructure, and biochemical composition of cartilage ECM can significantly alter the metabolic activity and phenotype of chondrocytes^{22, 29}. To more accurately recapitulate the *in vivo* osmotic environment that chondrocytes experience, it is essential to study their activity in a native environment.

In this study, we hypothesized that mature chondrocytes will demonstrate altered spontaneous $[Ca^{2+}]_i$ signaling when exposed to hypo/hypertonic stress compared with juvenile chondrocytes. The specific aims are 1) to investigate the interplay of age and mechanotransduction of chondrocytes by comparing the spontaneous $[Ca^{2+}]_i$ signaling of juvenile and adult bovine chondrocytes residing in cartilage explants, and 2) to compare the spontaneous $[Ca^{2+}]_i$ signaling of *in situ* chondrocytes at these two ages under three osmotic conditions (isotonic, hypertonic, and hypotonic).

Materials and Methods

Cartilage explants

Cylindrical cartilage explants (diameter: $\phi = 3$ mm) were harvested from the central region of femoral condyle heads of freshly slaughtered calf or adult bovine knee joints (Green village, NJ). The calf was ~3-6-months old and the musculoskeletal-mature adult bovine was ~18-months old. Both age groups were of mixed breed, mixed gender, and mixed sides. The thickness of cartilage explants were controlled at ~2 mm with the intact articular surface using a custom-designed cutting tool. Samples were then cultured at 37 °C and 100% humidity in chemically defined chondrogenic medium (DMEM, 1% ITS+Premix, 50 µg/ml L-proline, 0.1 µM dexamethasone, 0.9 mM sodium pyruvate, 50 µg/ml ascorbate 2-phosphate) for 3 days before initiating experiments ⁴³.

Calcium signaling of in-situ chondrocytes

To record the spontaneous $[Ca^{2+}]_i$ signaling of chondrocytes in cartilage explants, the cylindrical samples were halved axially with a cutting tool (ASI-Instruments, MI); one of the halves was used for hypotonic testing while the other half was used for hypertonic testing. The halved sample was first dyed with 5 µM Fluo-8 AM (AAT Bioquest, CA) in an incubator for 40 minutes and then washed in pure DMEM three times for 5 minutes each time ²⁸. With the cross-section area facing down, the halved cylindrical explant was placed in an imaging chamber containing DMEM medium and then mounted on a confocal microscope (Zeiss LSM510) (Fig. 1A). The cartilage was allowed a 15-minute resting period to enable chondrocytes to recover from any agitation during previous operations ^{25, 35}. The focal plane of the fluorescent image was ~30 µm deep into the cross-section surface to avoid damaged cells from cutting. The imaging area was located on the central axis of the cylindrical sample and ~200 µm below the articular surface, as illustrated in Fig. 1B. Initially, fluorescence images of chondrocytes' spontaneous $[Ca^{2+}]_i$ signaling were recorded every 1.5 seconds for 15 minutes while the sample was undisturbed in 310 mOsm DMEM medium (isotonic). After this imaging session, the osmolarity of the imaging chamber was changed to either 165 mOsm (hypotonic) or 600 mOsm (hypertonic) by adding deionized water or 890 mOsm NaCl solution, respectively, as described previously ^{7, 15, 23, 44, 46, 50}. A theoretical simulation, by modeling the cartilage as a triphasic material with fiber reinforced solid matrix ²¹, estimated the tissue principle strain under the osmotic stress is ~1%. The strain difference between juvenile and adult tissue should be smaller than 0.5%. To avoid recording the immediate active $[Ca^{2+}]_i$ response upon osmotic shock, recording of calcium images in the new osmotic environment was started 5 minutes after supplementation of respective solutions ³² with the same imaging profile. According to the measurement of

solutes diffusion in cartilage, the time for the osmolarity at the focal plane ($\sim 30 \mu\text{m}$ inside tissue) to reach equilibrium should be within the order of seconds³. The change of cell volume under osmotic stress, such as shrinking³⁰ or swelling⁴, could take 1-4 minutes in chondrocytes. A comparison of the calcium responsive rates between the first and second half of the recording time showed no significant difference. Thus, the medium mixing process should be relatively fast and have little or minor effects on the outcome.

Cell density of the cartilage explants, defined as the number of dyed live cells divided by the imaging area, was counted using the full calcium images of each sample. To extract the $[\text{Ca}^{2+}]_i$ signaling of individual cells, fluorescent images were analyzed as described in our previous studies^{25, 26, 28, 35}. In brief, oscillation of $[\text{Ca}^{2+}]_i$ concentration was represented by the average image intensity of each cell. A cell was defined as responsive if it displayed a $[\text{Ca}^{2+}]_i$ peak with a magnitude four times higher than its maximum fluctuation along the baseline¹⁴. The responsive percentage was defined as the fraction of cells with $[\text{Ca}^{2+}]_i$ peak(s) over the total number of cells. For responsive cells, the total number of $[\text{Ca}^{2+}]_i$ peaks were counted. To further compare the $[\text{Ca}^{2+}]_i$ signaling between groups, spatiotemporal features of the $[\text{Ca}^{2+}]_i$ peaks including the magnitude of peaks, time to reach a peak, relaxation time from a peak, and time interval between two neighboring peaks were also extracted (Fig. 1C)^{26, 28, 35}. These spatiotemporal parameters are defined in Fig. 1C using a typical $[\text{Ca}^{2+}]_i$ oscillation curve. Overall, 5 juvenile (each from a different joint) and 7 adult halved cylindrical explants (randomly selected from 3 joints) were tested in each group under each osmotic condition. The total number of analyzed live cells in each group were: Juvenile Hypo: 1046, Juvenile Iso: 1070, Juvenile Hyper: 863; Adult Hypo: 482, Adult Iso: 493, Adult Hyper: 619.

Mechanical properties

Mechanical stiffness of juvenile and adult cartilage explants were measured by unconfined compression using another set of samples ($N=8$ for each group)³⁶. The original thickness of each cartilage explant was measured as the distance between the upper and lower loading platens with a 5-gram tare load. During the test, a 10% strain was applied on the tissue at a constant speed of $2 \mu\text{m/s}$. After the tissue reaction force reached an equilibrium state, sinusoidal dynamic loading was applied at 0.5 Hz with a magnitude of $\pm 1\%$ strain. Equilibrium Young's modulus and dynamic modulus of the sample was determined from the recorded force.

Statistical analysis

Student's t-test was employed to compare the mechanical properties and cell density between juvenile and adult groups, and the data are shown as mean $\pm$ SD. For the $[\text{Ca}^{2+}]_i$ responsive percentage, the significance of differences among groups was determined by two-way analysis of variance (ANOVA) followed by a Tukey post hoc test using Origin (OriginLab, Northampton, MA). To compare the spatiotemporal parameters of $[\text{Ca}^{2+}]_i$ signaling, nonparametric Mann–Whitney U test was utilized, and the data are shown as mean $\pm$ SEM. P-values less than 0.05 are considered significant.

Results

Cell density and mechanical properties

Cell density in the juvenile samples was 4.7 times higher than in the adult tissues (1572 ± 289 vs 337 ± 97 cell/mm²) (Fig. 1F). Mechanical stiffness of knee condyle cartilage also decreased with age. The young's modulus of adult cartilage was 58.2% lower than that of juvenile cartilage (0.27 ± 0.14 vs 0.65 ± 0.32 MPa) (Fig. 1G). Additionally, the dynamic modulus of the adult group was 74.3% lower than that of the juvenile group (0.55 ± 0.26 vs 2.12 ± 0.43 MPa) (Fig. 1H).

Responsive percentage of $[Ca^{2+}]_i$ signaling

Spontaneous $[Ca^{2+}]_i$ oscillations were observed in both juvenile (Fig. 1D) and adult (Fig. 1E) *in situ* chondrocytes under the isotonic condition with no extraneous stimuli. Typical $[Ca^{2+}]_i$ intensity oscillation curves for both age groups under each osmotic conditions are shown in Fig. 2. A large portion of responsive chondrocytes demonstrate repetitive, spike-like $[Ca^{2+}]_i$ peaks. Within the 15-minute imaging period, the number of peaks reached 11 and 15 for juvenile and adult chondrocytes, respectively. No attenuation in the frequency or amplitude of $[Ca^{2+}]_i$ oscillations were noted in the 15-minute recording period. In the isotonic environment, the responsive percentage in adult cartilage was significantly higher than that in juvenile cartilage ($45.8 \pm 2.2\%$ vs. $15.3 \pm 1.1\%$), and this significant difference between the two age groups was conserved under both the hypotonic ($58.5 \pm 2.2\%$ vs. $40.2 \pm 1.5\%$) and hypertonic ($24.8 \pm 1.7\%$ vs. $5.1 \pm 0.7\%$) conditions (Fig. 3). After balancing in the hypotonic environment, the responsive percentage significantly increased in both groups (juvenile: $40.2 \pm 1.5\%$; adult: $58.5 \pm 2.2\%$) where the adult group was still significantly higher than the juvenile group. In contrast, the hypertonic environment reduced the number of responsive cells in both age groups (juvenile: $5.1 \pm 0.7\%$; adult: $24.8 \pm 1.7\%$) where the juvenile samples were constantly lower than the adult samples. There was a significant difference between the two factors (age and osmotic condition) for the responsive percentage ($p < 0.001$, two-way ANOVA).

Spatiotemporal characteristics of $[Ca^{2+}]_i$ signaling

Under all three osmotic conditions, both juvenile and adult cells shared the same trend in the number of multiple peaks where hypotonic was significantly higher than isotonic and isotonic was significantly higher than hypertonic (Fig. 4A&C). For juvenile cells, the magnitude of $[Ca^{2+}]_i$ peaks decreased in the hypertonic condition (Fig. 4B). There was no similar trend detected in adult cells, which shows lower magnitude under the hypotonic condition but no difference between the isotonic and hypertonic conditions (Fig. 4D).

A full comparison of all the spatiotemporal parameters of $[Ca^{2+}]_i$ response under the three osmotic conditions between juvenile and adult chondrocytes are listed in Table 1. In the isotonic environment, the average magnitude of $[Ca^{2+}]_i$ peaks of juvenile cells was higher than adult cells (juvenile vs. adult: 2.92 ± 0.07 vs. 2.54 ± 0.04), and this trend was the same under both the hypotonic (juvenile vs. adult: 3.14 ± 0.04 vs. 2.13 ± 0.02) and hypertonic conditions (juvenile vs. adult: 2.75 ± 0.28 vs. 2.54 ± 0.06). Under the isotonic and hypertonic conditions, the average number of multiple $[Ca^{2+}]_i$ peaks in juvenile chondrocytes was not

statically different with adult chondrocytes (isotonic: 2.33 ± 0.12 vs. 1.98 ± 0.06 ; hypertonic: 1.36 ± 0.14 vs. 1.33 ± 0.06). However, in the hypotonic environment the responsive adult chondrocytes had more peaks than the juvenile chondrocytes (5.17 ± 0.16 vs. 2.94 ± 0.11).

In the isotonic environment, $[Ca^{2+}]_i$ peaks from juvenile cells were more spike-like than adult cells, *i.e.*, taking shorter time to reach a peak or to relax from a peak. However, an opposite trend was observed when the cells were under hypotonic or hypertonic condition. It took a shorter time for adult cells to release full $[Ca^{2+}]_i$ peaks than juvenile cells. Under all three osmotic conditions (Fig. 5), juvenile cells released $[Ca^{2+}]_i$ peaks fastest under the isotonic condition and slowest under the hypertonic condition, and the peak relaxation time share a similar trend in all three osmolarities. In contrast, adult cells released $[Ca^{2+}]_i$ peaks fastest under the hypotonic condition and slowest under the hypertonic condition. For both types of cells, the interval time between two neighboring $[Ca^{2+}]_i$ peaks increased with osmolarity (Fig. 5).

Discussion

Spontaneous calcium oscillations in chondrocytes

Spontaneous $[Ca^{2+}]_i$ signaling was first noted in “excitable” cells such as neurons, myocytes, and cardiomyocytes^{1,9}. Recently, spontaneous $[Ca^{2+}]_i$ signaling has also been observed in several other non-excitable cells found in the musculoskeletal system, such as bone cells²⁸, mammary mesenchymal stem cells³¹, and chondrocytes^{18,32,43}. Kono et al. observed “occasional” spontaneous $[Ca^{2+}]_i$ signaling in chondrocytes residing in sliced pieces of cartilage and those cultured in monolayer, but no responsive ratio of cells was reported³². O'Connor et al. found that ~20% of juvenile porcine chondrocytes embedded in agarose gel after a 3-day culture period showed $[Ca^{2+}]_i$ signaling without any osmotic stress⁴³. Using chicken chondrogenic cells, Fodor et al. recorded “spontaneous Ca^{2+} transients” in 19% of cells after 1 day of culturing¹⁸. In the present study, after 3 days of *in vitro* culture, 15% of chondrocytes in juvenile cartilage explants showed spontaneous $[Ca^{2+}]_i$ oscillations in the isotonic environment, which is in a similar range with those reported in previous studies. Using monolayer primary bovine chondrocytes, Yellowley et al. also observed spontaneous calcium transients in 8.9% cells⁵³. Therefore, chondrocytes are capable to release spontaneous calcium signaling with and without the presence of ECM. However, since ECM plays a critical role in the mechanotransduction of chondrocytes²⁰, interplay of the spontaneous calcium signaling and ECM properties may still exist and need further examination. The responsive rate and the spatiotemporal characteristics of $[Ca^{2+}]_i$ transients could be important indicative parameters for the change of ECM²⁰. Previous studies have demonstrated that fluid flow induced calcium responses of chondrocytes are dependent on the ligand and scaffold structures^{12,13}. Our recent study found that the spontaneous $[Ca^{2+}]_i$ signaling of each *in situ* chondrocyte has a unique pattern and behaves like a “fingerprint” of the cells⁵⁴.

In excitable cells, such as neuronal and cardiac cells, oscillations of $[Ca^{2+}]_i$ are often facilitated by Ca^{2+} influx via voltage-gated calcium channels, which themselves are regulated by the fluctuations of membrane potential⁹. The mechanisms of spontaneous $[Ca^{2+}]_i$ oscillations in chondrocytes, however, are largely unidentified. Initially, an

intracellular pulsatile Ca^{2+} pacemaker region was imagined that is responsible for the rhythmic Ca^{2+} oscillations in chondrocytes¹¹. It was later suggested that the underlying oscillation in membrane potential driven by K^{+} channels can activate the action potential and further the influx of extracellular Ca^{2+} ⁸. Ca^{2+} concentration in cartilage ECM is usually of the order of 10^{-3} M, while the cytosol calcium concentration can be as low as 10^{-7} M⁹. The 10,000 fold gradient of Ca^{2+} could also result in a direct calcium influx into cytosol to evoke a full $[\text{Ca}^{2+}]_i$ oscillation³⁸.

An important mechanism by which cells initiate a $[\text{Ca}^{2+}]_i$ oscillation is through the transfer of calcium related signals from neighboring cells via intercellular junctions or extracellular diffusion. This high-efficient cell-cell communication mechanism is termed as calcium wave propagation, which is related to cell-cell distance as revealed in our previous studies²⁵. Chondrocytes are generally isolated in cartilage and enclosed within a pericellular matrix. Thus, extracellular diffusion should be the dominant mechanism for calcium wave propagation, if there is any, between chondrocytes. Although the cell density in adult tissue is much lower than the juvenile tissue, chondrocytes in the adult tissue tend to form cell clusters as shown in Fig 1. Thus the calcium wave propagation could be efficient among the cells in a cluster. To test this hypothesis, the shortest distance between two responsive cells, defined as the distance from a responsive cell to its closest responsive neighboring cell, is measured under hypotonic condition. The distance in adult cartilage is significantly higher than that in the juvenile tissue (25.2 ± 13.2 vs 19.5 ± 10.7 μm , $p < 0.01$). In adult tissue, less than 40% of responsive cells have another responsive cell < 20 μm away. For juvenile tissue, this percentage is over 60%. Therefore the higher responsive rate in adult tissue is unlikely due to the shorter distance between cells in a cluster. It should be noted that this result is not necessarily ruling out the role of calcium wave propagation in adult cells. A systematic pathway study could be performed in the future to identify the roles of gap junctions and extracellular messenger diffusion in chondrocyte calcium signaling.

Osmotic stress and calcium signaling

In this study, both juvenile and adult chondrocytes demonstrated significantly more $[\text{Ca}^{2+}]_i$ oscillations under the hypotonic condition. The effect is more prominent in juvenile cells with the response percentage increasing to 40.2% from the 15.3% under the isotonic condition. It is important to note that our experiments recorded the spontaneous $[\text{Ca}^{2+}]_i$ activity without the initial $[\text{Ca}^{2+}]_i$ response to the osmotic shock, which is usually released in a few seconds⁵². Studies found that a higher percentage of chondrocytes responded with an increase of $[\text{Ca}^{2+}]_i$ upon the exposure of hypotonic stress, *e.g.*, 73.2% for bovine chondrocytes (-150 mOsm)⁵² and 92.3% for porcine chondrocytes (-100 mOsm)⁴⁵. In this study, magnitudes of the two osmotic stresses are selected to cover the possible range of osmolarity oscillation experienced by chondrocytes within the extracellular matrix. Although these two values may be larger than the physiologically relevant variations in daily activities⁵⁰, they have been widely adopted in literature to reveal the effects of osmotic stress in chondrocyte mechanobiology, such as the volume change of cells^{6, 7, 15, 23}, metabolism and gene expression of chondrocytes in 2D and 3D culture^{16, 27, 42, 46}, extracellular matrix accumulation in tissue engineering applications^{42, 43}, and calcium signaling of cells^{2, 7, 10, 15, 52}.

In the isotonic environment, spherical-shaped chondrocytes are featured with numerous membrane ruffles and microvilli²³, which can smooth out during hypotonic swelling associated with significant cell volume increase^{16, 42}. Swollen chondrocytes were markedly more sensitive with stretched plasma membrane while hypotonic stress could open the stretch-activated cation channels, the transient receptor potential vanilloid receptor (TRPV) channels, and activate the PLC-IP₃ pathway⁴⁸. All of these effects may contribute to the enhanced [Ca²⁺]_i oscillations of both juvenile and adult chondrocytes in the hypotonic environment observed in this study. For adult cells, drastic changes were also noted in the spatiotemporal parameters of [Ca²⁺]_i peaks such as shorter rising and recovery time (Fig. 5). The interval time decreased by ~ 50% and the average number of peaks increased from 1.98 (isotonic) to 5.17 (hypotonic) for responsive cells. Thus, exposure to the hypotonic environment induces a more vigorous spontaneous [Ca²⁺]_i signaling in adult chondrocytes compared those under the isotonic condition. Although the responsive percentage of juvenile cells increase in the hypotonic condition, the average number of [Ca²⁺]_i peaks only changes from 2.33 to 2.94, a mild increase compared to adult cells. In addition, the duration and magnitude of [Ca²⁺]_i peaks in juvenile cells increased significantly under hypotonic conditions, which is opposite with adult cells.

In contrast to the promotion effect of the hypotonic condition, exposure to the hypertonic condition restrained the [Ca²⁺]_i oscillations in chondrocytes. Significant decreases were noted in the responsive percentage and the number of peaks for both juvenile and adult cells. Furthermore, all three measured temporal parameters of [Ca²⁺]_i peaks had increased values, indicating slower [Ca²⁺]_i oscillations, which could be related to the reduced numbers of active cation channels and pumps on ruffled cell membrane²³. It has been shown that hypertonic shock could induce chondrocyte hyperpolarization, leading to a fast increase of [Ca²⁺]_i, which usually lasts for less than a few minutes before recovering to its initial level. Afterwards, as demonstrated by previous studies, chondrocytes can barely release [Ca²⁺]_i peaks in hypertonic medium. For *in situ* chondrocytes in mouse cartilage, only a few cells displayed multiple [Ca²⁺]_i peaks in 400 mOsm medium¹⁰. Even the initial [Ca²⁺]_i responses decreased when the osmolality was increased from 450 to 550 mOsm¹⁵.

A major osmolality-regulated ion channel on chondrocytes' membrane is the transient receptor potential vanilloid 4 (TRPV4) channel⁴⁴. Researchers found after short-term osmolality treatment on isolated equine chondrocytes that hypotonic stimulation upregulated TRPV4 expression while hypertonicity decreased its expression²⁴. Our results also showed that [Ca²⁺]_i response of *in situ* chondrocytes are osmotic-dependent and that hypotonic stress increased the [Ca²⁺]_i response while hypertonic stress decreased the response. Thus, intimate connections may exist among the TRPV4 expression, environmental osmotic level, and spontaneous [Ca²⁺]_i signaling of chondrocytes.

Age-related alterations in chondrocyte calcium signaling

Little is known about the interplay between the aging process and [Ca²⁺]_i signaling in chondrocytes. In this study, adult chondrocytes demonstrated more active, but less intensive, spontaneous [Ca²⁺]_i signaling than juvenile cells, and this difference was retained in both hypo- and hyper-tonic environments. It is well established that aged chondrocytes have a

diminished capacity to produce extracellular matrix^{33, 49}. Thus, the intensity of $[Ca^{2+}]_i$ signaling in chondrocytes is not necessarily a positive indicator of its synthesis rate. More importantly, when exposed to the hypotonic environment, adult chondrocytes responded with high-frequency spontaneous $[Ca^{2+}]_i$ oscillations but with dampened peak magnitudes. In contrast, juvenile cells showed increasing duration and magnitude of each peak. The chondrocytes at two different ages obviously have distinct metabolic profiles in the same low osmolarity environment.

When comparing the gene expression profiles in knee joint tissues from juvenile and adult male mice^{34, 40}, several sets of genes involved in calcium signaling were found to be significantly upregulated in older mice vs. younger mice. These include the purinergic receptors P2X1, P2X4, and P2X7 as well as the transient receptor potential cation channels TRPC1 and TRPC6. P2X family include several ligand gated cation channels in response to extracellular ATP, while TRPC channels are activated by receptors coupled to PLC, mechanical stimulation, and depletion of intracellular calcium stores. Thus, the increased expression of these two families of cation channels in adult chondrocytes could be particularly related to the more frequent but weaker $[Ca^{2+}]_i$ oscillations observed in this study, when compared to juvenile chondrocytes. Another potential factor here is the oxidative stress, which is able to cause rapid increase in $[Ca^{2+}]_i$ concentration¹⁷. Increased oxidative stress with aging could also promote the $[Ca^{2+}]_i$ signaling in adult chondrocytes.

This study confirmed that the compressive stiffness and cell density of adult cartilage is significantly lower than juvenile tissue^{39, 47}. With increasing age, the collagen network and proteoglycan content gradually change and decrease leading to altered cartilage biomechanical properties, which further changes the physicochemical environment of cells including ion concentrations, pH, osmolality, and stiffness of ECM^{34, 39}. Moreover, reorganization of the cytoskeleton with age can also change the cytoplasmic appearance, stiffness, and size of cells⁵. Under osmotic stress, the $[Ca^{2+}]_i$ response were intimately linked with the alteration of the structure and strength of the actin cytoskeleton¹⁶. In summary, the age-dependent extracellular oxidative stress, age-related change in ECM, membrane, and cytoskeleton could all contribute to the differences of $[Ca^{2+}]_i$ signaling between juvenile and adult chondrocytes observed in this study.

Limitations

A few limitations should be noted for this study. Firstly, cartilage is a heterogeneous tissue and the calcium signaling experiments were performed on cells located in superficial zone. Thus, the conclusions drawn here may not be applicable for cells in the middle or deep zones where the cell phenotype could be different. Secondly, only two age groups, juvenile and adult samples, were investigated. A full knowledge about the interplay between aging and calcium signaling in chondrocytes may require the usage of dedicated animal model or human tissues with aging control, which could be an essential future direction of this study. Thirdly, the adult explants were randomly selected from three knee joints and pooled together for statistical analysis without the consideration of biological replicates. This may have some minor effects on the statistical outcome. Lastly, the mechanisms for the spontaneous $[Ca^{2+}]_i$ oscillations in chondrocytes or “non-excitable” cells remain largely

elusive⁹, although it has been observed in several types of cells in musculoskeletal system. This further hinders the thorough understanding of the differences noted between juvenile and adults cells' spontaneous $[Ca^{2+}]_i$ signaling in this study.

Conclusions

In summary, chondrocytes are capable of “sensing” their osmotic environment in terms of the frequency and spatiotemporal characters of spontaneous $[Ca^{2+}]_i$ oscillations, which may play essential roles in enabling cells to adapt their phenotype and metabolic activity according to the environmental cues. Furthermore, the spontaneous $[Ca^{2+}]_i$ signaling of *in situ* chondrocytes under all osmotic conditions appears to be a function of tissue age. The osmolality environment surrounding chondrocytes is gradually altered during cartilage degeneration or aging. Therefore, the osmotic environment may serve as a target to modulate chondrocytes' behaviors for the prevention and treatment of osteoarthritis.

Acknowledgments

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Figure 1.

(A) Illustration of $[Ca^{2+}]_i$ imaging. The chondrocytes in a cartilage explant were dyed with Fluo-8 AM. Fluorescent $[Ca^{2+}]_i$ images of chondrocytes were recorded using a confocal microscope (20 $\times$ objective, Zeiss LSM510). (B) Location of the imaging zone was marked with a dash-line square on the cross-section area of a cartilage explant. (C) A typical $[Ca^{2+}]_i$ intensity oscillation curve of a chondrocyte and the definitions of spatiotemporal parameters including the number of $[Ca^{2+}]_i$ peaks, magnitude of peak m_1 , time to peak t_1 , relaxation time of peak t_2 , and time interval between two neighboring peaks t_3 . (D) Typical $[Ca^{2+}]_i$ image series from juvenile cartilage sample. (E) Typical $[Ca^{2+}]_i$ image series from adult cartilage sample. A few responsive cells are marked with white arrows. (F) Cell density ($/\text{mm}^2$) of total dyed cells in $[Ca^{2+}]_i$ images. (G) Equilibrium Young's modulus of cartilage explants. (H) Dynamic modulus of cartilage explants. (*: $p < 0.05$)

Figure 2. Typical $[Ca^{2+}]_i$ intensity curves from juvenile (top) and adult (bottom) *in situ* chondrocytes under three different osmotic conditions.

Figure 3.

Comparison of $[Ca^{2+}]_i$ responsive percentage (number of cells with $[Ca^{2+}]_i$ peaks divided by number of total dyed cells) under different osmotic stress. Cells with single $[Ca^{2+}]_i$ peak are separated from those with multiple peaks. The number of total analyzed live cells are: Juvenile Hypo: 1046, Juvenile Iso: 1070, Juvenile Hyper: 863; Adult Hypo: 482, Adult Iso: 493, Adult Hyper: 619. (Mean $\pm$ S.D., *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$)

Figure 4.

(A, C) Average number of $[Ca^{2+}]_i$ peaks released by responsive cells and (B, D) the magnitude of $[Ca^{2+}]_i$ peaks in three different osmotic environments for juvenile (top) and adult (bottom) chondrocytes. (Mean $\pm$ S.E.M., *: $p<0.05$, **: $p<0.01$, and ***: $p<0.001$.)

Figure 5.

Temporal parameters of $[Ca^{2+}]_i$ peaks. (A, D) Time to reach a $[Ca^{2+}]_i$ peak, (B, E) Relaxation time from a $[Ca^{2+}]_i$ peak, and (C, F) Time interval between two neighboring $[Ca^{2+}]_i$ peaks. (Mean $\pm$ S.E.M., *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.)

Table 1

Comparison of all spatiotemporal parameters of $[Ca^{2+}]_i$ signaling between juvenile and adult chondrocytes under three osmotic conditions.

		Juvenile	Adult	P value
Hypotonic	average number of peaks	2.94 ± 0.11	$5.17 \pm 0.16^{***}$	0.000
	magnitude of peaks	3.14 ± 0.04	$2.13 \pm 0.02^{***}$	0.000
	peak rising time	42.20 ± 2.18	$31.13 \pm 1.57^{**}$	0.003
	peak relaxation time	43.94 ± 1.70	$32.73 \pm 1.84^{***}$	0.000
	time between peaks	151.50 ± 4.12	$116.92 \pm 2.33^{***}$	0.000
Isotonic	average number of peaks	2.33 ± 0.12	1.98 ± 0.06	0.196
	magnitude of peaks	2.92 ± 0.07	$2.54 \pm 0.04^{**}$	0.002
	peak rising time	26.32 ± 1.87	$52.80 \pm 2.18^{***}$	0.000
	peak relaxation time	32.51 ± 2.05	$46.52 \pm 2.34^{***}$	0.000
	time between peaks	202.26 ± 11.56	$236.45 \pm 7.28^{***}$	0.000
Hypertonic	average number of peaks	1.36 ± 0.14	1.33 ± 0.06	1.000
	magnitude of peaks	2.75 ± 0.28	$2.54 \pm 0.06^*$	0.023
	peak rising time	84.95 ± 9.13	52.70 ± 3.46	0.535
	peak relaxation time	106.96 ± 9.41	$51.06 \pm 2.55^*$	0.033
	time between peaks	285.74 ± 50.93	374.61 ± 26.38	0.540