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Alteration of Viscoelastic Properties is Associated with a Change in Cytoskeleton Components of Ageing Chondrocytes from Rabbit Knee Articular Cartilage

Wangping Duan[*], Lei Wei[†], Juntao Zhang[*], Yongzhuang Hao[*]
Chunjiang Li[*], Hao Li[*], Qi Li[*], Quanyou Zhang[‡]
Weiyi Chen[‡], Xiaochun Wei[*,§]

Abstract: The cytoskeleton network is believed to play an important role in the biomechanical properties of the chondrocyte. Ours and other laboratories have demonstrated that chondrocytes exhibit a viscoelastic solid creep behavior *in vitro* and that viscoelastic properties decrease in osteoarthritic chondrocytes. In this study, we aimed to understand whether the alteration of viscoelastic properties is associated with changes in cytoskeleton components of ageing chondrocytes from rabbit knee articular cartilage. Three age groups were used for this study: young (2-months-old, N=23), adult (8-months-old, N=23), and old (31-months-old, N=23) rabbit groups. Cartilage structure and proteoglycan and type II collagen content were determined by H&E and Toluidine Blue staining, and type II collagen antibody. The detailed structure of the chondrocytes in all groups was visualized using transmission electron microscopy (TEM). Chondrocytes were isolated from full-thickness knee cartilage of rabbits from all groups and their viscoelastic properties were quantified within 2 hours of isolation using a micropipette aspiration technique combined with a standard linear viscoelastic solid model. The components and network of the cytoskeleton within the cells were analyzed by laser scanning confocal microscopy (LSCM) with immunofluorescence staining as well as real time PCR and western blotting. With ageing, articular cartilage contained less chondrocytes and less proteoglycans and type II collagen. TEM observations showed that the cell membranes were not clearly defined, organelles were fewer

[*] Department of Orthopaedics, The Second Hospital of Shanxi Medical University; Shanxi Key Laboratory of Bone and Soft Tissue Injury Repair. Taiyuan 030001, China
[†] Department of Orthopaedics, The Warren Alpert Medical School of Brown University/Rhode Island Hospital, Suite 402A, 1 Hoppin Street, Providence RI 02903, USA
[‡] Institute of Applied Mechanics and Biomedical Engineering, Taiyuan University of Technology, Taiyuan 030024, China
[§] Corresponding author: Xiaochun Wei. No. 382, Wuyi Road, Taiyuan City, Shanxi Province, China 030001. Tel: 86-351-3365760; Fax: 86-351-3365118; E-mail: weixiaochun06@163. com

and the nuclei were deformed or shrunk in the old cells compared with the young and adult cells. In suspension, chondrocytes from all three age groups showed significant viscoelastic creep behavior, but the deformation rate and amplitude of old chondrocytes were increased under the same negative pressure when compared to young and adult chondrocytes. Viscoelastic properties of the old cells, including equilibrium modulus (E_∞), instantaneous modulus (E_0) and apparent viscosity (μ) were significantly lower than that those of the young and adult ones ($P < 0.001$). No significant differences were detected between young and adult chondrocytes ($P > 0.05$). Moreover, we found that the cytoskeletal networks of old cells were sparser, and that the contents of the various components of the intracellular networks were reduced in old cells, compared with adult and young cells. Aged chondrocytes had a different response to mechanical stimulation when compared to young and adult chondrocytes due to alteration of their viscoelastic properties, which was in turn associated with changes in cell structure and cytoskeleton composition.

Keywords: Chondrocyte, Micropipette aspiration, Chondrocyte Viscoelastic properties, Chondrocyte Cytoskeleton, Ageing cartilage

1 Introduction

Osteoarthritis (OA) is an age-related disease mainly affecting articular cartilage. Studying the age-related changes of articular cartilage is therefore essential for understanding the pathogenesis of OA [1-3]. Many studies have shown age-related changes in the mechanical properties of articular cartilage, particularly decreased modulus in old age [4-7]. Chondrocytes are the only cell type present in articular cartilage and therefore they are responsible for the production of extracellular matrix (ECM) and cartilage repair [8, 9]. Better understanding of the biomechanical properties of chondrocytes and the intracellular mechanisms that govern them may provide new insight into the role of mechanical factors in the pathogenesis of OA [10, 11].

The cytoskeleton's three-dimensional network of tubulin, phalloidin, vinculin, and vimentin plays an important role in the physical interactions between chondrocytes and their ECM [12, 13]. It is also believed that this network contributes to the biomechanical properties of chondrocytes [10, 14, and 15]. Ours and other laboratories have previously demonstrated that chondrocytes exhibit a viscoelastic solid creep behavior under micropipette aspiration, and that viscoelastic properties decrease in OA chondrocytes when compared to healthy chondrocytes [16-18]. However, it is unclear whether changes in the viscoelastic biomechanic behavior of aged cartilage are associated with changes in cytoskeleton composition and structure. We hypothesize that changes in cytoskeleton composition are responsible

for the alteration of the viscoelastic properties that occur in articular chondrocytes with ageing. In this study, we measured the viscoelastic properties and the composition of the cytoskeleton of chondrocytes from the knee articular cartilage of rabbits from three different age groups using micropipette aspiration and confocal microscopy, respectively. We found that the viscoelastic properties are dependent on the cytoskeleton composition and structure, and that these change with ageing. These changes in viscoelastic properties may contribute to the progress of cartilage degeneration processes that take place with ageing.

2 Materials and methods

2.1 Animals

New Zealand White rabbits were acquired from Shanxi Medical University Experimental Animal Center and approved by the Shanxi Medical University Institutional Animal Care and Use Committee. A total of 69 rabbits were divided equally into 3 groups according to their age: young (2-month-old, N=23), adult (8-month-old N=23), and old (31-month-old N=23) groups. From each group, 6 rabbits were used for histological and TEM visualization and analyses of knee articular cartilage. The remaining 17 animals in each group provided the chondrocytes used to study viscoelastic properties and measure cytoskeleton properties.

Histology

The entire femoral condyle and tibial plateau were harvested from the knee joints of rabbits (N=3 per age group) immediately following euthanasia. Harvested tissues were fixed overnight in 10% phosphate-buffered formalin and decalcified in ethylene diamimeteraacetic acid for about 3 months. Sagital sections of 5-μm thickness were prepared from the inner one-third region of the medial femoral condyles and tibial plateau, and stained with Hematoxylin-Eosin (H&E) and Toluidine Blue (TB). Type II collagen was detected in these sections through immunohistochemistry with Collagen-2 antibody (CP18 Merck). The tissue structure and its content of proteoglycans and type II collagen were qualitatively evaluated with optical microscopy.

Transmission electron microscope

Several cartilage blocks from the medial femoral condyles were taken from each of the different age groups (N=3 per age group). The blocks were fixed in 2% purified glutaraldehyde and post-fixed in 1% osmium tetroxide, followed by dehydration in a series of alcoholic solutions, and embedding in epoxy resin. The sections for electron microscopy were cut with an ultrotome using a diamond knife, and then stained with uranyl acetate and lead citrate. The ultrastructure of chondrocytes from each group was observed in a JEOL 100-CX transmission electron microscope (TEM).

2.2 Measurement of viscoelastic properties of chondrocytes

Chondrocyte isolation

Full-thickness articular cartilage was removed from the femoral condyles and tibial plateau of young, adult, and old rabbit knee joints immediately following euthanasia (N=5 per age group). Chondrocytes were harvested using sequential 0.4% protease (P6911, Sigma, Saint Louis, USA) and 0.025% collagenase-2 (C6885, Sigma, Saint Louis, USA) digestions in Dulbecco's Modified Eagle Medium-F12 (DMEM-F12, HyClone, Beijing, China) at 37˚C. After straining through 100μm nylon cell strainer (352360 BD, Massachusetts, USA) and centrifugation, isolated chondrocytes were resuspended at a density of 2×10^5 cells per mL in culture medium DMEM-F12 at 37˚C and 5% CO_2. The isolated chondrocytes were prepared for micropipette aspiration experiments immediately.

Measurement of viscoelastic properties

The viscoelastic properties of the isolated chondrocytes were tested using the micropipette aspiration technique, in a similar fashion to that described previously [16]. Briefly, the micropipettes were pulled from borosilicate capillaries (Olympus, Tokyo, Japan) using a Pipette Puller (P-97, Sutter, San Francisco, USA) with a range of inner tip diameters from 4 to 5 microns, and soaked in 5% acetum for at least 48 hours before use.

One mL of chondrocyte suspension (2×10^5 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 at first. The negative pressure was fixed to a desired value (0.3-0.4 kPa), which caused a time-dependent aspiration deformation of a portion of the cells. The cells were visualized throughout the deformation procedure using an inverted microscope (IX71, Olympus, Tokyo, Japan) equipped with a temperature-controlled stage kept at 37°C, which maintained good viability and hydration of the cells.

The length of chondrocyte aspiration vs. time was determined from optical imaging recorded every 2 s until 200 s total time, which represented an instantaneous deformation. The cell size and time-dependent aspiration were measured in each graph by Olympus Measurement Software (DP-70, Olympus, Tokyo, Japan) as shown in Fig. 3A and an exponential curve was fitted by Origin 8.0 Software. The viscoelastic parameters, including E_∞ (equilibrium modulus), E_0 (instantaneous modulus), and μ (apparent viscosity), were calculated coupled with the standard linear viscoelastic solid model as an earlier theoretical study described [16].

All micropipette aspiration experiments were performed within 2 hours after isolation. Approximately 50 chondrocytes from 5 experiments of each group were

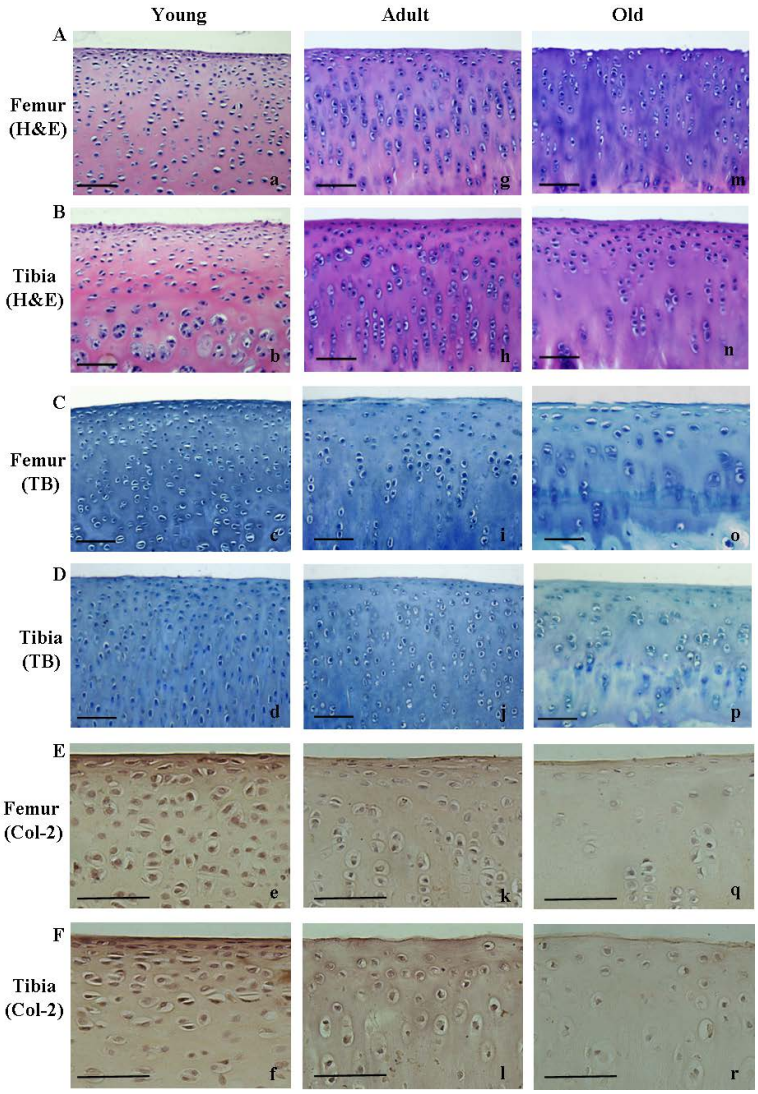

Figure 1: Changes in articular cartilage structure and composition with ageing in the rabbit knee. Results of H&E staining (A and B), Toluidine Blue staining (C and D), and immunohistochemistry for type II collagen (E and F) for cartilage harvested from the inner one-third region of the medial femoral condyles and tibia plateaus respectively. In young (a-f) rabbits, cartilage is highly cellular with strong proteoglycan and type II collagen staining, and the borders between cartilages are hard to define. In adult (g-l) rabbits, cartilage is less cellular and highly oriented in the palisade spatial arrangement in the deep zones. In old rabbits (m-r) the cells are sparser and the proteoglycan and type II collagen staining is decreased (m-r). Scale bars=50μm.

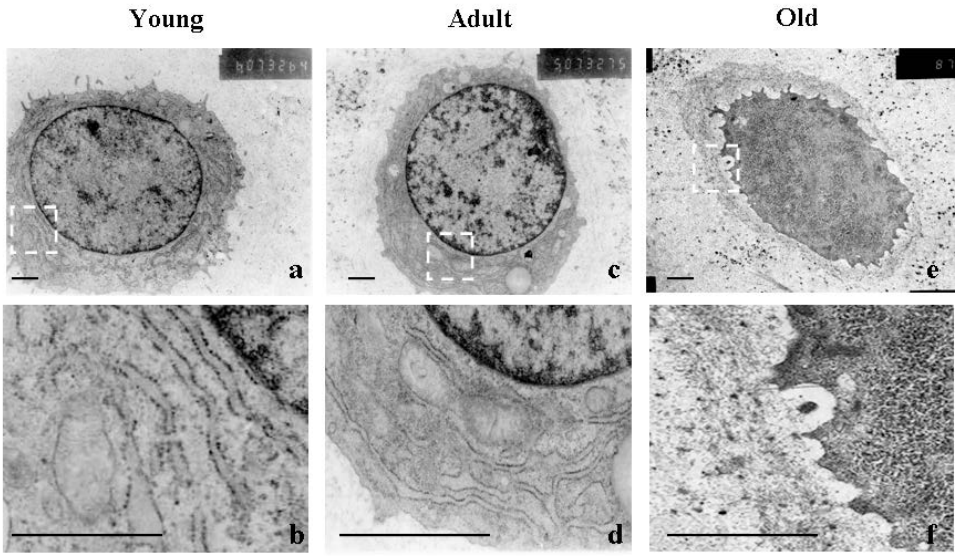

Figure 2: Loss of cell organelles and changes in cell structure with ageing. Transmission electron micrographs of chondrocytes from the medial femoral condyle of young (a, b), adult (c, d) and old (e, f) rabbits. Chondrocytes from young and adult animals are more oval-shaped, their cell and nuclear membranes are complete (a and c), and they show a lot of organelles in the cytoplasm (b and d). The boundaries of the membranes are not clear and the nuclear is shrunk (e) with an obvious loss of cytoplasmic organelles and an increase of the electron density (f) in cells from the old group. Scale bars=1μm.

measured and analyzed.

2.3 Visualization of the cytoskeletal structure with confocal microscopy

Chondrocytes were isolated as described above (N=5 per age group) for observation of the cytoskeleton under confocal microscopy.

Chondrocyte culture

Isolated chondrocytes were seeded in 24 Well Cell Culture Clusters (Costar, New york, USA), where the aseptic D's circular coverslip was placed in the bottom of the well, at a density of 1×10^5 cells/well in 1 mL DMEM-F12 with 10% fetal bovine serum at 37°C and 5% CO_2. Fluorescence immunolabeling was used to visualize the structure of the cytoskeleton under confocal microscopy 2 days after culture.

Immunofluorescence staining and confocal microscopy

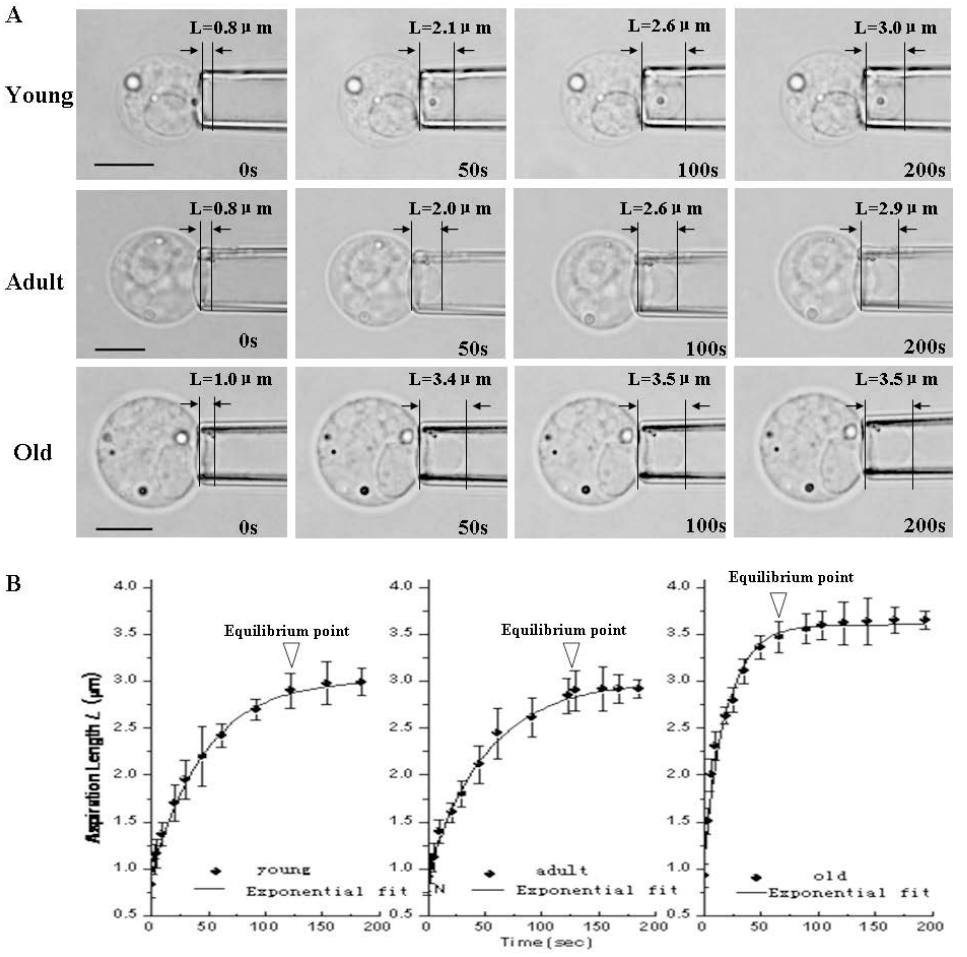

Figure 3: Increase in the aspirated length of aged rabbit chondrocytes. Viscoelastic creep behavior of chondrocytes in response to a negative pressure of 0.3-0.4 kPa. In A, the instantaneous length of time-dependent (0-200s) aspiration into the micropipette was measured in young, adult and old chondrocytes. An increase in the aspirated length was only found in the old cells. Scale bars=5μm. B: average time course changes of the aspirated length L in the chondrocytes from young (n=47, R^2=0.995), adult (n=50, R^2=0.983), and old (n=49, R^2=0.995) rabbits.

The primary antibodies employed were: mouse monoclonal anti-tubulin (T-9028, Sigma), anti-phalloidin (p39, Sigma), anti-vinculin (V-9131, Sigma), and anti- vimentin (3B4, Sigma) at a dilution of 1:100. The fixed cells with glutaraldehyde

were washed 3 times for 10 min each in 0.5% Triton/PBS, exposed to these primary antibodies for 1 h in 37˚C, washed 3 times as described above, and then exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG, at a dilution of 1:200, F8521, Sigma) for 1h in 37˚C, and then DAPI (nucleus staining, at a dilution of 1:2000, 32670, Sigma) for 3 min, washed 3 times and coated with 2.5% glycerin. The immunostained chondrocytes were observed and photographed using a Lecia SP5 laser scanning confocal microscopy (LSCM, λ=567nm, 20$\pm$2mw).

Semi-quantitation of cytoskeleton component intensity

To determine the intensity of cytoskeleton components present in chondrocytes from the different age groups, the immunostained chondrocyte boundary was fingered and the intensity of each cytoskeleton component labeled by fluorescence in each cell was analyzed qualitatively by Leica SP5 Confocal Software in LSCM (Fig. 5A). Fourty chondrocytes for each cytoskeletal protein in each different age group were measured and analyzed in this study.

Real time PCR

To further test whether the mRNA levels of aggrecan (AGG), type II collagen (Col-2), and cytoskeleton composition of tubulin and vinculin were different in aged cartilage, the mRNA levels of the target genes were quantified by real-time PCR (RT-PCR). Total RNA was isolated from young, adult, and old rabbit chondrocytes (N=4 per age group) using Trizol reagent (15596-026, Invitrogen, California, USA). 1 μg total of RNA was reverse transcribed with the iScript™ cDNA Synthesis Kit (K1642, Fermentas, Maryland, USA). Real time quantitative PCR amplification was performed using the QuantiTect SYBR Green PCR kit (K0251, Fermentas, Maryland, USA). mRNA levels were normalized to GAPDH and calculation of mRNA values was performed as previously described [19, 20]. The cycle threshold (Ct) values for GAPDH and for chondrocyte mRNA samples were measured and calculated by computer software (IQ50, Bio-Rad, California, USA). Relative transcription levels were calculated as $x = 2 - \Delta\Delta C_t$, in which $\Delta\Delta C_t = \Delta E - \Delta C$, and $\Delta E = C_t \exp - C_t G$; $\Delta C = C_t \mathrm{ctl} - C_t G$. The primer sequences used for real time-PCR were shown in Table 1.

Western-blotting for cytoskeleton proteins

Total proteins were extracted from young, adult, and old chondrocytes (N=3 per age group) and the protein concentration was assessed using a Bradford assay kit and a spectrophotometer (NanoDrop 2000, THERMO, Massachusetts, USA).

The initial total protein concentration was interpolated to 20 mg/ml in the three age groups, and was electrophoresed in 10% SDS-PAGE (P0012A, Beyotime, Shanghai, China) under reducing conditions. After electrophoresis, proteins were transferred onto Immobilon-PVDF membrane (FFP30, Beyotime, Shanghai, China).

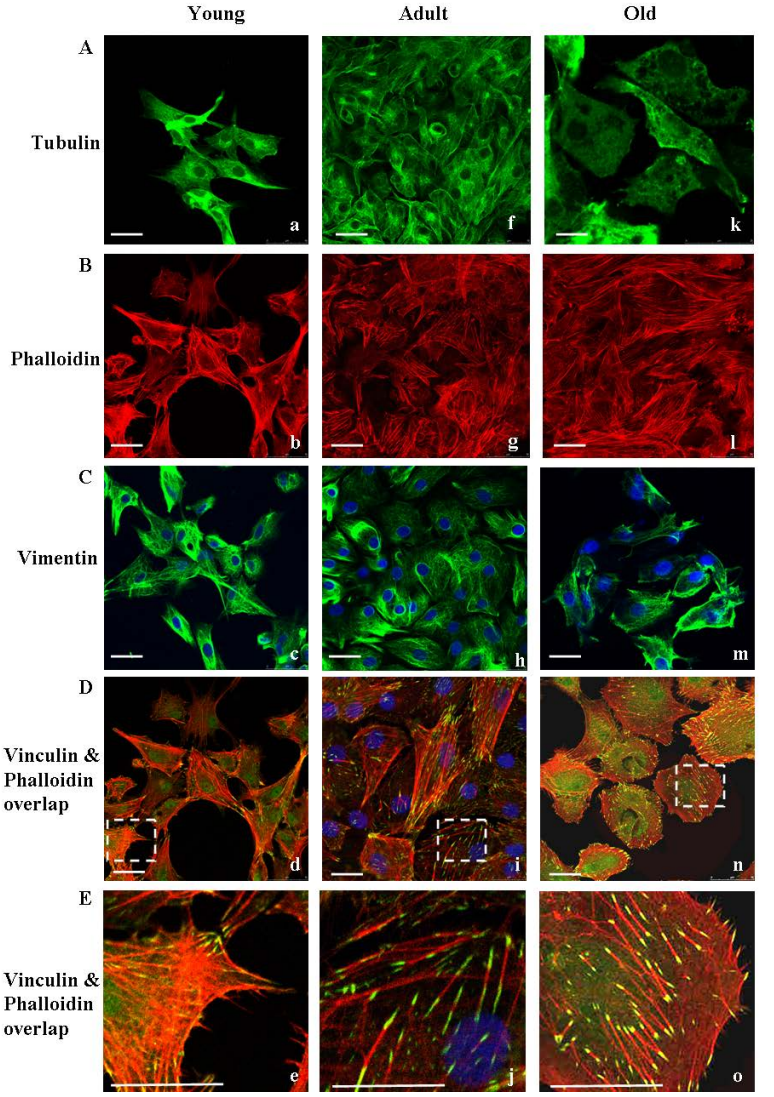

Figure 4: Changes in the cytoskeleton structure and composition with ageing. Cytoskeleton structure and components labeled by fluorescence and observed by LSCM: tubulin (A, green), phalloidin (B, red), vimentin (C, green), and vinculin (green) & phalloidin (red) overlap (D and E, with orange interpreted as the overlap or co-localization of vinculin and phalloidin). Young (a-e), adult (f-j), and old (k-o) rabbits. Scale bars=10μm.

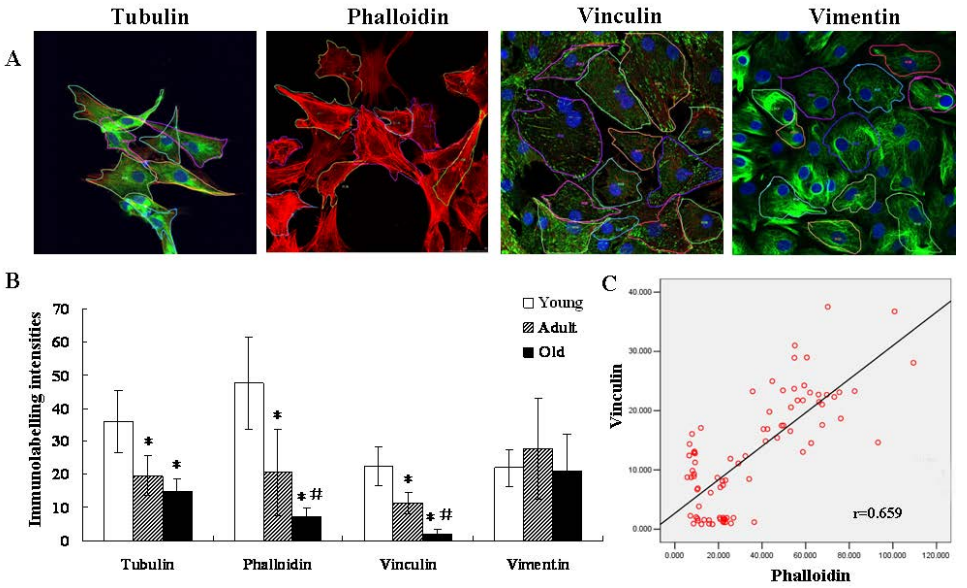

Figure 5: Decreased tubulin, phalloidin, and vinculin with ageing in rabbit chondrocytes. The four cytoskeleton components were quantified by LSCM. In A, a single cell was drawn and its intensity was calculated by an Leica SP5 Confocal Software. In B, a histogram was plotted for these cytoskeleton component intensities in young (n=40), adult (n=40), and old(n=40) chondrocytes, *compared with young group $p<0.05$, $^{\#}$compared with adult group $p<0.05$. In C, phalloidin intensity is correlated with vinculin intensity for all aging chondrocytes.

The primary antibodies employed were mouse monoclonal anti-tubulin (T-9028, Sigma), and anti-vinculin (V-9131, Sigma), at a dilution of 1:400 in PBS-T containing 1% bovine serum albumin. β-actin (sc69879, Santa Cruz, dilution 1:400) was used as a loading control. Values of quantitation of tubulin and vinculin protein levels after normalized to β-actin were presented. The secondary antibody was Horseradish peroxidase-conjugated goat anti-mouse IgG (A4416, Sigma), at a dilution of 1:500 in PBS-T. Visualization of immunoreactive proteins was achieved by using the ECL western blotting detection reagents (CW0049, Cwbio, Beijing, China), and band densities were quantified using Image Acquisition and Analysis Software (Chemi DocTMXR, Bio-Rad). The relative tubulin and vinculin densities in the adult and old groups were normalized to the densities in young animals.

2.4 Statistical analysis

All data in this study are presented as the mean $\pm$ standard deviation (SD). Statistical analysis between the groups was performed using analysis of variance (ANOVA) in SPSS 13.0 software. Statistical significance (α) was set at 0.05.

3 Results

3.1 Morphological differences in cartilage from different age groups

In young animals, the chondrocytes were more rounded in shape and arranged in a more isotropic fashion through the full thickness of the articular cartilage samples. The cells became large in the lower radial zone. The ECM was homogeneous in composition and structure, and there was significantly higher synthetic activity for proteoglycans and type II collagen (Fig.1a-f). In the adult group, a dramatic change in the cartilage structure was apparent with respect to the young group. In the radial and calcified layers, chondrocytes followed a highly oriented spatial arrangement, with three or more cells in a straight column perpendicular to the articular surface. Staining for proteoglycans and type II collagen was reduced in the femur and tibia of adult animals when compared to that in young animals (Fig.1g-l). In the old group, the cells were sparser and the total content of proteoglycans and type II collagen was decreased compared to the young and adult groups (Fig.1m-r).

To quantify the expression levels of proteoglycans and type II collagen in knee cartilage from different age groups, the mRNA levels of aggrecan (AGG) and type II collagen (Col-2) were determined by real-time PCR. Data in Fig.6A indicate that the AGG mRNA expression in the old group was approximately 4.79-fold that in the young group ($p<0.05$). AGG expression was decreased in the old group compared to the adult group ($p<0.05$), but there was no significant difference detected between the young and the old groups ($P>0.05$). Quantitative real time PCR analysis indicated that Col-2 mRNA was most highly expressed in young chondrocytes, while it was expressed at very low levels in the adult or old chondrocytes ($P<0.05$) (Fig. 6B).

3.2 Ultrastructural differences in chondrocytes from different age groups

To further observe the detailed changes that occur in chondrocytes with ageing, we analyzed the ultrastructure of the cells under TEM (Fig.2). Chondrocytes from the young and adult groups were oval-shaped, with integral cell and nuclear membranes. Nuclear membranes were clear and the density of cytoplasm was uniform. There were a lot of organelles in the cytoplasm, such as mitochondria and endoplasmic reticulum (Fig.2a, b). Chromatin was uniformly distributed in sections from

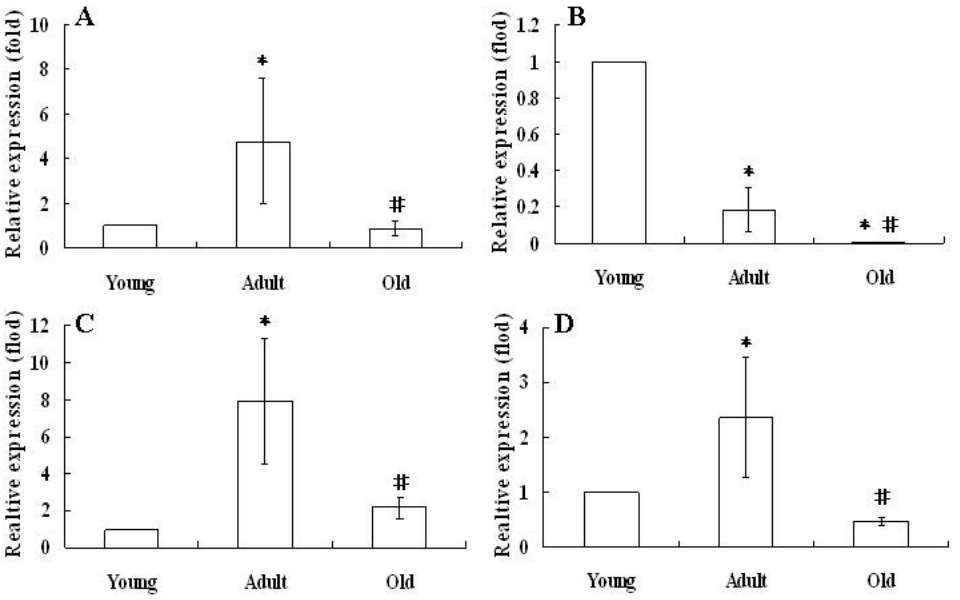

Figure 6: Changes in gene expression with ageing. Expressions of AGG (A), Col-2 (B), tubulin (C), and vinculin (D) mRNA were determined by real time PCR in different age chondrocytes. The expression of AGG, tubulin, and vinculin was higher in the adult group and decreased in the old cells. Type II collagen expression decreased gradually with ageing. *compared with young group $p<0.05$, #compared with adult group $p<0.05$.

the young group, however, increased heterochromatin and vacuoles (lipid droplet) appeared in the cytoplasms from the adult group (Fig.2c, d). A striking finding was a decrease in the cytoplasm organelles, including the mitochondria and endoplasmic reticulum, in the cells of old animals. These chondrocytes also exhibited irregular shape and increased electron density. The boundary of their cell membranes was not clear and their nuclei were deformed or shrunk, in some cases even absent. We also observed chromatic agglutination in the cells' nuclei (Fig.2e, f).

3.3 Differences in viscoelastic properties of chondrocytes from different age groups

In response to an applied constant, negative 0.3–0.4 kPa aspiration pressure, all chondrocytes exhibited standard linear viscoelastic solid properties. Namely, the cells 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 (Fig.3A).

Serial visualization under the inverted microscope showed that the instantaneous length of time-dependent aspiration into the micropipette was significantly increased for old chondrocytes, but the time of viscoelastic creep reaching equilibrium was reduced to (40±10 s) compared with the young and adult cells (Fig. 3A, B). There were no significant differences detected in the amplitude aspirated into the micropipette and the time to equilibrium (110±18 s) between young and adult chondrocytes (Fig.3A, B). Meanwhile, the size of cells in suspension significantly increased with ageing from 8.84±0.97 μm in young animals to 9.77±1.41 μm in adult animals, and 11.50±1.76 μm in old animals ($P < 0.05$) (N=90 cells per age group).

Model predictions of the creep data showed that the average instantaneous modulus (E_0), the equilibrium modulus (E_∞) and the apparent viscosity (μ) of old chondrocytes were significantly lower than those of young and adult ones ($P < 0.001$). No significant differences were detected between young and adult chondrocytes ($P > 0.05$) (Table 2).

Table 1: Primer sequences used for real time PCR

Gene	**Primer nucleotide sequence**	**Accession No.**
AGG	Forward 5'-TCTACCGCTGTGAGGTGATGC-3' Reverse 5'-TTCACCACGACCTCCAAGG-3'	L38480
Col-2	Forward 5'-ACACTGCCAACGTCCAGATG-3' Reverse 5'-GTGATGTTCTGGGAGCCCTC-3'	D83228
Tubulin	Forward 5'-CAAGCGTGCCTTTGTTCACT-3' Reverse 5'-CTTCCTCCTCACCCTCACCTT-3'	TaKaRa
Vinculin	Forward 5'-TACCAAGCGGGCACTCATTC-3' Reverse 5'-TGGGATTCGTTCACACACCT-3'	TaKaRa
GAPDH	Forward 5'-GGTGAAGGTCGGAGTGAACG-3' Reverse 5'-AGTTAAAAGCAGCCCTGGTGA-3'	L23961

3.4 Differences in cytoskeleton network and Content in Chondrocytes from Different Ages

Laser scanning confocal microscopy

Typically, chondrocytes cultured on cover slips became confluent after 1–2 days in culture, exhibiting a monolayer of flat cells with several cytoplasmic processes. All

Table 2: Viscoelastic parameters of chondrocytes from the articular cartilage of the knees of rabbits from three age groups (Mean ± SD)

	n	E_0(kPa)	E_∞(kPa)	μ(kPa·s)
Young	47	0.67±0.10	0.37±0.09	6.29±0.92
Adult	51	0.65±0.07	0.35±0.05	6.01±0.89
Old	49	0.55±0.05*#	0.28±0.04*#	4.10±0.61*#

* compared with young group p<0.05, # compared with adult group p<0.05.

cytoskeletal proteins displayed a positive immunofluorescent reaction and revealed a network of polygonal arrays within the cytoplasm of chondrocytes (Fig. 4).

Tubulin was distributed completely throughout the cells, with clear radial filamentous structures that extended from the nuclei to the cell membranes (Fig. 4A). Data from cytoskeletal fluorescence quantitative analysis showed that the average tubulin intensities in adult and old chondrocytes were significantly lower than that in the young ones (P<0.05). There was no significant difference detected between the adult (19.60±5.85) and the old (14.88±3.91) groups (P >0.05) (Fig. 5B).

Phalloidin in young chondrocytes was distributed as networks throughout the cytosol, and mainly near the cellular membranes and cell processes. In adult and old chondrocytes, phalloidin was distributed in a disorganized manner and its content around the cell membrane and cell processes appeared to decrease with ageing (Fig. 4B). The average phalloidin cytoskeletal intensity was reduced gradually with aging from 47.6±23.9 in young cells, 20.6±13.1 in adult cells and 7.0±2.5 in old cells (P<0.05) (Fig. 5B). Vinculin participates in the anchoring of phalloidin in all ageing chondrocytes (Fig. 4D, E). The data in Fig.5B showed that the average vinculin fluorescence intensity was reduced gradually with aging from 22.40±5.81 in young cells to 11.30±3.35 in adult cells and 1.99±0.55 in old cells (P<0.05), and there was a positive direct correlation between vinculin fluorescence and phalloidin intensity (r=0.659) (Fig. 5C).

Similar to phalloidin, vimentin was distributed as networks throughout the cytosol, and was found to be enriched mainly near cell membranes. There was little or no change in the distribution and fluorescence intensity of vimentin with ageing (P>0.05) (Fig. 4C, 5B).

Real time PCR

Real time PCR analysis indicated that tubulin and vinculin mRNAs were most highly expressed in the adult group, which showed 7.89-fold expression of tubulin (p<0.05) and 2.36-fold expression of vinculin (p<0.05) when compared with the

young group. Both tubulin and vinculin mRNA expression decreased in the old group when compared to the adult group ($p<0.05$), but there were no significant differences between the young and old groups ($P>0.05$) (Fig.6C, D).

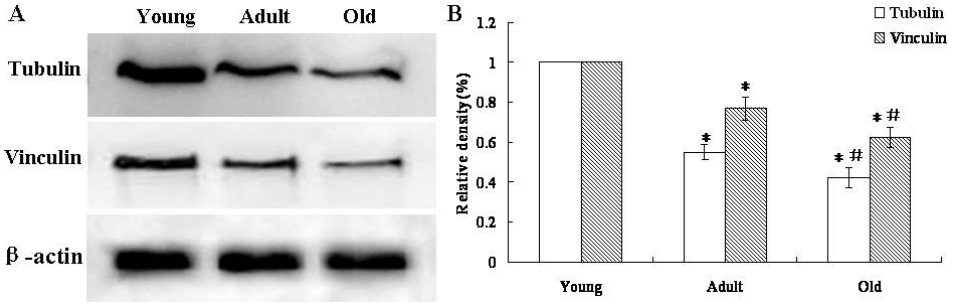

Figure 7: Decreased tubulin and vinculin with ageing. Cytoskeleton protein content determined by western blotting in different age chondrocytes (A), relative density of tubulin and vinculin from three age groups (B) (n=3).*compared with young group $p<0.05$,#compared with adult group $p<0.05$.

Western-blotting

Expression of tubulin and vinculin proteins decreased gradually with ageing, so that tubulin protein was reduced to 55% in adult chondrocytes ($P<0.05$), and to 42% in old cells ($P<0.05$) when compared to young cells, and there was also a significant difference between the adult and old chondrocytes ($P<0.05$) (Fig.7). The protein level of vinculin decreased to 77% in adult cells ($P<0.05$) and 62% in old cells ($P<0.05$) compared with young cells, and also in old chondrocytes compared to adult ones ($P<0.05$) (Fig.7).

4 Discussion

Studies have demonstrated that alterations of the viscoelastic properties of chondrocytes occur in osteoarthritis [16, 18, 21], but it is unclear whether similar changes take place with ageing. Our findings in this study indicate that chondrocytes in suspension showed significant viscoelastic behavior under a constant negative pressure. But, the viscoelastic properties of chondrocytes from the knees of old rabbits were significantly reduced when compared with those from chondrocytes derived from the knees of young and adult animals, whereas no discernible differences were observed between young and adult chondrocytes.

Previous studies have shown that the changes in the mechanical properties of articular cartilage are due to alterations in the matrix that occur with age [22-25].

Other studies also indicated that a stiffer and more cross linked collagen network owing to Maillard reaction, with advancing age, may become more brittle and more prone to fatigue [26, 27], and that accumulation of advanced glycation end products increases cartilage brittleness, while decreasing the synthesis of cartilage matrix constituents [28]. Chondrocytes are the only cells that maintain normal articular cartilage's homeostasis. Therefore, their function and mechanics are of utter importance to the health of normal aged articular cartilage.

Recent studies have shown that the cytoskeleton may play a critical role in the viscoelastic properties of chondrocytes [10]. Within articular cartilage, the chondrocyte cytoskeleton comprises a dynamic three dimensional network consisting principally of the proteins actin, vimentin, and tubulin, which are organized into microfilaments, intermediate filaments, and microtubules, respectively [29]. The microfilaments and intermediate filaments provide the viscoelastic properties of cell, whereas the microtubules do not contribute to viscoelasticity [10]. Other studies showed that an intact cytoskeleton is essential to maintain chondrocyte metabolism [30]. Another group reported a similar finding in which the cytoskeleton not only contributed to the biomechanical properties of the chondrocyte, but also influenced the interactions between the cell and its pericellular and extracellular matrix [31, 32]. Thus, changes in cytoskeleton components and structure with age may lead to alterations of viscoelastic properties by modifying the interactions between chondrocytes and matrix. Modulation of the cytoskeletal network changes the mechanical properties of the cell that are essential for functions such as locomotion [33, 34] and cytokinesis [35]. The evidence indicates that the cytoskeleton serves an important biomechanical function in governing cell–matrix interactions in articular cartilage, and also plays a role in response to mechanical stimuli.

Our next question was whether the changes in viscoelastic properties and metabolism observed in chondrocytes with ageing are actually associated with changes in the skeletal components. Our results suggest that the decrease in viscoelastic properties of aged chondrocytes is associated with a decrease of skeletal components including proteoglycan and type II collagen. TEM further demonstrated the decrease of organelles in the cytoplasm of aged chondrocytes, such as endoplasmic reticulum and Golgi apparatus. These structural changes in aged chondrocytes result in decreased ability to produce cell components, which in turn may be responsible for the change in viscoelastic properties [36]. In our study, we noticed that mRNA expression and levels of particular proteins were different with ageing, with mRNAs highly expressed but poorly translated into proteins in adult animals. These finding implicates that aged cartilage may try to produce more cartilage matrix, but matrix degradation remains beyond repair. Further study in this area is needed.

Studies indicate that the mechanical behavior of chondrocytes may be governed

primarily by their membrane at small deformation [37, 38], which may be considered to be largely elastic [16, 39]. As the deformation is increased, some other intracellular components such as the cytoskeleton, organelles, and even nucleus may play an increasingly significant role. These components show increased viscoelastic behavior than the membrane [37, 40-43]. In our study, we found that the early deformation rate and amplitude in old cells were increased under the same negative pressure, and the instantaneous modulus (E_0) in old cells was reduced to 82% compared with young and adult cells. These findings indicated that the cell membrane of old animals may have been different than that in young and adult animals. Our TEM findings further support this notion of changes in the cell membrane. Meanwhile, the equilibrium length of time-dependent aspiration into micropipette was increased in old cells, and data (table 2) showed that the viscoelastic properties of old chondrocytes, including the equilibrium modulus (E_∞), and apparent viscosity (μ) were reduced to 75and 65% compared to young and adult ones respectively. These biomechanical findings indicated that the cytoskeletal network and organelles, even nuclear components were changed in old chondrocytes. Moreover, we found that the cytoskeletal network was sparser, and its contents were reduced in old cells. This finding was corroborated by immunohistochemistry staining, LSCM, real time PCR and western blotting. TEM showed few organelles and deformed or shrunk nuclei in old cells. These findings further indicated that the cytoskeletal network, cellular organelles, and even the nucleus play an important role in the changes of the whole-cell viscoelastic properties with aging [41, 42, 44].

It is well known that the cytoskeleton plays a crucial role in signal transduction from the extracellular matrix to the cell nucleus and it also can be transformed from a signal into a corresponding cascade of biochemical events [9, 45]. With ageing, the changes of cytoskeleton may result in a transformation of signal transduction, and even lead to a series of changes that occur in the cell nucleus. As a result, we found that the decrease of the viscoelastic properties of chondrocytes is associated with the loss of the skeletal components and the changes of nuclear membrane observed by TEM in old cells.

5 Conclusions

Our study demonstrated that the viscoelastic properties of chondrocytes from the knees of old rabbits are decreased when compared with chondrocytes from young and adult animals. The changes of viscoelastic properties in aged chondrocytes may play an important role in the changes of biomechanics of articular cartilage with ageing. Our findings further indicate that the viscoelastic properties of chondrocytes are associated with the changes of the cytoskeleton structure and components, including cell membrane, nucleus, and matrix proteins that occur with aging.

Further understanding of how the components of the cytoskeleton are regulated by other factors that affect chondrocyte behavior such as growth factors and cytokines may help to explain the changes of the viscoelastic properties of chondrocytes with ageing.

Competing interests

The authors declare that they have no competing of interest.

Authors' contributions

Wangping Duan participated in the acquisition of biopsies and TEM, performed all the analyses except for biomechanics and participated in the design and drafting of the manuscript. Lei Wei participated in the design, in the interpretation of results, and in the revision of the manuscript. Juntao Zhang, Yongzhuang Hao and Qi Li participated in cell cultivation, real time PCR and western-blotting, and data acquisition. Chunjiang Li, Quanyou Zhang and Weiyi Chen participated in the viscoelastic properties analyses for chondrocytes. Hao Li participated in the cytoskeleton structure analyses by LSCM, and data acquisition. Xiaochun Wei participated in the design and coordination, help to draft the manuscript, interpret results and revised the manuscript. All the authors gave their final approval of the version to be published.

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