Loss of autophagy in tibial plateau chondrocytes causes increased apoptosis of chondrocytes in spontaneous osteoarthritis of guinea pigs

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Abstract

Objective

To observe the effect of autophagy in tibial plateau chondrocytes on apoptosis in spontaneous knee osteoarthritis (OA) in guinea pigs.

Methods

Fifty 2-month-old female Hartley guinea pigs were divided into a normal group, which was euthanized after 7 months, and an OA group, ten of which were euthanized after 10 months. Immunohistochemistry, PCR and western blotting were used to evaluate the level of autophagy, intracellular glycogen accumulation and apoptosis in tibial plateau chondrocytes in vivo and in vitro. The remaining 30 guinea pigs in the OA group were divided into 3 groups: a rapamycin group, a normal saline group and a 3-MA group. Intracellular glycogen accumulation and chondrocyte apoptosis were observed by changing the level of autophagy in tibial plateau chondrocytes in vivo.

Results

When spontaneous OA occurred in the guinea pigs, the level of autophagy in tibial plateau chondrocytes decreased, and intracellular glycogen accumulation and the rate of chondrocyte apoptosis increased. After enhancing the autophagy level of tibial plateau chondrocytes in OA guinea pigs, the intracellular glycogen accumulation and the rate of chondrocyte apoptosis decreased. When the autophagy level of the chondrocytes was weakened, intracellular glycogen accumulation was further increased, and the apoptosis rate was higher.

Conclusions

The autophagy function of chondrocytes may be at least partly involved in the catabolism of glycogen in chondrocytes. In OA guinea pigs, the autophagy level in tibial plateau chondrocytes decreased, and the chondrocytes were unable to degrade intracellular glycogen into glucose, leading to less energy for the chondrocytes and increased apoptosis.

Introduction

Knee osteoarthritis (OA) is a degenerative disease that seriously reduces the physical and mental health of middle-aged and elderly individuals; it is a high-prevalence disease with substantial socio-economic impact (1–5). The main manifestation of this disease is the degeneration of cartilage in the knee joint (6–9). Cartilage primarily comprises chondrocytes and the cartilage matrix secreted by the chondrocytes.
(10, 11). Therefore, the physiological state of the chondrocytes determines the degree of cartilage degeneration.

Autophagy is a self-regulatory process of cells that occurs in response to harmful external stimuli (12–16). It primarily produces autolysosomes that combine with and degrade intracellular metabolites or redundant organelles allows cells to adapt to changes in the external environment and maintain homeostasis. The process of autophagy is divided into three steps: initiation and elongation, phagocytosis, fusion and degradation (17). Initially, autophagic vacuoles are gradually isolated from the rough endoplasmic reticulum. Autophagic vacuoles encapsulate intracytoplasmic substances and then combine with lysosomes to form autolysosomes. The hydrolytic enzymes carried by lysosomes can degrade the substances in the autolysosomes. Previous studies have found that autophagy primarily degrades metabolites and damaged organelles (18, 19). However, further research has found that autophagy vacuoles can devour and further degrade nutrients in the cytoplasm, including amino acids, fats and glycogen granules (20–25). Chondrocytes exhibit a high level of autophagy because cartilage tissue is a nutrient-poor medium (26). The key proteins related to chondrocyte autophagy are light chain-3 (LC-3), autophagy-related protein-5 (ATG-5), ATG-7 and Beclin-1 (27–29). Studies have shown that when OA occurs, the level of autophagy in articular chondrocytes significantly decreases (30–32). Moreover, when the level of autophagy was reduced, the occurrence and development of knee OA were also more rapid (33–35). Targeted deletion of Atg-5 in chondrocytes promotes age-related osteoarthritis. It should be noted that because chondrocytes exist in nutrient-poor medium, energy intake is essential for their survival. Glucose is the most direct energy source of chondrocytes. If glucose is lacking for an extended time, it will lead to apoptosis of chondrocytes. Most intracellular glucose is stored in chondrocytes in the form of glycogen, which is degraded into glucose for chondrocyte use. Recent studies have shown that autophagy is involved in chondrocyte energy metabolism, and autolysosomes can degrade intracellular glycogen into glucose (36–38).

The aim of our study was to detect changes in the level of chondrocyte autophagy, intracellular glycogen accumulation and apoptosis in tibial plateau chondrocytes during spontaneous knee OA to further explore the relationship between autophagy and glycogen metabolism.

**Materials And Methods**

**Animal handing**

This study was approved by the Ethics Committee of Shanxi Medical University (approval number:SXMUE2019004). Fifty 2-month-old female SPF (specific-pathogen free) grade Dunkin Hartley (DH) albino guinea pigs (Animal Experiment Center of Shanxi Provincial People's Hospital, China) were housed in pairs and given 2 weeks to acclimate to the housing facility. Environmental conditions included a temperature of 24°C ± 1°C, humidity 53% ± 15%, and a 12:12 light:dark cycle with lights on at 07:00 and off at 19:00. The guinea pigs were given access to a sterilized diet and water. The guinea pigs were divided into two groups: a normal group (10 animals), which were euthanized after 7 months and an
osteoarthritis (OA) group, ten of which were euthanized after 10 months. The remaining 30 guinea pigs were divided into three groups: a rapamycin group, a normal saline (NS) group and a 3-methyl adenine (3-MA) group. Solutions were prepared according to the drug instructions. Guinea pigs in the rapamycin group were intraperitoneally injected with 6mg/kg/d rapamycin for 30 d. Guinea pigs in the NS group were intraperitoneally injected with an equal-volume of NS for 30 d. Guinea pigs in the 3-MA group were intraperitoneally injected with 2.5mg/kg/d 3-MA for 30 d. After 30 d of intraperitoneal injections, all guinea pigs were sacrificed and bilateral knee joint specimens were taken. No animals died during the experiment.

Guinea pigs tibial plateau chondrocytes

A sterile scalpel was used to cut the corresponding layers of cartilage, and chondrocytes were obtained after tissue clipping and collagenase type II digestion. The isolated chondrocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The chondrocytes were cultured to the third generation (P3) for experiments.

Evaluation of knee degeneration

Safranin O staining, India ink staining, and fluorescence molecular tomography (FMT) were used to evaluate knee joint degeneration. Routine histological sections were made, and the degree of degeneration in tibial plateau cartilage tissue of the knee joint was observed after safranin O staining and compared between groups using the Osteoarthritis Research Society International (OARSI) score. After the knee joints were opened and disarticulated, the gross morphological lesions were visualized with India ink staining. FMT (ViSen, Waltham, MA, USA) was used to detect the expression of MMPs in the articular cartilage tissue.

Immunohistochemistry

Immunohistochemistry (IHC) staining was used to detect Aggrecan (1:100, PAB908Ra02, Cloud-Clone Corp., USA), matrix metalloproteinase-13 (MMP-13) (1:100, PAA099Ra01, Cloud-Clone Corp., USA), LC-3 (1:200, ab48394, Abcam, USA), glycogenin-1 (1:100, sc-271109, Santa Cruz, USA), caspase-3 (1:200, PAA626Ra01, Cloud-Clone Corp., USA), and proliferating cell nuclear antigen (PCNA) (1:10000, ab29, Abcam, USA) in cartilage tissue. We quantitatively scored the IHC results based on the percentage of positive chondrocytes and the staining intensity as described below (39). We rated the intensity of staining on a scale of 0 to 3: 0, negative; 1, weak; 2, moderate; and 3, strong. We assigned the following proportional scores: 0, if 0% of the chondrocytes showed positive staining; 1, if up to 1% of the chondrocytes were stained; 2, if 2% to 10% were stained; 3, if 11% to 30% were stained; 4, if 31% to 70% were stained; and 5, if 71% to 100% were stained. We then combined the proportion and intensity scores to obtain a total score (range: 0-8) as described previously. The results were assessed by 2 experienced pathologists in a blind manner.
Polymerase chain reaction (PCR)

Total RNA was extracted from cartilage tissue and chondrocytes by using TRIzol reagent (Thermo Fisher Scientific). The quality and quantity of total RNA samples were tested using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific). A preparation of RNA with an A260/A280 ratio of 1.8~2.0 was used for analysis of mRNA expression. Individual RNA samples (1 μg per sample) were reverse transcribed into cDNA using the PrimeScript RT Master Mix kit (Takara, Shiga, Japan) according to the manufacturer's instructions. The relative expression levels of target gene mRNA to the control 18SrRNA transcripts were determined by RT-PCR using SYBR Premix Ex TaqTM (Takara) and the specific primers in the IQ5 Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA). The sequences of the primers were forward 5’-ACTCTGGGTTTTCGTGACTCT-3’ and reverse 5’-ACACTCAGCGAGTTGTCATGG-3’ for Aggrecan; forward 5’-CAGGAATGGTGATAAAGTAGAT-3’ and reverse 5’-CTGTATTCAAACTGTATGGGTC-3’ for MMP-13; forward 5’-TGGACGATCAGGCGAAACC-3’ and reverse 5’-GCTGCGGATGCTCTCAATCT-3’ for LC-3; forward 5’-ATGCTGCCACAAATACCCTTT-3’ and reverse 5’-GGTAGTGGGCCCTTTATGCCT-3’ for Glycogenin-1; forward 5’-TTGCACTGAGGATCTCACTTG-3’ and reverse 5’-CCTTCTTCATCCTCGATCT-3’ for Caspase-3.

Western blot

The cartilage tissue and chondrocyte samples were lysed in RIPA lysis buffer containing PMSF, protease and phosphatase inhibitors (Keygen). Some of the protein was mixed with the loading buffer, boiled for 10 min and subjected to SDS-PAGE followed by transfer to PVDF membranes. After being blocked with 5% fat-free dry milk in TBST, the bolts were probed with primary antibodies. The expression level of the target protein relative to the control β-actin was determined by western blot analysis. The bound antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized using the enhanced chemiluminescence reagent. The data were analyzed using densitometric analysis with IMAGEJ software.

Statistical analysis

SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. All data in this study are expressed as the mean ± standard deviation (SD). A P value of less than 0.05 was considered statistically significant.

Results

Changes in chondrocyte autophagy and glycogen accumulation in OA cartilage tissue
To observe the degree of degeneration of knee tibial plateau articular cartilage tissue, we compared the OARSI scores of the two groups after safranin O staining. The results showed that the surface of the normal cartilage tissue was intact, without damage, and with deep safranin O staining. In the OA group, the surface of the cartilage tissue was broken, the longitudinal fissure was as deep as the middle zone, the number of superficial chondrocytes was significantly reduced, and the safranin O staining was very shallow in the damaged area [Figure 1A].

To further quantify the changes in chondrocyte autophagy and glycogen accumulation in OA cartilage tissue, immunohistochemistry was used to detect the level of LC-3, a biomarker of chondrocyte autophagy, and the level of glycogenin-1, a biomarker of glycogen accumulation. We also examined a number of indicators related to cartilage degeneration, such as Aggrecan, MMP-13, and Caspase-3. The results showed that, compared with the normal group, the levels of the LC-3 protein and the Aggrecan protein decreased, the glycogenin-1 protein, MMP-13 protein and Caspase-3 protein increased in the cartilage tissue of the OA group. The TUNEL technique was used to detect the articular cartilage tissue of the two groups, and the results showed that the rate of apoptosis of chondrocytes in the damaged area of the OA group was higher than the normal group [Figure 1B].

After scraping the tibial plateau cartilage tissue of the two groups, PCR was used to detect the levels of LC-3 mRNA, glycogenin-1 mRNA, Aggrecan mRNA, MMP-13 mRNA, and Caspase-3 mRNA, and a western blot analysis was used to detect the corresponding protein levels. The PCR results showed that the levels of LC3 mRNA and Aggrecan mRNA decreased, and the levels of glycogenin-1 mRNA, MMP-13 mRNA, and Caspase-3 mRNA increased in the OA group. The western blot results showed that the levels of LC-3 and Aggrecan decreased, and the levels of glycogenin-1, MMP-13 and Caspase-3 increased in the OA group [Figure 1C, Figure 1D].

**Changes in chondrocyte autophagy and glycogen accumulation in OA chondrocytes in vitro**

The cartilage tissue was scraped from sterile tibial plateau specimens, and chondrocytes were extracted for in vitro culture to generation 3. Immunohistochemistry was used to detect the expressions of the LC-3 protein, glycogenin-1 protein, Aggrecan protein, MMP-13 protein, and Caspase-3 protein in chondrocytes. The results showed that, compared with the normal group, the levels of the LC-3 protein and the Aggrecan protein decreased, and the glycogenin-1, MMP-13 and Caspase-3 proteins increased in the chondrocytes of the OA group [Figure 2A]. The TUNEL technique was used to detect the articular cartilage tissue of the two groups, and the results showed that the rate of apoptosis of chondrocytes in the OA group was higher than that in the normal group [Figure 2B].

The mRNA and protein were extracted from lysed chondrocytes. The PCR results showed that the levels of LC-3 mRNA and Aggrecan mRNA decreased, and the levels of glycogenin-1 mRNA, MMP-13 mRNA and Caspase-3 mRNA increased in the OA group. The western blot results showed that the levels of LC-3 and Aggrecan decreased, and the levels of glycogenin-1, MMP-13 and Caspase-3 increased in the OA group [Figure 2C, Figure 2D].
Effects of changing autophagy levels on glycogen accumulation in the presence of OA in vivo

To observe the effect of chondrocyte autophagy level on glycogen accumulation, we gave OA guinea pigs continuous intra-abdominal injections of the autophagy activator rapamycin and the autophagy inhibitor 3-MA. The results showed that after 30d of continuous intra-abdominal injection of rapamycin, the autophagy level of tibial plateau chondrocytes in guinea pigs was significantly increased as indicated by the level of the LC-3 protein autophagy biomarker. The accumulation of glycogen in the chondrocytes of the rapamycin group was mild as indicated by the level of the glycogenin-1 protein glycogen accumulation biomarker. However, after 30 d of 3-MA intra-abdominal injection, the autophagy level of tibial plateau chondrocytes in guinea pigs decreased significantly [Figure3B]. At the same time, the results showed that the injection of rapamycin attenuated the degeneration of tibial plateau cartilage tissue (as indicated by safranin O staining, India ink staining, and FMT) [Figure3A]. The injection of 3-MA promoted the degeneration of cartilage tissue. These results were consistent with the reports from previous studies [Figure4].

Discussion

We found that autophagy of tibial plateau chondrocytes was at least partially involved, in intracellular glycogen degradation and energy supply. Autophagy vacuoles can devour intracellular glycogen granules, and then bind to the lysosome to form the autolysosome. Hydrolase in the autolysosome degraded the glycogen granules into glucose for chondrocyte use. When knee OA occurs, the level of autophagy in the tibial plateau chondrocytes decreases, and sufficient autophagic vacuoles cannot be formed in the chondrocytes to consume the glycogen granules. As a result, there was insufficient glucose in the chondrocyte, which lead to apoptosis of the chondrocyte due to insufficient energy. The apoptosis of OA tibial plateau chondrocytes as a result of the decrease in autophagy may be related to the failure of glycogen degradation in chondrocytes.

Knee tibial plateau cartilage is primarily composed of chondrocytes and extracellular matrix secreted by the chondrocytes; therefore, chondrocytes determine the physiological state of cartilage tissue [10,11,40,41]. Research on chondrocytes is a hot spot in the field of knee OA [8,12-14,18,30,31,34]. To date, there are many experimental methods to simulate human knee OA, such as surgical application of traumatic OA, drug injection (such as papain) into the joint to produce knee OA, and external fixators to break the knee joint, causing OA [42-45]. However, these are all exogenous interventions. The ideal way to study the occurrence and development of knee OA is to find an animal model that can simulate the natural degeneration process of the human knee joint. The guinea pig is a typical animal model in OA-related studies, and spontaneously exhibits degeneration, degradation and loss of knee cartilage with the increase in monthly age [46-48].

Two-month-old guinea pigs were sacrificed after 7 months. Knee joint specimens were taken for histological sections and stained with safranin O. No obvious change in OA was found in the articular cartilage. However, after 10 months, there were significant changes in the cartilage of the tibial plateau of...
the guinea pig knee OA. This spontaneous cartilage tissue degeneration model in guinea pigs can better simulate the development process of human knee OA; OA progresses very slowly, such that markers for early and middle stage chronic inflammatory proteins of OA can be naturally expressed. The experimental results showed that when spontaneous OA occurred, the ability of chondrocytes to synthesize Aggrecan protein decreased, whereas the synthesis and expression of MMP-13 protein increased in the area of damaged cartilage tissue. This is consistent with the pathological changes in cartilage tissue in knee OA reported in the literature.

Autophagy is an important function that allows chondrocytes to maintain homeostasis. Autophagysomes can be produced to degrade intracellular metabolites, and excess organelles can be degraded when there is external malnutrition to reduce the energy consumption of the chondrocytes. Cartilage tissue is an avascular structure, suggesting that chondrocytes experience malnutrition and hypoxia. Chondrocytes always maintain a high level of autophagy under normal conditions\textsuperscript{14,15,18}. Traditionally, autophagy maintains cell homeostasis by degrading metabolites and redundant organelles. However, recent studies have found that autophagy is closely related to the energy metabolism of cells, particularly sugar metabolism. Glycogen is a long chain molecule formed by glucose after the shrinkage reaction, and can supply necessary energy to cells by degrading to form glucose molecules when energy is lacking. Glycogenin-1 plays an important role in the initial stage of glycogen synthesis, and its level in the chondrocyte can reflect the glycogen accumulation in the chondrocytes\textsuperscript{49}. The data showed that glycogen levels were high in liver cells and muscle cells. Studies have shown that there was also a certain amount of glycogen in chondrocytes\textsuperscript{12}. Glycogen exists in chondrocytes in two forms: the free form in the cytoplasm and the form consumed by autophagy vacuoles. In the free form in the cytoplasm, glycogen is phosphorylated and degraded under the action of a catabolic enzyme. In the latter form, autolysosomes are formed after the combining of autophagy vacuoles and lysosomes. Hydrolase in the autophagysomes directly degrades glycogen to glucose without phosphorylation. Studies have shown that glucose produced in this manner (without the phosphorylation of glycogen) is more direct and is important to the energy supply of the chondrocyte\textsuperscript{12,38}. We detected levels of the LC-3 protein, a biomarker of autophagy function, in the normal group using immunohistochemistry, and found that most chondrocytes expressed this protein, whereas the level and expression rate of the LC-3 protein in chondrocytes of the OA group decreased significantly. Simultaneously, glycogen accumulation was detected in the chondrocytes. It was found that the level of glycogenin-1, a marker of glycogen accumulation, was significantly increased in the OA group, whereas the level in the normal group was very low, which proved that the autophagy level decreased and glycogen accumulation increased in the chondrocytes of the OA guinea pigs. By detecting the level of the Caspase-3 protein, a biomarker of apoptosis, it was found that the rate of chondrocytes expressing caspase-3 protein was significantly increased in the OA group. Furthermore, the TUNEL technique was used to detect the apoptosis rate of tibial plateau chondrocytes in the two groups, which also proved that the apoptosis rate of chondrocytes in the OA group was higher in vivo and in vitro.

Why is suppression of autophagy accompanied by increased apoptosis? Based on these experimental results, we speculate that there is a certain level of glycogen storage in chondrocytes, as shown in Figure...
4. When autophagy function is normal, autophagy vacuoles consume glycogen granules and then combine with lysosomes to form autolysosomes. The hydrolase in autolysosomes degrades glycogen to glucose for chondrocytes to use. When the autophagy level of the chondrocytes decreases and the autophagy vacuoles decrease, glycogen cannot be consumed and degraded, which leads to its accumulation in the chondrocytes, which cannot obtain the necessary glucose, which eventually leads to a reduced energy supply and apoptosis.

Our experiment has some limitations. We found that when OA occurs, the autophagy level of chondrocytes decreases, which leads to the failure of glycogen granules in chondrocytes to degrade into glucose for chondrocytes use, and eventually leads to a lack of energy and apoptosis of chondrocytes. However, when OA occurs, there are many factors that cause chondrocyte apoptosis; therefore, we do not know what proportion of apoptosis is caused by decreased autophagy in chondrocytes. Chondrocytes exist in a hypoxic environment, and anaerobic glycolysis should be the primary form of nutrient metabolism; therefore, we do not know what proportion of this glycogen metabolism is autophagy-mediated. These issues require further research.

**Declarations**

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**Contributors** XJ Wang: study design, data acquisition, data analysis, wrote the manuscript. Y Xue, TY Chen and SJ Guo: data analysis, discussion of results. YF Gao, LJ Li: data acquisition, histochemistry and western blotting analysis. F Chang and XC Wei: statistical analyses. L Wei and RS Li: study design, manuscript correction. All authors have approved the submitted manuscript.

**Competing interests** None declared.

**Patient consent** Obtained.

**Ethics approval** This study was approved by the Ethics Committee of Shanxi Medical University [Approval number: SXMUE(2019004)] (Tai Yuan, China).

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**Figures**
Figure 1

Comparison of autophagy levels and glycogen accumulation between normal and OA chondrocytes in vivo. (A) Comparison of safranin O staining of knee tibial plateau cartilage tissue and OARSI scores. (B) Immunohistochemical results of the proteins Aggrecan, MMP-13, LC-3, glycogenin-1, and Caspase-3 and comparisons of tissue immunohistochemical scores of knee tibial plateau cartilage tissue. (C) PCR results for Aggrecan mRNA, MMP-13 mRNA, LC-3 mRNA, glycogenin-1 mRNA, and Caspase-3 mRNA from...
knee tibial plateau cartilage tissue. (D) Western-blot results of Aggrecan, MMP-13, LC-3, glycogenin-1, and Caspase-3 and comparison of gray values. Scale=100 μm. Statistical significance is shown for a two-tailed t-test. Bars represent mean±SEM, *p<0.05 compared with the normal group.

Figure 2

Comparison of autophagy levels and glycogen accumulation between normal and OA chondrocytes in vitro. (A) Immunohistochemical results of the proteins Aggrecan, MMP-13, LC3, glycogenin-1, and
Caspase-3 and the comparisons of tissue immunohistochemical scores of chondrocytes in vitro.

Scale=10 μm. (B) Results of TUNEL method to detect the 72 h apoptosis rate of chondrocytes in vitro. Scale=100 μm. (C) PCR results for Aggrecan mRNA, MMP-13 mRNA, LC3 mRNA, glycogenin-1 mRNA, and Caspase-3 mRNA of chondrocytes in vitro. (D) Western-blot results of Aggrecan, MMP-13, LC-3, glycogenin-1, and Caspase-3 and comparison gray values. Statistical significance is shown for a two-tailed t-test. Bars represent mean±SEM, *p<0.05 compared with the normal group.

Figure 3
Effect of changes in chondrocyte autophagy levels on articular cartilage. (A) Comparison of safranin O staining of the knee tibial plateau cartilage using chondrocyte autophagic agonist and antagonist. (B) Gross morphological cartilage lesions and fibrillation in the tibial plateau were visualized using India ink staining. (C) Knee articular cartilage degeneration was evaluated in vivo using FMT (MMPs probe). (D) Immunohistochemical results of the proteins Aggrecan, MMP-13, LC-3, glycogenin-1, and Caspase-3 and comparisons of tissue immunohistochemical scores. Scale=100 μm. Statistical significance is shown for a two-tailed t-test. Bars represent mean±SEM, *p<0.05 compared with the rapamycin group; #p<0.05 compared with the NS group.

Figure 4

Proposed model for the role of chondrocyte autophagy on intracellular glycogen degradation. (A) Normal chondrocytes form autophagic vacuole. (B) Normal chondrocyte autophagic vacuole encloses glycogen particles. (C) Autophagic vacuole fuses with lysosome to form autolysosome, and the hydrolases in the lysosome contact the glycogen particles. (D) The hydrolases in the autolysosome degrade glycogen particles into glucose, which can be directly used by the chondrocytes. (E) OA chondrocytes cannot form autophagic vacuoles and a substantial quantity of glycogen particles in the cytoplasm cannot be
engulfed. (F) OA chondrocytes do not have adequate glucose production in the cytoplasm for cellular functions, and chondrocyte apoptosis occurs due to insufficient energy.