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EFFECTS OF DIFFERENT MODE HIGH-INTENSITY MOVEMENT TRAINING ON ARTICULAR CARTILAGE IN HISTOLOGY - A RANDOMIZED CONTROLLED TRIAL ON RABBIT KNEE

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Abstract. Objective: To study the "starting mechanism" and pathological process of knee cartilage injury in different movement training with high intensity. Materials and Methods: 72 New Zealand white rabbits were divided into 3 groups randomly: Untreated Control Group (CTRL, n=8), animals were untreated by any intervention processing; Running Training Group (RG, n=32), animals were trained running in a treadmill daily; Jumping Training Group (JG, n=32), animals were trained jumping in an electric stimulation cage daily. Rabbits in RT and JT were euthanized at 4 week and 8 week respectively, and knee joints were taken out to be examined histologically. GAG content, thickness of cartilage and subchondral bone, dead cell ratio and Mankin grades were measured respectively. Results: By microexaminations, different pathological changes of early sport injury of knee cartilage in RG and JG were found. Animals receiving jumping training displayed a significantly higher degree of grossly and cartilage matrix detectable degeneration on the weight-loading region of the femur condyles than did animals receiving running training; on the contrary, animals receiving running training displayed a significantly higher degree of chondrocyte damage than did animals receiving jumping training in the earlier stage (4-week). Conclusions: Repetitive and high-intensity jumping exercise can do more harm to cartilage matrix than to chondrocyte in knee joint, the pathogenesis displays as "cartilage matrix starting mechanism"; on the contrary, running exercise can do more harm to chondrocyte than to cartilage matrix, the pathogenesis displays as "chondrocyte starting mechanism". In addition, the difference in pathogenesis and cartilage damage phenotype by different exercises may have a close correlation with the different loading rate of stress on knee cartilage surface.

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Key words: Knee – Cartilage – Overuse - Injuries exercise

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Introduction

Movement training is the very important way to improve physical quality, different training mode and intensity can lead to different effects to human body, while unreasonable movement training can even induce injury. Both excessive motion of the joint [2,3] and excessive exposure of load on the joint [8,9,16,21] are known clinically and experimentally to cause degeneration or damage of the articular cartilage.

Jumping can be found commonly in many exercises, such as basketball playing, leapfrog, high or broad jump and some special items in military training. The repetitive jumping involved in these activities has seen a large incidence of overuse injuries, especially in knee joint [5,10]. Running has been thought to be a very eligible and popular physical activity for many years; however, the repetitive nature of running can also lead to overuse injuries in the weighted joint [12]. The same character of both activities is the repeatability, which can contribute to the gradual accumulation of knee cartilage damage due to insufficient recovery time between demands theoretically, further lead to the overuse injuries of cartilage. What are on earth the pathological characters of cartilage injuries caused respectively by running and jumping, and if they are similar or not, is still a question which had not been well documented before.

While the exact mechanism and pathological changes responsible for overuse injury caused by different exercises is yet to be determined, some methods may be rose suitably which might protect players from the cartilage damage caused by repetitive overloading of the knee joint.

In this search, we built model of cartilage overuse injury in rabbit knee joint by daily high-intensity running and jumping training, evaluated the effects of these two high-intensity exercises on the knee joint. The another aim of the present study was to analyze the pathogenesis of overuse injury of knee cartilage induced by these two exercises - running and jumping.

Materials and Methods

Animals and grouping: 72 New Zealand white rabbits (irrespective of gender, body weight 2.5-3.0 kg, average 2.8 kg, 10-12 months age), were divided into 3 groups randomly: Untreated Control Group (CTRL, n=8 at 8-week sacrifice), animals allowed unrestricted activity in the cage, untreated by any intervention processing; Running Training Group (RG, n=32 and 16 at 4-week and 8-week sacrifice respectively), animals put into double-track treadmill. Treadmill has a

uniform motion, speed as 36m/min, and with the assembly of vocal, photic and electric stimulus at the end of the treadmill. Jumping Training Group (JG, n=32 and 16 at 4-week and 8-week sacrifice respectively), animals put into the electric stimulation cage with high voltage and low electric current (1.6m×0.85m×1.3m, length × width × height), frequency and time of the stimulation respectively 20 times/minute and 0.2~0.5 second, and rabbits jump and run promptly several steps in the cage after a electric stimulation. Rabbits in RG and JG were trained daily, training for two hours per day for a total, and had a half-hour rest in the training process. Animal weights were recorded weekly throughout.

Main instruments: The animal electric stimulation cage (Duanshi corporation, Hangzhou, China); Double-track rabbit treadmill (Duanshi corporation, Hangzhou, China); Scanning electron microscope S-520 (resolution, 6nm)(Nippon Electric company Ltd, Minato-Ku, Japan); Transmission electron microscope JEM-2000EX (accelerating voltage, 200 kV, resolution, 0.2 nm) (Nippon Electric company Ltd, Minato-Ku, Japan); Nikon TE300 fluorescence microscope (Nikon Co., Tokyo, Japan).

Histology observations: Because a rabbit's femora has a comparatively larger range of movement than that of the tibia, while the femora has a comparatively large loading and friction, we collected samples in the weight-loading region of the femur condyles. Both knees were opened immediately after sacrifice and examined grossly. We took left knee joint for making pathological sections and right knee joint for extracting GAG. Samples with an average thickness of 0.5cm for light microscope histological observation were prepared, including cartilage and subchondral bone. Samples with an average region of 2.0×2.0×1.0 mm (length×width×thickness) were prepared for electron microscope observation. All samples for light microscope histological observation were fixed in 4% paraformaldehyde and decalcified with hydrochloric acid decalcifying fluid; after being embedded in paraffin, 7μm sections were cut and stained with hematoxylin-eosin and Safranine-O stain. Samples for electron microscope were observed respectively by scanning electron microscope and transmission electron microscope after being fixed in 2.5% Glutaral for 2 weeks.

Measurement of glycosaminoglycan (GAG) content: Full thickness shavings of articular cartilage from the weight-loading region of the femur condyles were obtained for determination of GAG content. Cartilage samples were weighed and then digested in 1.0 ml of 50 mM phosphate buffer, pH 6.0, containing 10 mM EDTA, 10 mM cysteine and 27μg/ml papain for 8 h at 60°C. The digests were then subjected to the DMB assay as described in modified form [7]. Thus, cartilage GAG content was expressed as μg GAG/mg cartilage wet weight. ("GAG"

mentioned in this article all represent "Sulfated GAG")

Measurement of thickness of articular cartilage and subchondral bone: The thicknesses of articular cartilage [hyaline cartilage (from cartilage surface to the most superficial tidemark) + calcified cartilage (from the most superficial tidemark to the calcified cartilage – bone junction)] and subchondral bone (from the calcified cartilage–bone junction to the marrow space) were measured at 20 equidistant points (400μm apart) over the center of each weight-loading region of femur condyle using a 10×10 grid eyepiece micrometer and a ×2 objective. These measurements were then averaged.

Measurement of dead cell ratio in cartilage: A FDA/PI double fluorescence stain was used to observe chondrocyte viability. According to previously published methods, samples were cut perpendicular to the articular surface into 50μm sections, and slices were stained with 100 nm fluorescein diacetate (FDA, Sigma, USA) to visualize living cells and with 5μg/ml propidium iodide (PI, Sigma, USA) to stain dead cells [13]. After incubation for 5 min in the dark at room temperature, slices were washed three times with PBS to remove excessive dye, embedded and analyzed with a Nikon TE300 fluorescence microscope immediately. FDA and PI were viewed at 380 – 490 or 465 – 550nm excitation.

Referred to Pelletier's method [20] selected 6 fields of vision (×400) randomly from the reassembled pictures, 3 in the surface and upper layer, and 3 in the lower and under layer. Dead cell ratio was calculated in each field of vision. Dead cell ratio = dead cell/(dead cell + living cell) ×100%.

Mankin grades: The histological sections were evaluated using the Mankin scheme [17]. All sections were graded for structure, Safranine-O stain, tidemark integrity, and cellularity, and these grades were summed.

Statistical analyzes: All statistical analyses were performed using SPSS 10.0 software. The statistical significance of differences in the mean value between different groups was determined by nonparametric comparison procedures Mann–Whitney U-test (two-tailed). Data were considered statistically significant when $p<0.05$.

Results

All rabbits were maintained in good general health except 2 in RG, died at 2-week and 7-week respectively for unexpected event. There were 7 and 4 rabbits in RG excluded from the trail for rejecting running at 4-week and 8-week respectively. Animals exhibited a normal weight gain during the study. All knee joints were normal from exterior observation in every respect throughout the entire

experiment.

Histology observation in general: At sacrifice, all knee joints were opened and examined for gross morphological degeneration to the articular cartilage, inflammation and osteophytosis. Cartilage in CTRL appeared glossy and translucent, with a smooth surface. We could find low-quantity and icky SF in the joint cavity. At 4-week, in both RG and JG, the synovium of joint appeared slightly coarse and inspissate, and with a mild hyperemia dropsy display. SF was dilute, but the total quantity increased. At 8-week, articular cartilages lose normal gloss, appeared dim, but still kept smooth in joint surface. Inflammation performance aggravated, and superficial cartilage lesions in the weight-loading region of the femur condyles could be found, 1 example in RG and 2 examples in JG. Osteophytes were not seen in any of the knee joints, and no other gross abnormalities were noted in any of the knee joints.

Microexaminations: (Figs. 1a-1c) In CTRL, cartilage matrix stained with Safranine-O stain appears prunosus homogeneously. The superficial zone is covered by micro-collagen fibers, cells with fusiform shape and arrangement of approximately horizontal in it. Chondrocytes arrangement is regular, tidal line is clear, and there are no chondrocyte clusters or decrease of chondrocyte distribution density in each layer. Inspection with scanning electron microscope reveals that the cartilage surface is smooth, on which naked collagen fibers and chondrocytes cannot be seen. Inspection with transmission electron microscope reveals that normal chondrocytes have two kinds of shapes—round and ellipse. There are apophysis and reductus on the cells' surface and many vacuoles without limiting membrane in cytoplasm, while nucleus is partial and nuclear membrane is clear. Arrangement of the collagen fibers in each layer of cartilage is regular and compacted.

In JG, at 4-week, microscopic inspection of Safranine-O stained sections reveals that cartilage matrix dyes inhomogeneously and lightly, and now and then vessels even break through the tidal line. Using transmission electron microscope, we can find few degenerated and immature chondrocytes, and the collagen fibers are indiscriminate. At 8-week, Microscopic inspection reveals that the phenomenon of cartilage matrix dyeing inhomogeneous and light can been seen commonly. Chondrocytes arrangement appears indiscriminate, and tidal line appears deranged. Now and then fissures can be found on the surface of articular cartilage. Inspection with scanning electron microscope reveals that it is rough and uneven in the surfaces of most samples, on which naked collagen fibers and chondrocytes can be seen (Fig. 2). Collagen fibers arranging in an indiscriminate pattern, some of them swelling and/or breakage.

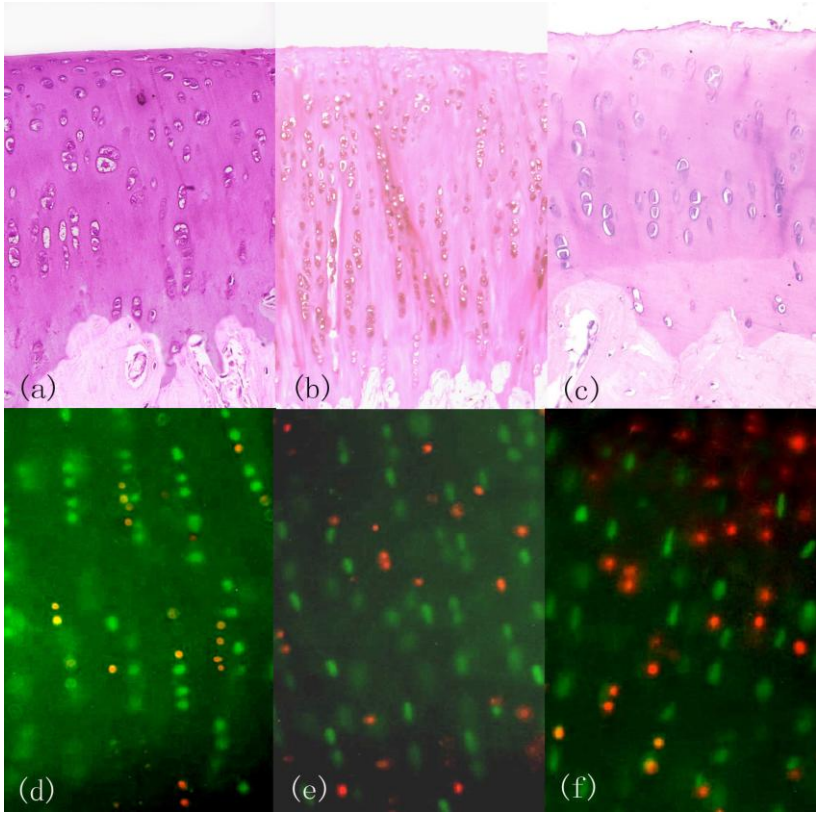

Fig. 1
Microexaminations of cartilage matrix and chondrocyte
Fig. 1a: Cartilage matrix appears prunosus homogeneously, with smooth surface and regular chondrocytes arrangement. (CTRL, Safranine-O staining, ×400)
Fig. 1b: Chondrocytes arrangement is irregular and much chondrocytes in the dividing phase. (RG at 8 week, Safranine-O staining, ×400)
Fig. 1c: Articular cartilage with rough and uneven surface as well as loss of surface structures. Cartilage matrix dyeing inhomogeneous and light. (JG at 8 week, Safranine-O staining, ×400)
Fig. 1d: Viability of chondrocytes in articular cartilage, dead cell in red and living cell in green. (CTRL, FDA/PI staining, ×400)
Fig. 1e: Viability of chondrocytes in articular cartilage, dead cell in red and living cell in green. (JG at 4 week, FDA/PI staining, ×400)
Fig. 1f: Viability of chondrocytes in articular cartilage, dead cell in red and living cell in green. (RG at 4 week, FDA/PI staining, ×400)

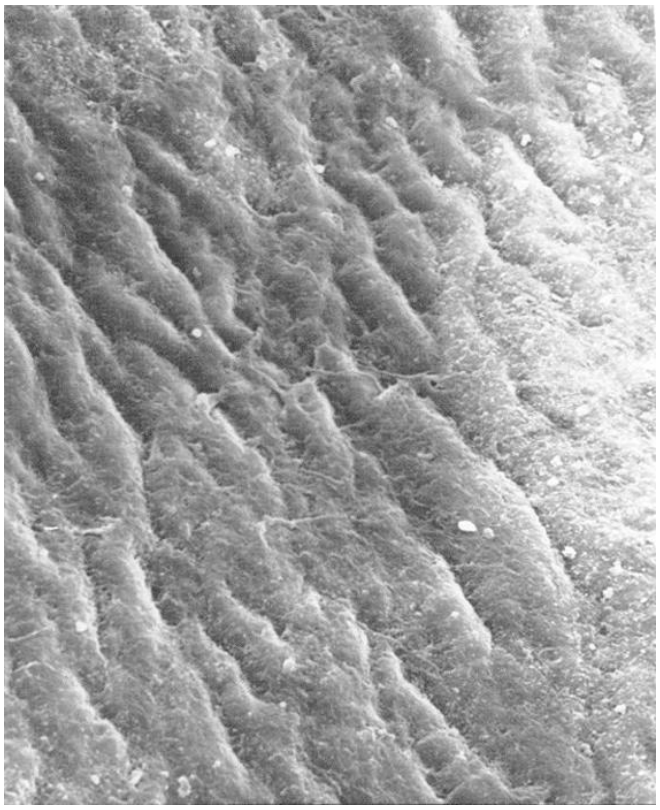

Fig. 2
Observation of cartilage surface with scanning electron microscope
Articular cartilage with rough and uneven surface. (JG at 8 week, Scanning electron microscope, ×1200

In RG, at 4-week, cartilage matrix stained with Safranine-O appeared mild prunosus, chondrocyte clusters and pyknosis can be seen. Sporadic or massive Cellular proliferation appears in layers of cartilage, much chondrocytes are in a dividing phase. Using transmission electron microscope, we find some degenerated chondrocytes with nucleus deformation, endoplasmic reticulum distension, heterochromatin increase and margination, cellular membrane microvillus decrease or disappearance (Fig. 3). At 8-week, Chondrocytes arrangement is much irregular, and normal columnar arrangement of chondrocytes in the radial zone disappears. Using transmission electron microscope, we find much more degenerated and immature chondrocytes than that at 4-week.

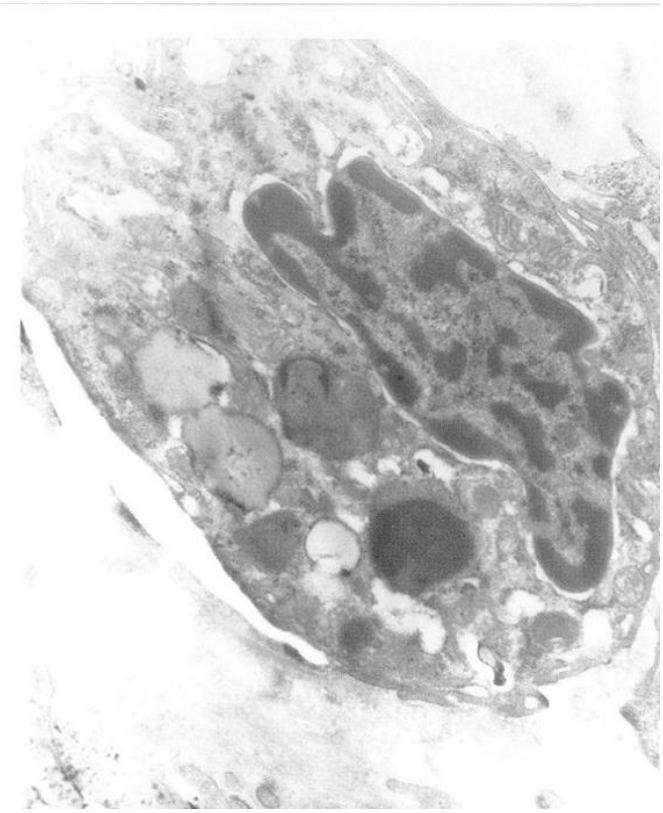

Fig. 3
Observation of degenerated chondrocyte with transmission electron microscope
Degenerated chondrocyte with deformed nucleus, dilated endoplasmic reticulum, heterochromatin lumping, chromatin margination, cellular membrane microvillus decrease or disappearance.
(RG at 8 week, Transmission electron microscope, ×6000)

GAG content (Fig. 4): In RG, GAG content had a significant drop (RG_{8W} vs. CTRL, 66.55±8.37 vs. 86.13±5.46, $p<0.01$) at 8-week; in JG, GAG content had a significant drop (JG_{4W} vs. CTRL, 64.88±5.97 vs. 86.13±5.46, $p<0.01$) at 4-week, and had a more significant drop (JG_{8W} vs. CTRL, 51.01±5.34 vs. 86.13±5.46, $p<0.01$) at 8-week. Furthermore, GAG contents in JG were significantly lower than that in RG at 4-week and 8-week respectively (JG_{4W} vs. RG_{4W}, 64.88±5.97 vs. 77.03±9.56, $p<0.01$; JG_{8W} vs. RG_{8W}, 51.01±5.34 vs. 66.55±8.37, $p<0.01$).

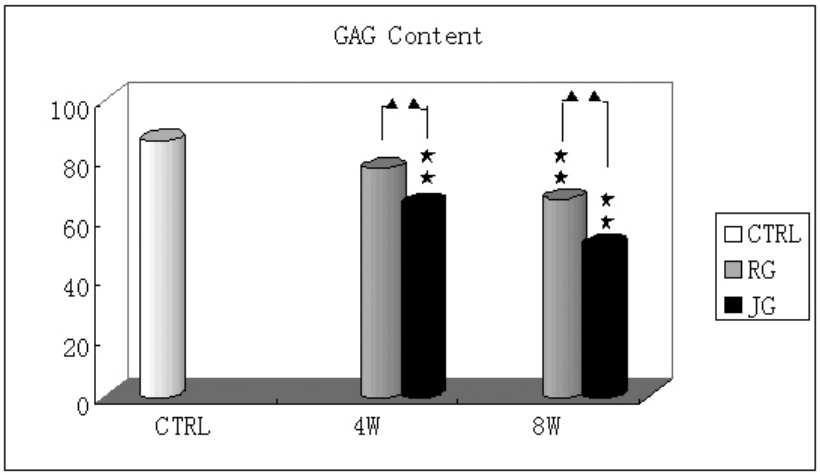

Fig. 4
GAG content (μg GAG/mg cartilage wet weight)
★ p<0.05 vs.CTRL; ★★▲ p<0.01 vs.CTRL; p<0.05 RG vs. JG; p<0.01 RG vs. JG

Thickness of cartilage (Fig. 5): In JG, Thickness of cartilage was reduced significantly (JG_{8W} vs. CTRL; 239.63±85.93 vs. 334.09±37.84; p<0.01) at 8-week. There was not significant difference between RG and JG in the thickness of cartilage.

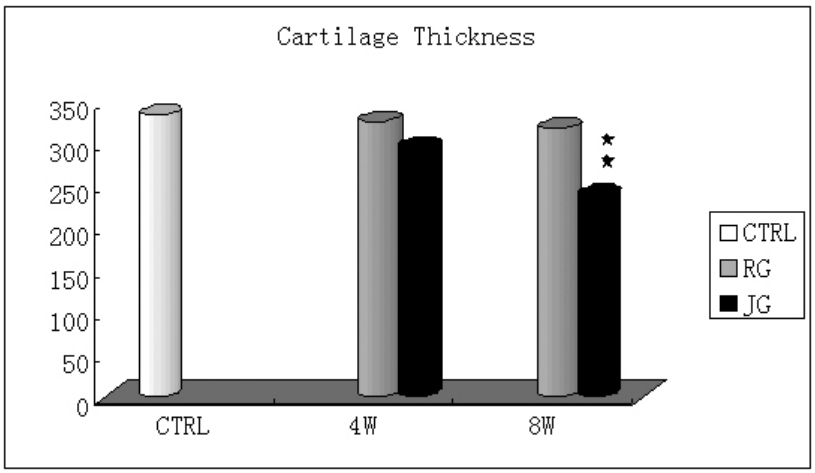

Fig. 5
Thickness of cartilage (μm)
★ p<0.05 vs.CTRL; ★★▲ p<0.01 vs.CTRL; p<0.05 RG vs. JG; p<0.01 RG vs. JG

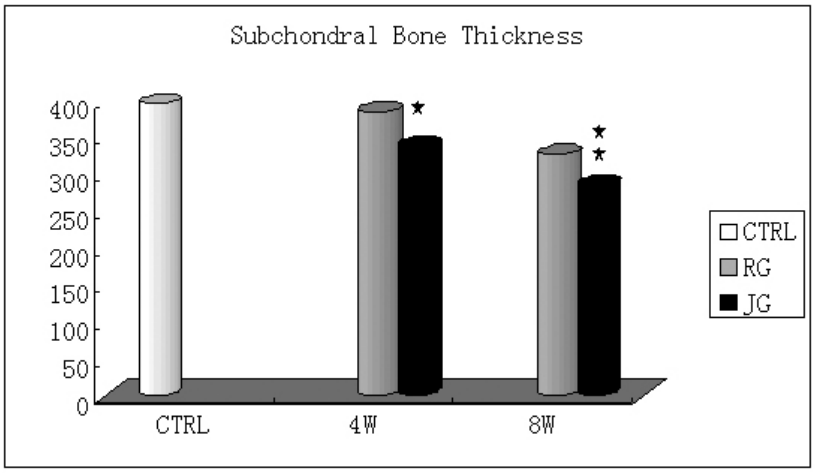

Fig. 6
Thickness of subchondral bone (μm)
★ p<0.05 vs.CTRL; ★★ p<0.01 vs.CTRL
▲ p<0.05 RG vs. JG; ▲▲ p<0.01 RG vs. JG

Thickness of subchondral bone (Fig. 6): In JG, thickness of subchondral bone had a significant drop (JG_{4W} vs. CTRL, 336.41±69.88 vs. 395.26±69.24, $p<0.05$) at 4-week, and had a more significant drop (JG_{8W} vs. CTRL, 283.92±68.59 vs. 395.26±69.24, $p<0.01$) at 8-week. There was not significant difference between RG and JG in the thickness of subchondral bone

Dead cell ratio (Fig. 1d-1f, Fig. 7): In RG, dead cell ratio had a significant elevation (RG_{4W} vs. CTRL, 4.13±1.81 vs. 1.72±0.99, $p<0.01$) at 4-week, and had a more significant elevation (RG_{8W} vs. CTRL, 7.22±1.58 vs. 1.72±0.99, $p<0.01$) at 8-week; in JG, dead cell ratio had a significant elevation (JG_{8W} vs. CTRL, 8.15 ±2.79 vs. 1.72±0.99, $p<0.01$) at 8-week. Furthermore, dead cell ratio in RG were significantly higher than that in JG at 4-week (RG_{4W} vs. JG_{4W}, 4.13±1.81 vs. 2.23 ±1.45, $p<0.01$).

Mankin grades (Fig. 8): In RG, Mankin score had a significant elevation (RG_{4W} vs. CTRL, 2.98±1.31 vs. 1.25±0.89, $p<0.05$) at 4-week, and had a more significant elevation (RG_{8W} vs. CTRL, 5.12±2.17 vs. 1.25±0.89, $p<0.01$) at 8-week; in JG, Mankin score had a significant elevation (JG_{4W} vs. CTRL, 3.63± 1.09 vs. 1.25±0.89, $p<0.01$) at 4-week, and had a more significant elevation (JG_{8W} vs. CTRL, 5.88±1.41 vs. 1.25±0.89, $p<0.01$) at 8-week. There was not significant difference between RG and JG in the Mankin score.

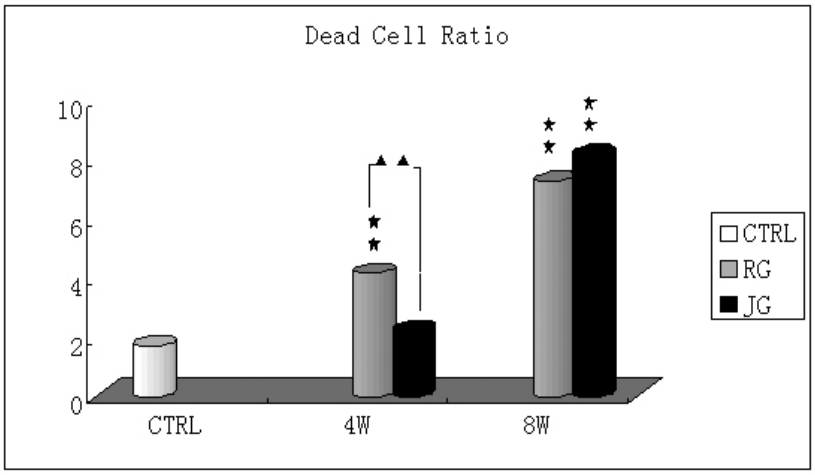

Fig. 7
Dead cell ratio (%)
★ p<0.05 vs.CTRL; ★★ p<0.01 vs.CTRL
▲p<0.05 RG vs. JG; ▲▲p<0.01 RG vs. JG

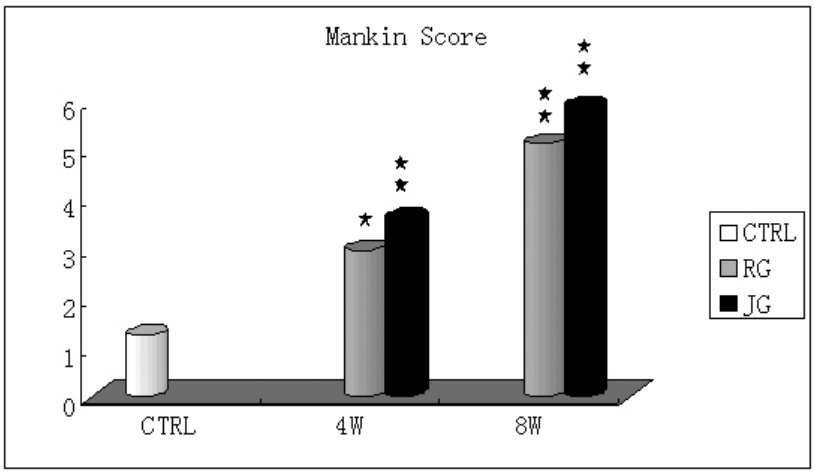

Fig. 8
Manikin Score
★ p<0.05 vs.CTRL; ★★ p<0.01 vs.CTRL
▲p<0.05 RG vs. JG; ▲▲p<0.01 RG vs. JG

Discussions

Excessive and repetitive use of joint, may cause partial load and attrition increasing on articular cartilage surface, stimulates the inflammatory reaction, or even cartilage tissue degeneration [1]. To study what kind of changes will be appeared on articular cartilage under different modes of training, has a very significant sense in adjusting training modes, rationalizing exercise intensity, and decreasing the incident of sport injuries. In this research we trained rabbits in high-intensity jumping and running mode respectively, and observed the pathological changes of articular cartilage in knee joint at 4-week and 8-week. Moreover, we analyzed in high-intensity jumping and running mode, the "starting mechanism" and pathological process of knee cartilage injury.

GAG is the essential component of articular cartilage, its content is very essential in maintaining articular cartilage function [6]. GAG physiological functions are mainly in: adjustment of Cellular proliferation and differentiation, conjunction between cells and matrix, modulation of macro-molecules diffusion in the intercellular space, mediation in cell-cell interaction, adjustment of cytokines and inflammatory products in partial inflammation reaction and adherency of cytokines. In this research, we found GAG loss in the knee joint cartilage after 4 weeks' jumping training and further aggravated after 8 weeks' training. In high-intensity movement training process, many kinds of mechanisms are involved in the destruction of GAG. Mechanical agent is the first. The excessively stress as well as revolving and shearing force, induce the gradual destruction of cartilage collagen, the loss of network structure which supports the normal cartilage shape, and the change of chondrocyte phenotype [15]. All of these cause the abnormality of GAG metabolism and loss of GAG in the later stage. Second, many enzymes [11,14] are involved in the degeneration process of GAG. In addition, the destruction of matrix structure may also cause immune response, which can also intensify GAG destruction [19]. Some works showed that, cartilage matrix may transmit the joint surface mechanical power signal to chondrocyte, and the distortion of cartilage matrix may also produce the electric, chemical signal which can stimulate chondrocyte [18]. Chondrocyte responses to the signal through autocrine or paracrine of cell factors, and further influence the metabolism of matrix ingredients. These appear to be reciprocal effect between chondrocyte and matrix.

When jumping, pressure load on articular cartilage surface reaches the peak in the extremely short time, and interstitial fluid pressure rises rapidly. While collagen structure bears a rapidly increasing expansile tension. When partial tension

surpasses the limitation of collagen bearing capacity, destruction of collagen structure appears, which further leads to the loss of matrix ingredients (e.g. GAG). In this research, we found cartilage matrix dyeing unevenness and inadequacy, tidal line derangement in JG 4 weeks after training, which indicated that cartilage matrix appeared damage in the early period under stress load with high rate of loading and high intensity, and articular cartilage damage displayed as "cartilage matrix starting mechanism".

When running, the change of the loading on the surface of articular cartilage is relatively slow, and the loading proportion on cartilage matrix and matrix deformation degree are great. Cartilage structure deformation mainly depends on matrix liquid flowing out. Collagen framework deforming slowly, related liquid flowing thoroughly, stress distributing in a balance mode, all of these prevent partial hypertension from happening. For this reason, damage of cartilage matrix structure is relatively slight in exercise of excess running. However, when running, much liquid drains out from cartilage matrix, collagen framework deforms lastingly, and spatial structure of cartilage matrix changes greatly, which lead to great deforming force effecting on the chondrocyte embedded in the matrix. Therefore, author considers that damage of chondrocyte is the main aspect in the cartilage injury induced by excess running. This view had been proved in our study: dead cell ratio had a significant elevation after 4 weeks of high-intensity running training, and much degenerated chondrocytes with deformed nucleus, expanded endoplasmic reticulum, and increased heterochromatin were found in observation with transmission electron microscope. The cartilage damage displayed as "chondrocyte starting mechanism".

In this research, we also found at later time (8-week), GAG content had an obvious drop in RG, and dead cell ratio had an obvious elevation in JG, which gave us a suggestion that damage of cartilage matrix and chondrocyte have reciprocal effect to each other. In one side, low-activity chondrocyte lost the ability to secrete sufficient cartilage components; in another side, chondrocyte who lost the protection of matrix can be injuried easily.

Subchondral bone plays an extremely vital role in the load conduction system of body. On one hand it anchors cartilage and underneath bone together, on the other hand it conducts the stress to cortical bone via bundle fibers, which avoids over stress concentration in deep layer of cartilage. Thickening of the subchondral bone used to be known as a lesion that was characteristic of chronic OA in humans, and appeared to be a relatively early lesion in naturally occurring OA in cynomolgus monkeys [4]. But our study showed a reversed result, in JG at 4-week, mean of subchondral bone thickness was obviously lower than untreated control; in JG at

8-week, mean of subchondral bone thickness descended to an even lower level. The possible reasons could be in some aspects blow: first, deformation and destruction of matrix collagen structure directly had a negative influence on the structure as well as the function of weight bearing and loading conducting of subchondral bone; second, the experimental animals in this study were obviously different from that in the former studies in structure and function of articular cartilage and subchondral bone; third, the change of subchondral bone thickness should be observed in quite a long period, yet observation duration in this study was somewhat short. Thicknesses of articular cartilage in JG significantly reduced after 8 weeks, which agrees well with the microstructural studies done in this research. In observation with light microscope and electron microscope, we found mild articular cartilage fibrosis and surface structure loss in JG in the later training period, which offered the evidences that high-intensity jumping training could lead to articular cartilage destruction and thickness descent.

Mankin grades is a common used scoring standard for the evaluation of cartilage injury degree, which grades the examples for structure, matrix dyeing, tidemark integrity, and cellularity. This study showed Mankin grades in RG and JG at 4-week were significantly higher than CTRL, and had an even more significant elevation at 8-week, which indicated that high-intensity jumping and running exercises could induce cartilage injury in the knee joint directly, and along with the lasting of training, articular cartilage injury also had a trend to aggravate gradually, finally led to an irreversible result.

By histological observations and measurement of GAG content, thickness of cartilage and subchondral bone, dead cell ratio and Mankin grades, this work provides sufficient evidence that high-intensity movement training, running and jumping, can reliably induces cartilage damage in rabbit knee joint. But the pathogenesis and the main phenotype of cartilage damage induced by these two exercises are distinguishing. In summary, high stress mechanical loads induced by jumping exercise can do more harm to cartilage matrix than to chondrocyte when affecting on knee cartilage, the pathogenesis displayed as “cartilage matrix starting mechanism”; on the contrary, loads induced by running exercise can do more harm to chondrocyte than to cartilage matrix, the pathogenesis displayed as “chondrocyte starting mechanism”. In author’s opinion, the difference in pathogenesis and cartilage damage phenotype by different exercises may have a close correlation with the loading rate of stress on articular cartilage, and in order to draw a more persuasive conclusion, further researches should be taken.

Conclusions

Repetitive and high-intensity jumping exercise can do more harm to cartilage matrix than to chondrocyte in knee joint, the pathogenesis displays as "cartilage matrix starting mechanism"; on the contrary, running exercise can do more harm to chondrocyte than to cartilage matrix, the pathogenesis displays as "chondrocyte starting mechanism". In addition, the difference in pathogenesis and cartilage damage phenotype by different exercises may have a close correlation with the different loading rate of stress on knee cartilage surface.

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