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ORIGINAL PAPER

Section of the anterior cruciate ligament in the rabbit as animal model for osteoarthritis progression

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Abstract
Purpose The purpose of this study was to determine whether instability of knee is a risk factor in the progression of osteoarthritis (OA).
Methods Twenty-four mature New Zealand White rabbits were randomly divided into four groups. The control group received 0.3 ml saline in the first, fourth and seventh days in the right knee, while the other three groups received the same dosage 4 % papain and its activator 0.03 M L-cystein. The P3w group knees were harvested at three weeks after the last papain injection, the P6w group knees received a sham surgery at three weeks and were harvested at six weeks after the last papain injection, while the P+ACLT group knees received ACL transection at three weeks and were harvested at six weeks after the last papain injection. Cartilage degradation of femoral condyles and tibial plateaus were evaluated by X-rays, macroscopy, light microscopic and transmission electron microscopy (TEM).
Results According to X-rays grade scale, macroscopic grade scale, light microscopic modified Mankin scale and TEM, in the P3w knees, cartilage degeneration of femoral condyles and tibial plateaus were significantly severe compared to those of the control group ($P<0.05$), but the differences were not apparent in comparison with the P6w knees ($P>0.05$). However, in P+ACLT knees, cartilage degeneration of femoral condyles and tibial plateaus appeared more severe in comparison with P6w knees, and the difference was significant ($P<0.05$).
Conclusions Instability of knee plays a significant role in increasing the severity of cartilage degradation in rabbit knees and should be considered as a risk factor in OA knee progression. Our data may suggest that reconstruction of knee stability may prevent or delay the progression of OA.

Keywords Instability · Osteoarthritis · Cartilage · ACL transection · Risk factor

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Introduction

The knee is a complicated weight-bearing joint which links the bones of the upper and lower leg. Under a normal physiology condition, the knee maintains dynamic stability at a functional position and allows for flexion/extension and some rotational movements [1]. The stable joint can carry required and normal intensity functional loads on the articular cartilage surface throughout its range of motion [2]. When the stability of the knee joint is broken, the unstable joint will lose the self-stabilization function and therefore be at a high risk of abrupt repositioning events which are associated with altered joint movement patterns and contact mechanics [3]. Therefore, the instability-associated changes of the joint may result in abnormal mechanical loading on the articular surface and subsequent lesions of the articular cartilage [1]. If the articular cartilage damage is not successfully and timely repaired, the affected joint will progress to osteoarthritis (OA).

OA is a major cause of disability in older adults [4]. Although the pathogenesis of OA has not been made clear, there are some known risk factors for OA onset, such as aging [5], post trauma [6], obesity [7], sex (women) [8] and genetics [9]. In addition, these risk

factors which promote the initiation of OA might also accelerate its progression. There has been some evidence that indicated that the risk factors for progressive joint damage were different from those of incident OA [10, 11]. Although an army of risk factors for the initiation of incident OA have been confirmed, far fewer could be found arousing OA progression [12]. It was widely considered that risk factors allocation between them may overlap but not be identical [13]. For instance, a mechanical study of risk factors of knee OA has shown that muscle weakness not only caused the disease occurrence but also worsened its progression [14, 15]. Obesity has long been considered as a risk factor for OA initiation as well as the disease progression. Particularly, adipokines might be a potential biomarker for OA progression and classify the disease severity [16]. However, Harvey et al. [17] explored the association of leg-length inequality with knee OA, whereby the result for progressive knee OA was less clear compared with incident knee OA. Furthermore, it was reported that increased bone mineral density (BMD) reduced the risk of knee OA progression although it was considered increasing the risk of incident knee OA [18, 19]. Reasons may be probed into that a knee OA sample has many other risk factors for the disease progression or a risk factor may fail to show effects on progression even when it increase the risk of incidence. A system synthesis also suggested few consistent risk factors for OA worsening [20]. Taken together, risk factors between OA onset and progression should be specifically and separately examined. Several researches have concluded that instability is an important risk factor for knee OA onset but whether it is a risk factor for OA knee development has not been demonstrated, though it is very common seeing OA patients with knee instability. Therefore, there is a great necessity to identify the role of instability in knee OA progression for better understanding of the disease pathogenesis and progression.

In this study, an animal model was designed to identify the effect of knee instability as a risk factor in OA progression in vivo, although the influence has been taken for granted, but as yet scientifically unproven.

Materials and methods

Animals

Twenty-four rabbits (males or females) weighing approximately 2.5–3 kg were purchased from the animal centre of Xi'an Jiaotong University. The experimental protocol was in accordance with the NIH guidelines of laboratory animals and approved by the Ethics Committee of the first affiliated hospital of Xi'an Jiaotong University. The rabbits were equally and randomly divided into four groups: the control group (control), the papain injection group harvesting three weeks after the last injection (P3w), the papain injection group harvesting six weeks after the last injection (P6w) which received sham surgery three weeks after the last injection, and the papain injection group harvesting six weeks after the last injection which received ACLT three weeks after the last injection (P+ACLT).

The right knee joint of rabbits in the control group received 0.3 ml saline in the first, fourth and seventh days. In the other groups, right rabbit knees received the same dosage 4 % papain and its activator 0.03 M L-cystein (Sigma-Aldrich) accordingly with the control group. Particularly, the ACL of each rabbit in the P+ACLT group was transected via general anaesthesia by pentobarbital (1 ml/kg).

Radiography

Conventional radiography is the available method for imaging joint structure in persons with OA. The presence and severity of disease typically is determined through use of the Kellgren and Lawrence (K&L) grading system [21], which greatly depends on the osteophyte status and the joint narrowing for classification of disease. Radiographs of the knees were obtained just before rabbits were sacrificed, graded by a radiologist blinded to groups. The score can be scaled from 0 to 4: Grade 0, normal, no features of OA; Grade 1, doubtful, minute osteophyte, doubtful significance; Grade 2, mild, definite osteophyte, normal joint space; Grade 3, moderate, moderate joint-space reduction; and Grade 4, severe, joint space greatly reduced, subchondral sclerosis.

Gross morphological assessment

After sacrificed, both femurs and tibias were cleaned and the gross appearance was recorded with a digital camera (Canon, Japan). The degree of degradation was graded on Pelletier JP score [22] as follows: 0–4, where 0=normal appearing surface; 1=minimal fibrillation or a slight yellowish discoloration of the surface; 2=erosion extending into superficial or middle layers; 3=erosion extending into the deep layers; and 4=erosion extending to the subchondral bone.

Histological evaluation

Tissue samples were fixed in 4 % paraformaldehyde for 48 hours. After decalcification in buffered EDTA (20 % EDTA, pH 7.3) for three weeks, the samples were dehydrated and embedded in paraffin, and 5-μm microsections in the sagittal plane were prepared. Sections were stained with haematoxylin and eosin and safranin O-fast green to evaluate histologic changes in the cartilage and bone tissue according

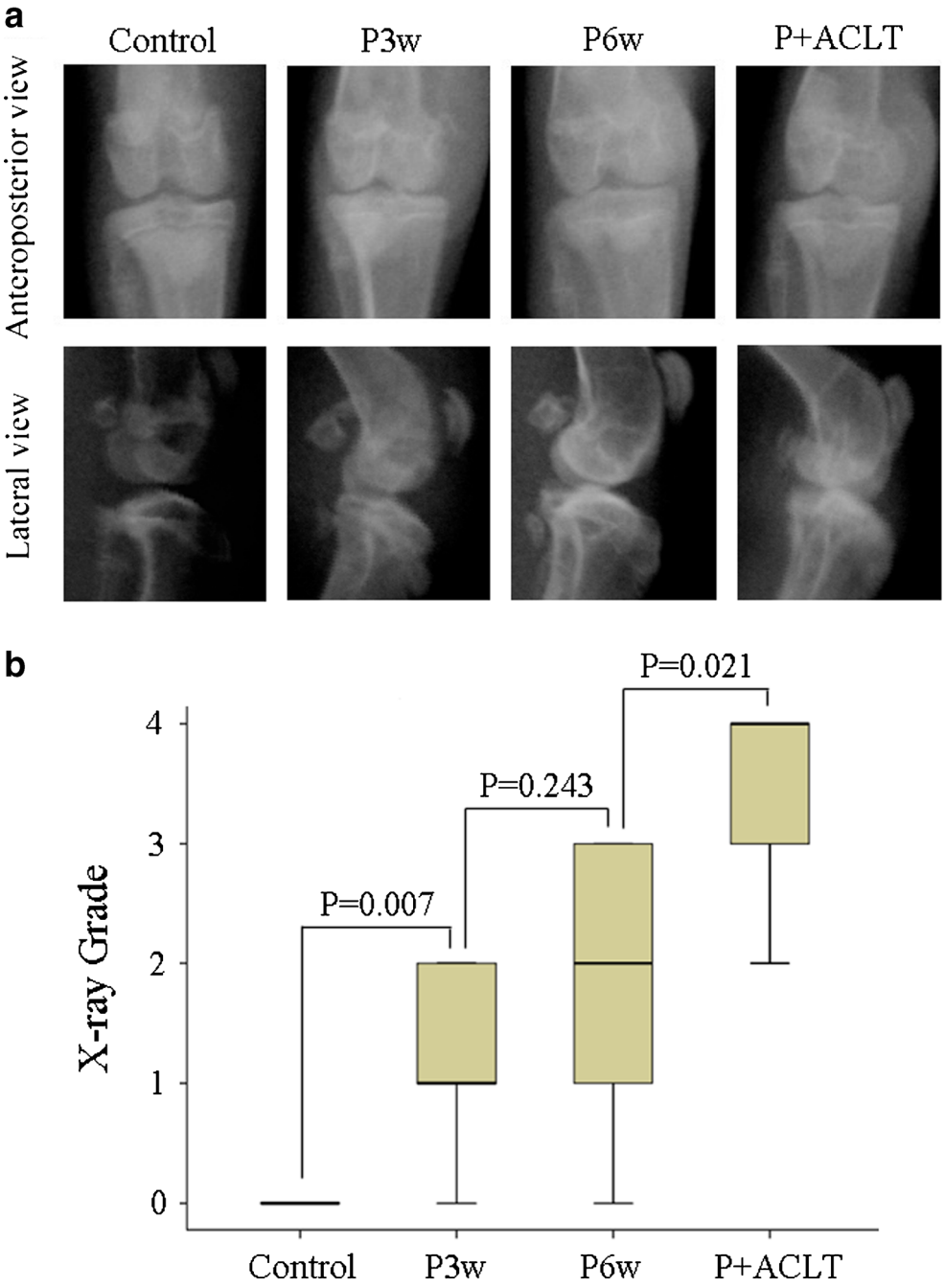


Fig. 1 **a** Representative X-ray images from each group. Control: saline; P3w: papain injection for three weeks; P6w: papain injection for six weeks; P+ACLT: papain injection for six weeks + ACLT for three weeks after the last injection. **b** X-rays according to K&L scale. Data were analysed with Kruskal-Wallis test. A box-whisker plot was shown to describe the data differences

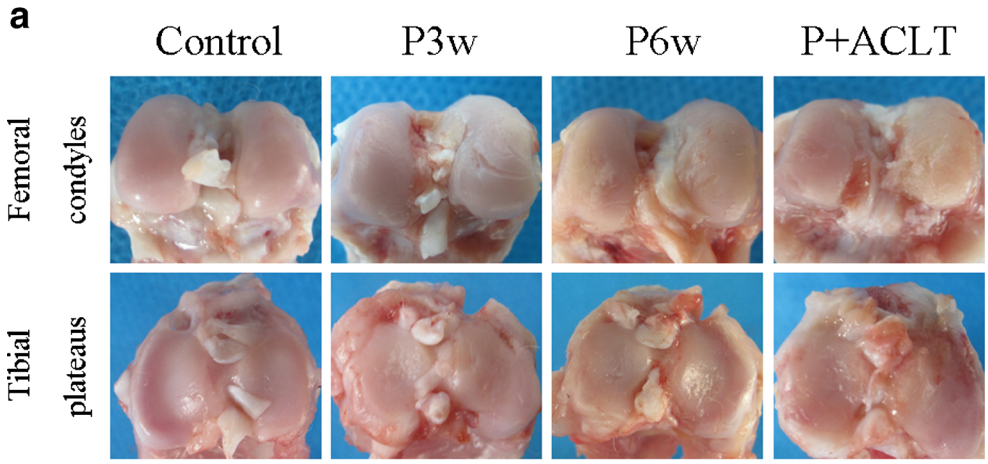
to the modified Mankin scale [23], ranging from 0–14 (best to worst). Sections were observed by two blinded pathologists, and the scores for the femoral condyles or tibial plateaus of each joint were averaged separately.

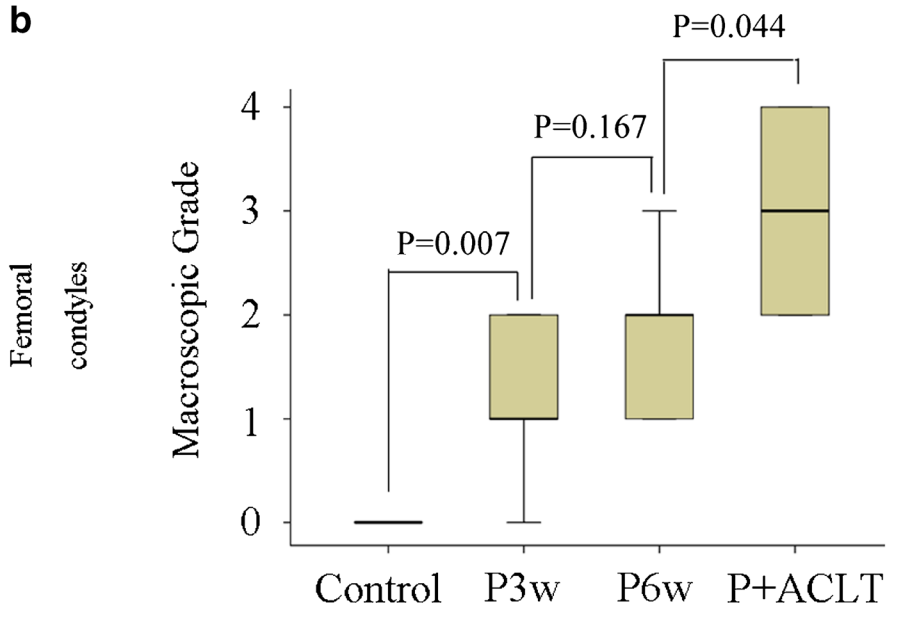
Ultrastructural changes

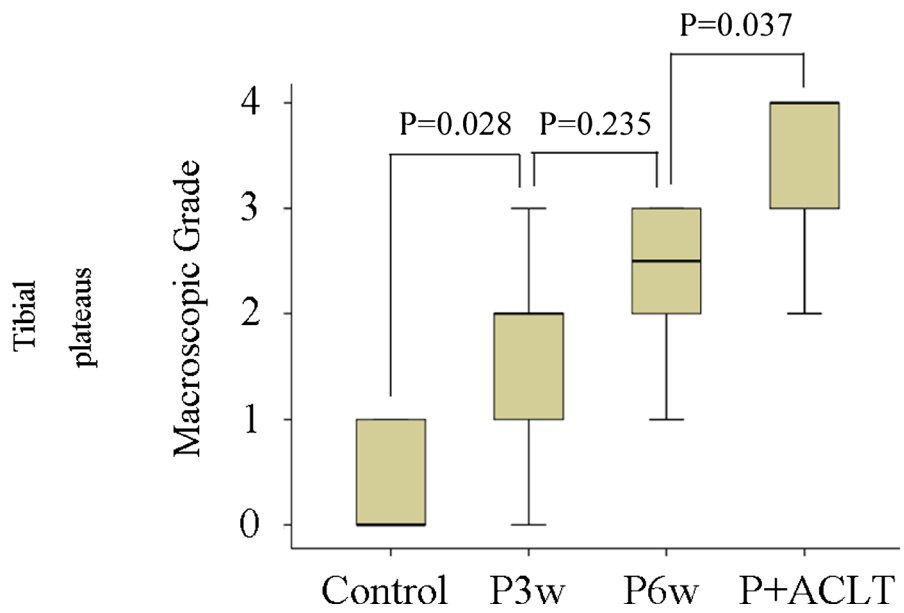
A 1 mm×1 mm×2 mm section of articular cartilage was fixed in 4 % glutaraldehyde for two hours. After being washed in PBS, the specimens were postfixed in 1 % (v/v) osmium tetroxide for one hour, rewashed, dehydrated in ethanol, and embedded in epon-araldite resin. Ultrathin sections were cut on a Leica ultramicrotome and stained with 2 % aqueous uranyl acetate, counterstained with 0.3 % lead citrate, and next, the specimens were observed with transmission electron microscopy (TEM) (H7650-Hitachi).

Statistical analysis

Data for each group are given as mean (averages±standard deviation [SD]) and differences were presented as box and whisker plots. Statistical analysis of the data was performed


a
Control
P3w
P6w
P+ACLT
Femoral condyles
Tibial plateaus


b
Femoral condyles
Macroscopic Grade
P=0.044
P=0.167
P=0.007
0
1
2
3
4
Control
P3w
P6w
P+ACLT


Tibial plateaus
Macroscopic Grade
P=0.037
P=0.028
P=0.235
0
1
2
3
4
Control
P3w
P6w
P+ACLT

◀ **Fig. 2** **a** Macroscopic appearance of articular cartilage from femoral condyles (*upper panels*) and tibial plateaus (*lower panels*). **b** Data were assessed according to Pelletier JP score. Data were analysed with Kruskal-Wallis test. Two box-whisker plots were shown to describe the data differences. Control: saline; P3w: papain injection for 3 weeks; P6w: papain injection for 6 weeks; P+ACLT: papain injection for six weeks + ACLT for three weeks after the last injection

with Kruskal-Wallis test and one-way ANOVA followed by the Bonferroni multiple comparison test. Differences were considered as statistically significant when $P<0.05$.

Result

Radiography

Representative X-ray images of each group are shown in (Fig. 1a), and data were compared among groups (Fig. 1b). Radiographs of the knee joints acquired showed subtle changes in the P3w and P6w groups compared to control knees ($P<0.05$), but the two groups did not present obvious differences ($P>0.05$). However, radiographs of the knee joints showed advanced OA changes in the P+ACLT group, which displayed severe joint space narrowing, structure destruction and osteophyte formation ($P<0.05$).

Gross morphological assessment

As shown in (Fig. 2a), control group knees maintained smooth articular surface and no ulceration formation both in femoral condyles and tibial plateaus, but in the P3w group, the cartilage displayed fibrosis changes, present a rough articular surface, many ulcerations, and osteophytes formation could be found in some parts of femoral condyles or tibial plateaus (Fig. 2b, $P<0.05$, respectively), particularly on the medial side. In P6w knees, the degeneration of cartilage showed similar changes compared to P3w knees (Fig. 2b, $P>0.05$, respectively). But in the P+ACLT group, compared with P6w knees, many parts of the cartilage surface were rough, deep ulcerations, osteophyte formation, and pieces of cartilage loss (Fig. 2b, $P<0.05$, respectively). In particular, cartilage loss was frequently found in the margin of femoral condyles or tibial plateaus.

Histological evaluation

Histological changes of the femoral condyles and tibial plateaus were examined by light microscopy (Fig. 3a). The cartilage of the control group was not degenerated. In the P3w group, compared to the control group, the cartilage surface was irregular. Chondrocyte loss in full thickness and reduced stain of safranin O-fast green were observed (Fig. 3b, $P<0.05$, respectively). Analogously, histological observation in the P6w group didn't present obvious deterioration compared to the P3w group (Fig. 3b, $P>0.05$, respectively). However, in the P+ACLT group, the upper layer of cartilage was almost lost, articular cartilage could not be collected in the partial region, and reduction of safranin O-fast green stain was significant (Fig. 3a, $P<0.05$, respectively). The result indicates that instability gives rise to more severe cartilage damage during OA development.

Ultrastructural changes

Ultrastructural changes in chondrocytes were observed by TEM. As shown in (Fig. 4), in control group, chondrocytes appeared to have an oval shape or a triangular shape with many microvilli protrusions. And organelles in the cells were rich and normal, such as a mature golgi apparatus, an abundant rough endoplasmic reticulum, some mitochondrias and glycogen particles scattered in the cytoplasm. What's more, the collagen network appeared regular, showing a tightly crowded and highly cross-linked network. In the P3w and P6w groups, similarly, the number of cytoplasmic organelles reduced. Nuclei became irregular and nuclear pyknosis appeared serrated and chromatin condensation irregular. There was some swelling mitochondria, lipid droplets, vacuoles and glycogen particle aggregation in the cytoplasm. The content of collagen fibres decreased, and the network was malaligned and loose. In the P+ACLT group, many chondrocytes appeared denatured and necrotic and the organelles were destroyed or had disappeared. There was significant swelling of mitochondria, large lipid droplets and a lot of vacuoles. The collagen fibre network was broken, and messy collagen texture was observed. In particular, collagen fibre content has greatly reduced.

Discussion

Several researches suggested that the incidence and progression of knee OA involved in different processes, and these differences may partly attribute to different risk factors [10, 12, 13]. Some clinical researches for OA progression suggested that many patients remained a long-time stability, while others developed rapidly [24–27]. The finding prompted that individual OA prognosis is fugacious. Therefore, there is a great necessity to identify the risk factors for OA progression. However, the precise risk factors for OA progression remain seriously speculative and only some clinical studies discuss it. Risk factors for OA progression is hard to identify because of formidable methodological

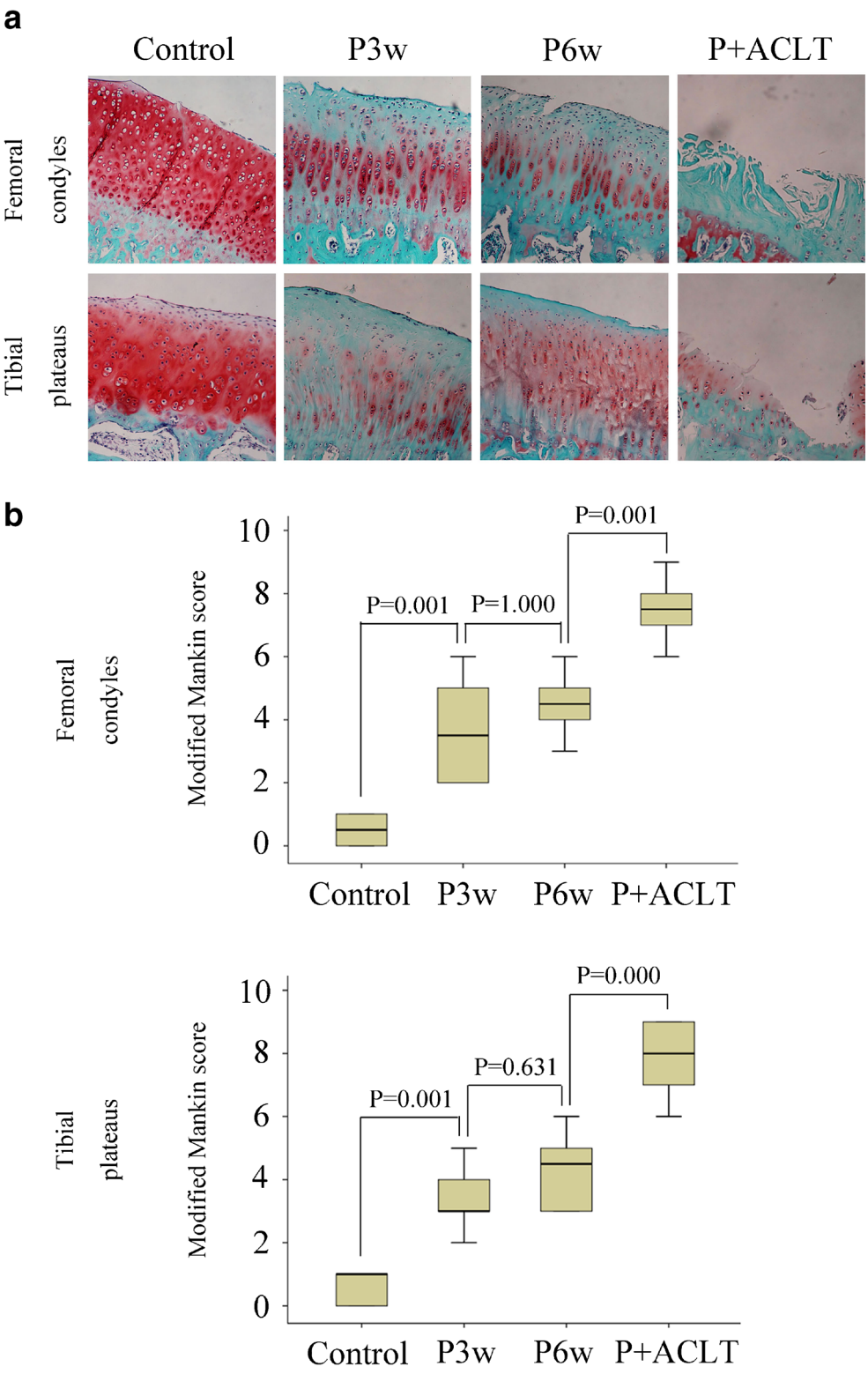


Fig. 3 **a** Safranin O-Fast green staining. **b** The modified Mankin score showed a significant increase in cartilage destruction for the P3w and P6w knees compared to the control knees. Cartilage destruction evidence for P+ACLT knees was more severe compared with P3w and P6w knees. Data were analysed with one-way ANOVA followed by the Bonferroni multiple comparison test. Two box-whisker plots were shown to describe the data differences. Magnification:×100. Control: saline; P3w: papain injection for three weeks; P6w: papain injection for six weeks; P+ACLT: papain injection for six weeks + ACLT for three weeks after the last injection

difficulties, such as index event bias and collider selection bias of the selected literatures [28, 29]. Instability induced by ACL transection has long been known as a risk factor for incident knee OA and was the most widely used mechanical instability-induced OA model of animal knee. However, as far as we know, the role of instability absence or not in OA progression has not been identified either in clinical research or in animal model.

Normally, ligaments are pivotal for the stability of the joint because they offer mechanical reinforcement and throughout control the range of motion [1]. In patients with a total knee replacement (TKA), retaining PCL can assist in maintaining the natural knee movements and increasing the 'normal' feeling [30]. A clinical study demonstrated that retaining the PCL in TKA performed better than posterior-stabilised TKA in terms of achieving an equalized rectangular gap which contributed to ensure proper kinematics of the knee joint [31]. The result indicated that, in patients with severe OA knees who received TKA, retaining the ligament of the joint may conduce to the dynamic stability of the joint and help to improve the patient satisfaction of sensation. Therefore, therapeutic methods avoiding instability of the knee joint may be a preferable choice for the treatment of patients with OA.

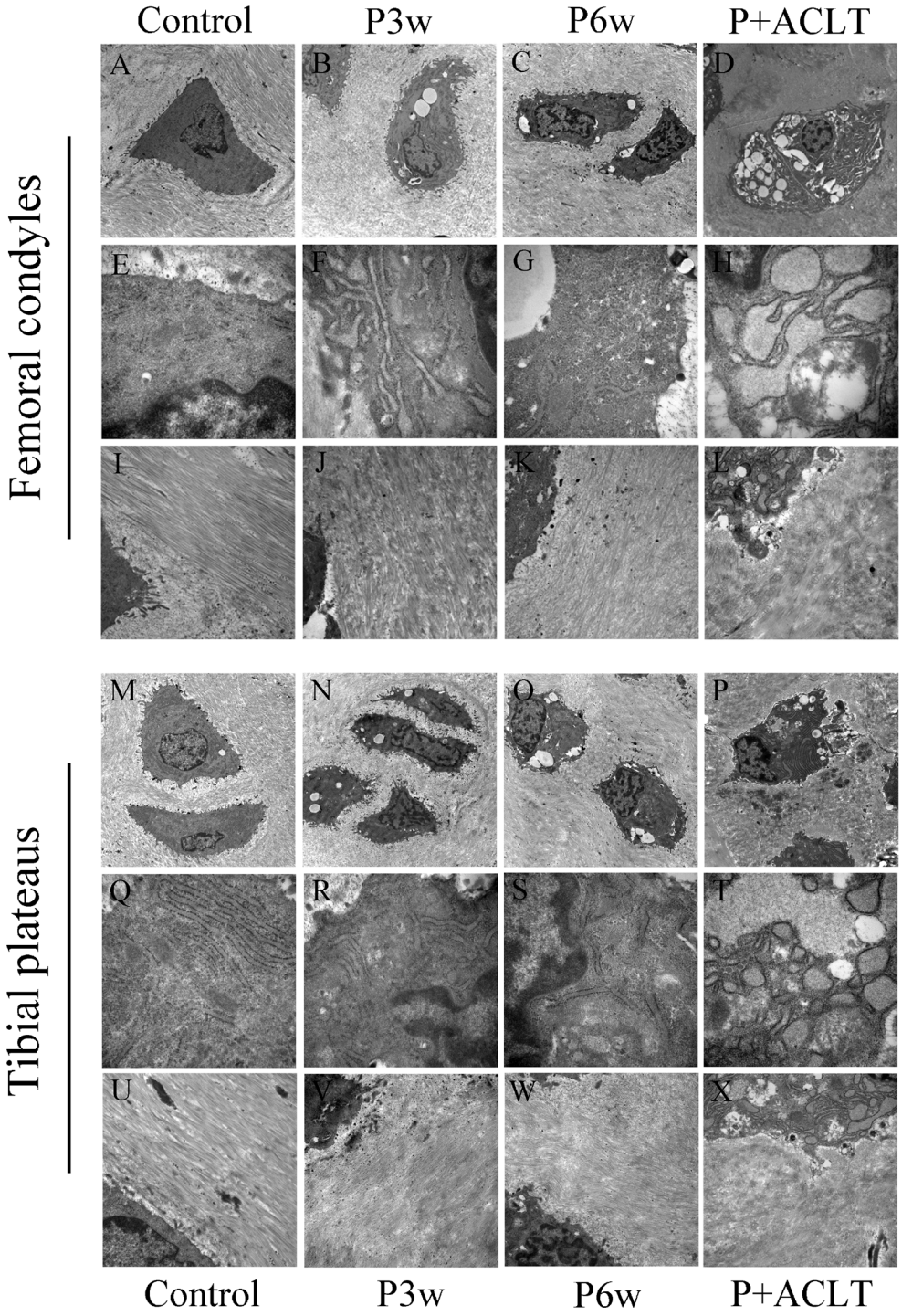
Taken together, investigation of the role of instability in OA knee progression may provide a clue in better understanding of pathogenesis and progression of OA, as well as its treatment and prevention.

In this study, we used papain as an initiate factor to set up OA model. Papain, which is one of the proteolytic enzymes, causes OA by releasing of chondroitin sulphate from the protein-polysaccharide complex of the matrix of articular cartilage [32]. It also effects on collagen integration while has no direct influences on chondrocytes [33]. The model, produced by chemical ferment, mimics the clinical condition in a normal rabbit joint for OA onset, avoiding any variables resulted by the time the knee joint becomes OA occurrence. Then, after the disease onset was evidenced by macroscopic and microscopic assessments, joint instability was induced by ACL transect. Indeed, both macroscopic and microscopic assessments in papain injected rabbit knees in this study were consistent with previous studies evaluating OA changes following papain injection [34, 35]. X-rays indicated that three weeks papain injection bring about joint space narrowing and structure destruction, and the changes had significantly difference compared with control group. Cartilage lesion of gross morphological assessment and histological evaluation was also obvious three weeks after the last papain injection. Furthermore, changes of chondrocytes and collagen also present OA characteristics in an ultrastructural perspective. All together, this evidence could demonstrate that OA has been onset. However, in P6w group knees, the severity of cartilage degeneration has not specific progression compared with the P3w group. In conclusion, the timing of radiography evidence and histological evaluation of OA changes were consistent with previous models which further supports the consistency and totality of this model.

The present results indicated evident differences between the injection of papain alone or with ACL transection. Actually, X-ray changes suggested that papain injection with ACL transection bring about severe joint space narrowing and structure destruction. In addition, based on the histological evaluation, joints with papain injection as well as instability induced by ACL transection presented a significant cartilage structural destruction. Particularly, collagen type II, a biomarker for cartilage degradation [36] which withstands mechanical tension strength for articular chondrocytes, was chaotic and the content of it reduced heavily in P+ACLT knees even though the P6w group have to a certain extent structure disorder and content loss. The changes of articular cartilage resulting from papain injection of a rabbit model have been interpreted as cartilage destruction induced by chemical fermentation while joint mechanical loading still remains normal [37]. Because cartilage showed more serious destruction when it received papain injection combined with instability, this difference should be explained because instability accelerated the development of OA for its non-physical loading, acting as a risk factor for the disease progression.

Here, we specifically pointed out this study demonstrated first that instability resulted in apparent damaging effects in the progression of OA, destroying cartilage structure, bringing about cartilage loss and predominately disintegrating collagen type II. Indeed, instability induced by ACL transection resulting in abnormal mechanical loads for articular cartilage could give rise to disruption of the collagen-proteoglycan solid matrix. Therefore, the non-physiological loads should be responsible for the collagen content reduction and collagen cross-linking destruction. Furthermore, the abnormal mechanical loads may also be a key factor in chondrocyte disruption and dismission in the OA knees progression. Additionally, this experimental design first discusses the risk factor for OA progression in an animal model. Both macroscopic and microscopic evidence could be acquired from animal knees, including conventional images, histological pictures and ultrastructural features, remedying the insufficient clinical research [17–19] restricted to imageological examination.

This study reported herein also had limitations. First, the mechanism of collagen disintegration was unclear. Because collagen is secreted primarily by chondrocytes, evidence should be taken to identify the reasons for collagen decreases either by wear and tear resulting from instability, or by instability induced chondrocytes reduction, or by both of them. Second, there was only one-time point evaluation for the damage of articular cartilage after the foundation of instability and no dynamic test performed to measure the range of instability.



It would be important to provide long-term changes of the cartilage and dynamic stress test for the measurement of the range of instability in order to provide more abundant and comprehensive materials about OA knee progression. Last, instability induced in this animal model was restricted to ACL transection. Although such instability models induced by ACL transection are known and reliant, and even commonly used, it likely does not occur on behalf of the entirety. Various injuries could lead to disparate instability of knee joint. Therefore, general instability, and its effect on joint health, should be studied systematically in the future.

◀ **Fig. 4** TEM images of ultrastructural characteristics of femoral condyles and tibial plateaus. The control femoral condyles and tibial plateaus presented normally. The cell membrane was intact, intracellular organelles were healthy and had a homogeneous distribution. Abundant rough endoplasmic reticulums in cytoplasm could be found. Lysosomes distribution were scattered. Mitochondria and golgi complex also could be found (*A*, *E*, *M* and *Q*). Stromal collagen fiber packed closely, showing a reticulate structure (*I* and *U*). The chondrocytes and collagen fiber of the P3w and P6w groups appeared to have pathology changes. The cell shapes became irregular. There were flocculent bulges on the cell surface. Scattered lipid vacuoles could be found. The nucleus biased to one side, showing a solidification contraction shape. Expansive organelles in cytoplasm were seen, such as dilation of endoplasmic reticulum and swelling of mitochondria (*B*, *C*, *F*, *G*, *N*, *O*, *R* and *S*). The regular collagen fiber network was broken, fractured and loose. The content of collagen decreased (*J*, *K*, *V* and *W*). But in the P+ACLT group, the integrity of cell structure was destroyed, and there was nuclear pyknosis or necrosis and many lipid vacuoles. There were highly swollen mitochondria and severely dilatate endoplasmic reticulum (*D*, *H*, *P* and *T*). The content of collagen fiber decreased severely, and the network was chaotic, and nearly couldn't be distinguished (*L* and *X*). Magnification: *A*, *B*, *C*, *D*, *M*, *N*, *O* and *P*×8000; *E*, *F*, *G*, *H*, *Q*, *R*, *S* and *T*×50000; *I*, *J*, *K*, *L*, *U*, *V*, *W* and *X*×20000. Control: saline; P3w: papain injection for three weeks; P6w: papain injection for six weeks; P+ACLT: papain injection for six weeks + ACLT for three weeks after the last injection

Conclusion

In summary, this study, in which the effect of instability induced by ACL transection, an abnormal mechanical load, on articular cartilage was investigated using papain injection rabbit, suggests that instability plays a crucial role in OA progression in the animal model. This evidence indicates that instability is a risk factor for OA progression, giving a new horizon in the epidemiology research of the disease. Even more important, this study demonstrates instability accelerating OA knee progression. Clinically, therefore, avoiding instability or restoring stability of OA knees may decelerate the disease progression.

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Competing interests The authors declare that they have no competing interests.

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