Moderate Intensity Treadmill Running Relieves Motion-Induced Post-Traumatic Osteoarthritis Mice by up-Regulating the Expression of lncRNA H19

Xuchang Zhou

Shanghai University of Sport

Hong Cao

Shanghai University of Sport

Miao Wang

Shanghai University of Sport

Jun Zou

Shanghai University of Sport

wei wu (✉ wuwei1@sus.edu.cn)

Shanghai University of Sport <https://orcid.org/0000-0003-0401-7363>

Research

Keywords: post-traumatic osteoarthritis, lncRNA H19, subchondral bone, cartilage, treadmill running

Posted Date: July 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-600744/v1>

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Version of Record: A version of this preprint was published at BioMedical Engineering OnLine on November 18th, 2021. See the published version at <https://doi.org/10.1186/s12938-021-00949-6>.

Abstract

Background The purpose of this study was to explore whether moderate-intensity exercise can alleviate motion-induced post-traumatic osteoarthritis (PTOA) and the expression change of lncRNA H19 during this progression.

Methods Twenty-week-old male C57BL/6 mice were randomly divided into five groups: model control group (CM group, n = 6), treadmill model group (M group, n = 6), rehabilitation control group (CK group, n = 6), treadmill model + rehabilitation training group (K group, n = 6) and treadmill model + rest group (J group, n = 6). Paraffin sections were used to observe the pathological changes in the mouse knee joint in each group. A micro-CT was used to scan the knee joint to obtain the morphological indexes of tibial plateau bone. Real-time PCR was used to detect the mRNA levels of inflammatory factors, synthetic and catabolic factors in cartilage.

Results After high-intensity exercise for four weeks, the inflammation and catabolism of the mouse knee cartilage were enhanced, and the anabolism was weakened. Further study showed that these results were partially reversed after four-week moderate-intensity training. The results of hematoxylin-eosin staining confirmed this finding. Meanwhile, high-intensity exercise reduced the expression of lncRNA H19 in cartilage, while the expression of lncRNA H19 increased after four weeks of moderate-intensity exercise.

Conclusion High-intensity treadmill running can cause injury to the knee cartilage in C57BL/6 mice which leads to PTOA and a decrease of lncRNA H19 expression in cartilage. Moderate-intensity exercise can relieve PTOA and partially reverse lncRNA H19 expression.

Background

Studies have shown that the knee cartilage bears about three times the body's weight while performing daily activities, about 10 times the body's weight during running, and about 20 times the body's weight when participating in jumping.(1–4) Under normal circumstances, chondrocytes are subjected to mechanical loads of millions of cycles per year.(5) However, an abnormal mechanical load that can be caused by factors such as obesity, joint instability, and excessive load increase in the activities of daily life may cause cumulative micro-injuries or trauma to the joints, and induce the conversion of healthy cartilage to degenerative joint disease phenotype-osteoarthritis (OA).(5–7) Post-traumatic osteoarthritis (PTOA) is an important subtype of OA, which is more common in adolescents, and is usually closely related to acute sports injuries or excessive exercise load.(8) It is believed that mechanical stimulation can affect biosynthesis of chondrocytes and alter cartilage morphology.(9) Previous studies have shown that there is a biomechanical "window" that is required to maintain cartilage homeostasis. A "too high" or "too low" load can cause a cartilage lesion,(10) while the appropriate load intensity can inhibit osteoclast activity in the subchondral bone, slow down the degeneration of the articular cartilage and prevent the development of PTOA. Numerous animal experiments have verified that treadmill running can relieve and treat PTOA. This relief includes a decline in the Mankin's score and OARSI score of the articular cartilage,

reduced bone mass loss of the subchondral bone, inhibition of proinflammatory factor production such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α), and increased IL-10 secretion to reduce joint inflammation. Also, moderate intensity exercise can inhibit the expression of matrix metalloproteinases (MMPs), and therefore reduce the death of articular chondrocytes and matrix degradation.(11) Therefore, in animal experiments, moderate intensity treadmill running is often used in research on the therapeutic mechanism of PTOA,(12, 13) while high intensity treadmill running is often used in research on the molding of PTOA.(14, 15)

Long non-coding RNAs (lncRNAs) are RNAs that have a length of over 200 nt and have no protein-coding function.(16) LncRNAs are widely distributed in the human genome.(17) Previous studies have focused on protein-encoded RNA, rather than on non-coding RNA, which is thought to be the “junk RNA” or “transcriptional noise.” Unlike miRNAs, lncRNAs can be folded into complex secondary or higher spatial structures to better identify targets.(18) With the development of high-throughput sequencing technology in recent years, more studies have shown that lncRNAs can regulate the expression of protein-coding genes in epigenetics, transcription, and post-transcription, and therefore affect a series of biological processes.(19–21)

LncRNA H19, located in human chromosome 11p15.5, is one of the most famous imprinted genes and is only transcribed from a maternally inherited allele(22). LncRNA H19 is highly expressed during the period of embryonic development, however after birth, it's expression is decreased in tissues other than skeletal muscle. It only increases again in a few tumor tissues.(23–25) LncRNA H19 is highly conserved in the evolutionary process, and has a very low mutation rate in exons, which indicates that it has important biological functions.(26) Studies have found that the high expression of lncRNA H19 is closely related to the proliferation, differentiation, and metastasis of tumor cells,(27) and the expression of lncRNA H19 is significantly correlated with the severity of tumor invasion.(28) In addition, lncRNA H19 is also involved in the regulation of atherosclerosis,(29) myocardial injury,(30) skeletal muscle regeneration,(31) and osteogenic differentiation.(32) Recent studies have shown that 73 lncRNAs were up-regulated and 48 lncRNAs were down-regulated in OA cartilage when it was compared with normal cartilage by microarray analysis. Among them, 21 lncRNAs, including lncRNA H19, were up-regulated by more than two times the normal level.(33) The subsequent RT-PCR further confirmed that pathological changes in OA may be related to the abnormal expression of lncRNAs. At present, several studies have confirmed that lncRNAs play an important role in OA lesions.(34) Steck et al. (35) studied the relationship between lncRNA H19 and the development of OA lesions. In this study, the expression of lncRNA H19 decreased in a culture environment with enhanced chondrocyte catabolism. Conversely, the expression of lncRNA H19 increased in a culture environment with enhanced chondrocyte anabolism. LncRNA H19 may be closely related to chondrocyte metabolism. In a normal culture environment without the induction of inflammatory factors, the levels of expression of lncRNA H19 and COL2A1 were significantly correlated, while in a culture environment induced by inflammatory factors, the levels of expression of lncRNA H19 and COL2A1 were not significantly correlated, which indicates that lncRNA H19 and COL2A1 were not simply co-regulated in the inflammatory environment.(35) However, current studies on the relationship between lncRNA H19 and OA are only focused on the cellular level, and further studies need to identify the specific regulatory

mechanism of lncRNA H19 on OA. This study is based on the clinical experience of exercise therapy in the treatment of PTOA and the early stage experimental knowledge of our team. We induced a mouse PTOA model through high intensity treadmill running, and then performed rehabilitation training on the PTOA mice with moderate intensity treadmill running. We noted the changes in the expression of lncRNA H19 during the modeling and rehabilitation training in order to provide a reference for the related research on sports treatment of PTOA.

Results

Weight changes

The weights of the mice in the CM group fluctuated dynamically as the mice aged but without significant change. During the modeling period of high intensity treadmill running, the weights of the mice in the M group continually declined, and there was a statistical difference when the mice were 23 weeks old (fourth week of treadmill running). Also, at the end of the modeling period (mice were 24 weeks at this time), the mice in the M group weighed less than those in the CM group, but there was no statistical difference (Fig. 1A). The weight change in the CK group was almost the same as that in the CM group, and also showed dynamic fluctuation without any significant change. The weights in the K group continued to decrease during the period of high intensity and moderate intensity treadmill running, and there was a statistical difference at the age of 26 weeks. The weight in the J group showed a continuous trend of weight loss during the high intensity treadmill running. However, the weight in the J group showed a trend of regaining weight in the first week after the end of the modeling period (mice were 25 weeks at this time). At the end of rehabilitation training (mice were 29 weeks at this time), the weights of mice in the K group and the J group were lower than in the CK group, but there was no statistical difference (Fig. 1B).

Pathological changes

Pathological changes of the mice knee joints after high intensity treadmill running

The paraffin sections of the knee joints of the mice were stained with hematoxylin-eosin and safranin O-fast green staining, respectively. The stained sections were observed and photographed using a 200 Olympus inverted microscope. The hematoxylin-eosin staining results showed that when compared with the CM group, the articular surface of the knee joint in the M group was rough, with a few irregular cracks on the surface and a decreased number of chondrocytes. The Mankin's score indicated that there was a significant statistical difference in the pathological changes of the this cartilage. The fast green staining results showed that the knee joint in the CM group had a smooth surface, while the knee joint in the M group had obvious cracks, and the hyaline cartilage layer in the M group was significantly thinner than in the CM group, with significant cartilage loss. There was a significant difference in the OARSI score between the two groups (Fig. 2).

Pathological changes of mice knee joints after moderate intensity rehabilitation training

The results of the hematoxylin-eosin staining showed that the articular cartilage surface of the CK group was smooth without cracks, the chondrocytes were evenly distributed and neatly arranged, and the tidal line was complete and visible. In the J group, there were irregular defects on the articular cartilage surface of the knee joint, the cartilage was somehow lost, and the distribution of chondrocytes was uneven and there was a decreased number of chondrocytes. When compared with the J group, the cartilage surface of the knee joint in the K group was smoother, the cartilage layer was thicker, and the distribution of the chondrocytes was normal. The results of the Mankin's score showed that the pathological changes of the knee cartilage in the K group were less severe than in the J group, and the difference was significant. The degree of the pathological changes of the knee cartilage in the K group was more serious than the changes in the CK group, but there was no significant difference. The safranin O-fast green stain showed that there was no obvious crack in the cartilage in the K group. Staining also showed that some cartilage fibrosis and volume loss were found in the K group when compared with the CK group, which indicates that the pathological changes in the cartilage in the K group were more serious than in the CK group, and the OARSI score of the two groups was significantly different. When compared with the J group, the pathological changes of the knee cartilage in the K group were less severe, but there was no significant difference in the OARSI score (Fig. 3).

Morphological changes of the subchondral bone of the knee joints in mice

Morphological changes of the subchondral bone of knee joints in mice after high intensity treadmill running

After scanning the knee joint, the medial and lateral subchondral bones of the tibial plateau were reconstructed separately to obtain the morphological indexes of the subchondral bone of the tibial plateau. The results showed that the BMD, BV/TV ratio and Tb.Th of the LM group was significantly higher than those in the LCM group. The above data in MM group was also higher than in the MCM group, but there was no significant difference. (Fig. 4 and Table 1)

Table 1

Morphological changes of the subchondral bone of the medial and lateral tibial plateau in mice after high intensity treadmill running

Group	BMD(mg HA/ccm)	BV/TV	Tb.N	Tb.Th	Tb.Sp
LCM	922.77 ± 4.282	0.544 ± 0.187	7.297 ± 0.218	0.087 ± 0.003	0.130 ± 0.005
LM	958.425 ± 18.716*	0.583 ± 0.031*	7.032 ± 0.349	0.100 ± 0.005*	0.131 ± 0.007
MCM	940.886 ± 5.236	0.619 ± 0.0226	7.199 ± 0.218	0.109 ± 0.005	0.133 ± 0.004
MM	974.429 ± 22.776	0.668 ± 0.033	7.131 ± 0.253	0.127 ± 0.139	0.130 ± 0.011
Comparison of BMD, BV/TV, Tb.N, Tb.Th and Tb.Sp of the subchondral bone of the medial and lateral tibial plateau in mice. LCM group: CM group lateral tibia plateau, LM group: M group lateral tibia plateau, MCM group: CM group medial tibia plateau, MM group: M group medial tibia plateau, n = 3,* $P < 0.05$ LM group vs LCM group					

Morphological changes of the subchondral bone of knee joint in mice after moderate intensity treadmill running

In the lateral tibial plateau of subchondral bone, the BMD, BV/TV ratio, Tb.N and Tb.Th of the K group mice were higher than in the CK group, when compared with the CK group, and only the difference of the BMD were statistically significant. The BMD and Tb.Th values of the lateral tibial plateau of the mice in J group were significantly higher than those in the CK group; In the medial tibial plateau of subchondral bone, the BMD values of the J group were higher than the CK group, and showed a statistical difference. Meanwhile, when compared with the CK group, there was no significant difference in the increase of BMD, BV/TV ratio, Tb.N and Tb.Th and the decrease of Tb.Sp of the K group mice, compared with the CK group, there was no significant difference. (Fig. 5 and Table 2)

Table 2

Morphological changes of the subchondral bone of the medial and lateral tibial plateau in mice after moderate intensity treadmill running

Group	BMD(mg HA/ccm)	BV/TV	Tb.N	Tb.Th	Tb.Sp
LCK	950.925 ± 27.040	0.5712 ± .033	7.166 ± 0.441	0.094 ± 0.008	0.131 ± 0.004
LK	999.071 ± 15.379*	0.5734 ± 0.037	7.370 ± 0.236	0.101 ± 0.010	0.128 ± 0.010
LJ	987.680 ± 5.319*	0.629 ± .0117	6.951 ± 0.375	0.111 ± 0.005*	0.134 ± 0.011
MCK	969.248 ± 21.332	0.616 ± 0.056	7.094 ± 0.337	0.104 ± 0.017	0.139 ± 0.013
MK	1008.845 ± 4.041	0.647 ± 0.054	7.655 ± 0.318	0.119 ± 0.011	0.123 ± 0.011
MJ	968.176 ± 4.204 ^{##}	0.572 ± 0.016	7.214 ± 0.268	0.092 ± 0.001	0.127 ± .006
Comparison of the BMD, BV / TV, Tb.N, Tb.Th and Tb.Sp of the subchondral bone under the medial and lateral tibial plateau in mice. (LCK group: CK group lateral tibia plateau, LK group: K group lateral tibia plateau, LJ group: J group lateral tibia plateau, MCK group: CK group medial tibia plateau, MJ group: J group medial tibia plateau, MK group: K group medial tibia plateau. n = 4, * <i>P</i> < 0.05, LK group or LJ group VS LCK group, ^{##} <i>P</i> < 0.01, MJ group VS MCK group).					

mRNA expression changes in the knee cartilage in mice

Changes in mRNA expression of the knee cartilage in mice after high intensity treadmill running

After modeling with high intensity treadmill running, the total RNA of the right knee cartilage in the CM group and the M group was extracted, and the expression of mRNA levels of the anabolic factors COL2 and ACAN, catabolic factors mmp-13 and proinflammatory cytokines TNF-α and lncRNA H19 in the cartilage were detected using fluorescence quantitative PCR. As shown in Fig. 6, when compared with the CM group, the levels of expression of COL2 and ACAN mRNA in the knee cartilage of the M group decreased significantly, and the levels of expression of MMP-13 and TNF-α mRNA increased significantly after the four weeks of high intensity treadmill running. Meanwhile, the expression of lncRNA H19 in the cartilage of the M group decreased significantly. These results demonstrated that high intensity treadmill running caused the occurrence of PTOA in mice, and the expression of lncRNA H19 in the cartilage of the PTOA mice was decreased due to the high intensity treadmill running.

Changes in mRNA expression in the knee cartilage in mice after moderate intensity rehabilitation training

As shown in Fig. 7, When compared with the J group, the levels of expression of COL2 and ACAN mRNA in the knee cartilage of the K group increased significantly, and the levels of expression of TNF-α mRNA decreased significantly after four weeks of moderate intensity treadmill rehabilitation training, but there was no significant difference in the decrease of MMP-13 expression. In addition, lncRNA H19 in the K group was significantly higher than in the J group. When compared with the CK group, the expression of COL2, ACAN and lncRNA H19 in the cartilage of the K group were all reduced, and there was no significant difference in the decrease of COL2, while the expressions of MMP-13 and TNF-α were

significantly increased. These results indicated that after the moderate intensity rehabilitation training, PTOA was alleviated to a certain extent, and the expression of lncRNA H19 was increased.

Discussion

The mouse model is a commonly used animal model used to study OA(38) and generally includes treadmill running, drug injection, surgery, etc.(39–41) The mice PTOA model can be successfully induced by treadmill running without the manual operation of the joint cavity, which avoids the difficulty of an operation on the joint cavity and has the advantage of being non-invasive. Therefore, treadmill running is a good modeling method in PTOA manufacturing. Meanwhile, as one of the effective methods to treat OA, exercise therapy has been widely used in clinical practice. Therefore, we used moderate intensity treadmill exercise as a rehabilitation training method for PTOA mice. Previous studies have shown that exercise therapy can effectively lead to weight loss, thereby reducing joint load and obesity, which is a high risk factor of OA, and thus alleviate the process of cartilage damage and degeneration.(42) In the present study, we found that the weight of mice showed a continuous downward trend during the four weeks of treadmill running, but the average weight loss of mice in the exercise group was within 3 grams, indicating that the weight loss is due to the promotion effect of exercise on the health of mice. The slight weight loss has very little effect on the knee joint load, so the influence of weight factors on the progression of PTOA can almost be ruled out in this experiment. In addition, obese mice often exceed the weight of normal mice by more than 20%, thus knee joints will have a significant increase in load, which will aggravate knee cartilage damage. In this experiment, all the mice were fed conventional, and the weights of mice in the exercise group and non-exercise group were in the normal range, which provide further confirmation of the weight changes is very little on PTOA progression in this experiment. Therefore, the damage of articular cartilage in CM group mice may only be induced by high intensity treadmill running, and has nothing to do with changes in body weight.

Many studies have shown that early OA lesions generally manifest as an inflammatory response, and the decrease of cartilage anabolism and the increase of catabolism lead to the degradation of the cartilage matrix, which results in cartilage damage.(43–45) In this study, the expression of the inflammatory factor TNF- α in the knee cartilage of high intensity treadmill running mice was significantly higher than in the blank control group, while the expression of COL2 and ACAN, which represent cartilage anabolism, were significantly down-regulated, and the expression of MMP-13, which represents catabolism, was also significantly up-regulated. Also, the Mankin's score and OARSI score were obtained from the pathological staining of sections of the knee joints of mice. The surface of the knee joint in M group was found to be uneven, with a few cracks and loss of cartilage volume. These results indicate that high intensity treadmill running successfully induced the early stage of a PTOA mouse model. Our experiment also conducted rehabilitation training on the PTOA mice through moderate intensity treadmill running. The results showed that when compared with the PTOA mice in the J group, moderate intensity treadmill running partially reversed the abnormal inflammatory and metabolic indicators. Meanwhile, the Mankin's score of the knee cartilage in the moderate intensity treadmill group was also significantly lower than in the convalescent group, although the reduction of the OARSI score was not statistically significant. These

results indicate that moderate intensity treadmill running can partially delay the course of motion-induced PTOA in mice.

LncRNAs are ribonucleic acids that are widely distributed in the human genome and they have no protein-coding function. Changes in LncRNAs can cause abnormal expression of genes and lead to various diseases and biological disorders. After using small interfering RNA to knock down the expression of lncRNA H19 in chondrocytes, Zhou et al. found that the proliferation of chondrocytes was significantly inhibited and the apoptosis of chondrocytes increased, while the overexpression of lncRNA H19 could significantly increase the proliferation of chondrocytes.(46) Meanwhile, other studies have found that overexpression of lncRNA H19 can significantly up-regulate the expression of COL2A1 in chondrocytes, while knockout of lncRNA H19 can reduce the expression level of COL2A1 in chondrocytes.(47) These experiments demonstrated from the cellular level that lncRNA H19 may be involved in the regulation of chondrocyte anabolism and apoptosis. Our animal experiment results showed that in the articular cartilage of PTOA mice induced by high intensity exercise, there was a significant low expression of lncRNA H19, which indicates that lncRNA H19 may participate in the pathogenesis of motion-induced PTOA. Further studies have shown that four weeks of moderate intensity treadmill running effectively alleviated the progression of motion-induced PTOA mice. At the same time, the expression of lncRNA H19 in the articular cartilage of moderate intensity exercise training mice was significantly higher than that of PTOA mice that were raised without exercise intervention. The above results indicate that lncRNA H19 may be closely related to the occurrence and development of motion-induced PTOA mice. It should be noted that the current main modeling method for OA in mice includes surgical induction, collagen induction, and the induction of high intensity exercise used in this experiment. These different induction methods may involve different pathogenesis. For example, a collagenase injection uses chemical factors to induce chondrocyte apoptosis and matrix degradation, which subsequently leads to subchondral bone disease.(48) Surgical induction induces mechanical homeostasis failure of the joints and results in the loss of mechanical properties of knee joint and a change in the subchondral bone microstructure, which increases the stress on the overburdened articular cartilage and leads to wear and degradation.(49) Therefore, the regulatory effect of lncRNA H19 on cartilage lesions in the above models may be different than the high intensity motion-induced PTOA mice model. Further research is needed to fully understand the regulatory effect of lncRNA H19 in motion-induced PTOA.

The articular subchondral bone is in the process of continuous and dynamic bone remodeling, which is regulated by osteoblasts and osteoclasts to ensure the stability of the subchondral bone microstructure. The occurrence of abnormal bone remodeling is considered to be a typical marker of OA.(50) In the early stage of OA, bone resorption is dominant. In the middle and later stages of OA, bone formation is dominant. The specific process is as follows: abnormal mechanical stress leads to increased bone resorption, and the subchondral bone presents an osteoporotic structure with reduced bone mass, increased trabecular space, and thinning of the subchondral bone plate.(51) In this study, micro-CT scanning of the M group knee joint showed that the BMD, BV/TV ratio and Tb.N were higher than the CM group. This suggests that high intensity exercise induced PTOA mice may be in the early stages of cartilage injury, while there is no obvious morphological change in the subchondral bone. Moreover, after

four weeks of moderate intensity treadmill rehabilitation training, the BMD, BV/TV ratio, Tb.Th, Tb.N of K group were higher than those in CK group, while Tb.Sp showed the opposite trend, which proved that the subchondral bone of mice does not show the bone resorption phenomenon that may appear in the early stage of OA. A possible cause of this is that mechanical overload on the subchondral bone may be compensated for with microcracks. A microcrack is a normal physiological activity of bone cells that is triggered by the mechanical load and is the result of a joint attempting to maintain its integrity, that is, a microcrack may protect the subchondral bone tissue from damage by distributing mechanical load.(52) The specific mechanism of this is as follows: the microcracks of the subchondral bone appear to reduce the elastic modulus of bone tissue, which affects its ability to resist the load of the bone cell. This reduces its ability to absorb mechanical strain, and leads to the subchondral bone having a higher mechanical load, resulting in mechanical overload which causes bone matrix damage activated bone cells to produce receptor activator of the NF- κ B ligand (RANKL). Also, down-regulated osteoprotegerin (OPG) (53) induces osteoclast activation, strengthens the bone absorption, and begins bone remodeling to promote bone repair.(54) Therefore, the subchondral bone may compensate for bone injury by early bone remodeling that is associated with microcracks, indicating that motion-induced early OA damage does not first occurs subchondral bone, but in articular cartilage. In addition, previous studies established a model of disuse osteoporosis (DOP) with mechanical stress reduction in rats and found that no mechanical load can induce decreased expression of lncRNA H19 in bone tissue and lead to reduced bone formation and DOP through a Wnt signaling cascade.(55) This suggests that lncRNA H19 may be involved in the regulation of mechanical stress-related bone metabolic processes. Unfortunately, this study failed to detect the expression of lncRNA H19 in the subchondral bone of motion-induced PTOA mice, which would have made it possible to determine if there is a difference or correlation between the regulatory effect of lncRNA H19 on bone tissue in DOP and on subchondral bone tissue in motion-induced PTOA. In the future, the specific regulatory effects of lncRNA H19 on subchondral bone in motion-induced PTOA mice also need to be further studied.

Conclusion

High intensity treadmill running can cause knee cartilage injury in C57BL/6 mice, induce PTOA, and decrease the expression of lncRNA H19 in the articular cartilage. Also, moderate intensity treadmill rehabilitation training can relieve motion-induced PTOA in mice, and increase the expression of lncRNA H19 in the articular cartilage.

Materials And Methods

This study was carried out in strict accordance with the recommendations from the Ethical Committee of Shanghai University of Sport on the Care and Use of Animal Subjects in Research (Approval Number: 2018013), following the protocols approved by the committee. Thirty specific pathogen-free wild-type C57BL/6 male mice (19 weeks old) were purchased from Shanghai Nanfang Biotechnology Co., Ltd. and used in the experiment. The mice were maintained in separate cages in a pathogen-free barrier system

animal house at the Shanghai University of Sport. Each of the cages contained five mice. The light inside the barrier was turned on at 8:30 a.m and turned off at 8:30 p.m, which simulated an alternating day and night for 12 hours. The temperature in the animal house was 22 ± 2 °C, the humidity was 40%-70%, and the mice were weighed every Monday.

Experimental grouping

All of the mice were acclimated to the new environment for a week. Then they were randomly divided into 5 groups as follows: blank model control group, (CM group, n = 6), treadmill model group (M group, n = 6), blank rehabilitation control group (CK group, n = 6), treadmill model + rehabilitation training group (K group, n = 6) and treadmill model + convalescent group (J group, n = 6). The mice were 20 weeks old when all of the groups began the experiment. The CM group and the CK group did not receive any intervention, and the remaining three groups (M group, K group, and J group) were pre-adapted to low intensity treadmill running for one week, and then high intensity treadmill running for four weeks to create a mouse model of PTOA. After four weeks of high intensity treadmill running, samples were collected from the CM group and the M group in order to test if high intensity treadmill running can successfully establish a PTOA model. Subsequently, the K group participated in four weeks of moderate intensity treadmill rehabilitation training, while the J group was conventionally raised in independent cages without any exercise intervention. After four weeks of moderate intensity treadmill running, samples were collected from the CK group, the K group and the J group in order to test the rehabilitation effect of moderate intensity treadmill running on mice with PTOA, as shown in Table 3.

Table 3
Grouping scheme of mice

Group	Abbreviation	Intervention scheme	mice age
Blank Model control group (n = 6)	CM	No intervention	25w
Treadmill model groups(n = 6)	M	4w high intensity	25w
Blank rehabilitation control group(n = 6)	CK	No intervention	29w
Treadmill model + rehabilitation training(n = 6)	K	4w high intensity+ 4w medium strength	29w
Treadmill model + convalescent group(n = 6)	J	4w high intensity+ 4w no intervention	29w

Treadmill running protocol

According to the literature (36, 37) and our research, the following experimental scheme was developed. The mice began running on the treadmill at 14:00, five times a week, for 80 minutes each time. The 80 minutes included 10 min of accelerated motion, 60 min of uniform motion, and 10 min of decelerated motion. During the pre-adaptation period, the uniform motion speed was gradually increased for five

days. The speed was 10 m/min on the first day, 12 m/min on the second day, 14 m/min on the third day, 16 m/min on the fourth day, and 18 m/min on the fifth day. The gradient of the treadmill was 5°. During the high intensity treadmill running, the uniform speed was 20 m/min and the treadmill slope was 5°. During the moderate intensity rehabilitation training, the uniform speed of movement was 8 m/min, and there were no acceleration and deceleration stages included in the scheme before or after the exercise. The exercise duration for this moderate intensity training was 40 min with no treadmill slope.

Sampling

When the treadmill running program was completed (at the fifth week for CM and M group; at the ninth week for CK, K and J group), the mice were anesthetized by ether inhalation after fasting overnight. After the mice were completely anesthetized, the mice were weighed, and the soft tissues around the knee joint such as the muscles and ligaments were cut off of the lower limbs of the mice with ophthalmic shears, and then the knee joint was cleaned with a PBS solution. The left knee joint was immediately scanned using a micro-CT, and then the samples were placed in the refrigerator with 4% paraformaldehyde solution at 4°C for 48 hours. Next, the samples were placed in the decalcination solution at room temperature for 30 days. During this time, the decalcination solution was replaced once a day. The right knee joint was wrapped with gauze, soaked with PBS and stored in a refrigerator at -80 °C for RNA extraction.

Paraffin embedding, sectioning, and staining

After the completion of the decalcification process, the knee joint samples were placed in a stainless steel dish and washed with running water for 5 min. The residual soft tissues around the knee joint were carefully trimmed with ophthalmic shears and the unrelated bone was cut around. Only the intact knee joint was retained for paraffin embedding which occurred at a later stage. Then, the knee joints of each group were placed in 50% alcohol at room temperature for 12 hours overnight, and the next day the knee joints were dehydrated using gradient ethanol, transparentized with xylene, and embedded in wax. Subsequently, a paraffin embedding machine was used for embedding and the popliteal fossa of the knee joint was aligned to the bottom of the embedding box. The section thickness of the paraffin was 5.0 µm. The paraffin sections were stained according to the standard instructions for hematoxylin-eosin staining and safranin O-fast green staining. The sections were scored by two independent observers under a 200-fold light microscope according to the Mankin's and OARSI scoring standards. The means of the two groups were used as the final score.

micro-CT scan

A micro-CT was used to scan the knees of the mice. The scanning parameters were as follows: FOV/diameter: 319480 µm, voxel size: 10.4 µm, energy: 55 kv, intensity: 72 µm, and sample time: 250,000 µs. When a knee was placed for scanning, we confirmed that the angle of the placement was the same each time, and it was adjusted again in the pre-scan window to ensure the effectiveness and accuracy of the scanning and post-reconstruction. After the scan, 100 layers were selected to reconstruct the medial and lateral tibial plateau of each group from the apex of the tibial plateau, and they were divided into the

following groups: CM group lateral tibia plateau (LCM group) and M group lateral tibia plateau (LM), CK group lateral tibia plateau (LCK group), K group lateral tibia plateau (LK), J group lateral tibia plateau (LJ); CM group medial tibia plateau (MCM group), M group medial tibia plateau (MM group), CK group medial tibia plateau (MCK group), K group medial tibia plateau (MK group), and J group medial tibia plateau (MJ group). At the same time, micro-CT software was used to analyze the subchondral bone structure. The following measurement parameters were used: (1) trabecular spacing (Tb.Sp); (2) trabecular thickness (Tb.Th); (3) trabecular number (Tb.N); (4) bone volume fraction (BT/TV); and (5) bone mineral density (BMD).

Real-time PCR detection

The knee joint samples were removed from the -80°C refrigerator and thawed. The soft tissues around the knee joint, including the joint capsule, were carefully removed and the articular cartilage on the tibial plateau and the femoral condyle were carefully detached. Then, the articular cartilage was put into a small mortar and liquid nitrogen was added to help grind the cartilage into a powder. The cartilage and remaining liquid nitrogen were quickly spooned into the 1.5 ml EP tube before the liquid nitrogen evaporated (if the cartilage was not ground into a powder before the liquid nitrogen evaporated or the powder was not scooped into the EP tube, a small amount of liquid nitrogen should be added again). The total RNA was extracted from the powdered cartilage according to previous studies. Subsequently, SYBR green from the Vazyme Company was used to prepare the corresponding working fluid system according to the given instructions and it was added to a 96-well plate for fluorescence quantitative amplification. With this, the expressions of lncRNA H19, inflammatory factor TNF- α , cartilage anabolic factor COL2, ACAN, and cartilage catabolic factor MMP-13 were detected. β -actin was used as an internal reference gene. The results were analyzed using $2^{-\Delta\Delta\text{Ct}}$. The primer premier 5.0 software was used to design all of the primers that were involved in the study. The primer sequences are shown in Table 4 below.

Table 4
primer sequence

Gene	Primer sequence (5'-3')
β-actin	Forward: 5'-CTGTCCCTGTATGCCTCTG-3'
	Reverse: 5'-ATGTCACGCACGATTTCC-3'
TNF-α	Forward: 5'-AGGCTGCCCCGACTACGT-3'
	Reverse: 5'-GACTTTCTCCTGGTATGAGATAGCAAA-3'
MMP-13	Forward: 5'-ACGTGTGGAGGTGAGGCATCC-3'
	Reverse: 5'-GCAGAAGGCAGACCGCAATGG-3'
COL2	Forward: 5'-CAT GAC CTC GTG ATG AAC GTG T-3'
	Reverse: 5'-CGG GTG AGG ACG TTT ACA AAG-3'
lncRNA H19	Forward: 5'-AGGCGAGGATGACAGGTGTGG-3'
	Reverse: 5'-GAACAGACGGCTTCTACGACAAGG-3'
ACAN	Forward: 5'-CACTCCTGCCTGCTATGGAATGC-3'
	Reverse: 5'-CCTGGTGATGCTGGTGCTGTTAG-3'

Data analysis

All of the results were expressed as mean $\pm$ standard deviation ($X \pm SD$), and SPSS 20.0 software was used for data analysis and processing. An independent sample T-test was used to compare the CM group and the M group in order to judge whether or not the PTOA modeling was successful. In order to judge the recovery effect, the CK group, the K group and the J group were compared using a one-way analysis of variance, and a post-hoc Bonferroni's correction for multiple comparisons was used for between-group pairwise comparisons. $P < 0.05$ indicates that the results are statistically different.

List Of Abbreviations

Osteoarthritis (OA)	Post-traumatic osteoarthritis (PTOA)
Long non-coding RNAs (lncRNAs)	Long non-coding RNA H19 (lncRNA H19)
Osteoprotegerin (OPG)	Receptor activator of the NF- κ B ligand (RANKL)
Disuse osteoporosis (DOP)	Interleukin-6 (IL-6)
Tumor necrosis factor alpha (TNF- α)	Matrix metalloproteinases (MMPs)
Trabecular spacing (Tb.Sp)	Trabecular thickness (Tb.Th)
Trabecular number (Tb.N)	Bone volume fraction (BV/TV)
Bone mineral density (BMD)	

Declarations

Ethics approval and consent to participate

This study was carried out in strict accordance with the recommendations from the Ethical Committee of Shanghai University of Sport on the Care and Use of Animal Subjects in Research (Approval Number: 2018013), following the protocols approved by the committee. All mice were anesthetized prior to the extraction of knee cartilage and were humanely euthanized after the procedures were completed.

Consent for publication

Not applicable.

Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Competing Interests

The authors declare that they have no conflict of interests.

Funding

The work is supported by funding from the National Natural Science Foundation of China (NO. 81572242) and Shanghai Key Laboratory of Human Sport Competence Development and Maintenance (Shanghai University of Sport, No.11DZ2261100).

Author's contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Xuchang Zhou, Hong Cao and Miao Wang. The first draft of the manuscript

was written by Xuchang Zhou and Hong Cao. Wei Wu and Jun Zou supervised the project and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

Figure 1

A: Weight changes in CM group and M group, B: Weight changes in CK group, K group, and J group. (CM group: blank model control group, M group: treadmill model group, CK group: blank rehabilitation control group, K group: treadmill model + rehabilitation training group, J group: treadmill model + convalescent group. N = 6, *P < 0.05, intra-group comparison of M group;△P < 0.05, intra-group comparison in J group;#P < 0.05, ##P < 0.001.)

Figure 2

The effect of high intensity treadmill running on the cartilage of mice knee joints (A: sections of the knee joint of the high intensity treadmill running mice with hematoxylin-eosin staining and safranin O staining under the 200-fold light microscope; B: Mankin's and OARSI scores of hematoxylin-eosin staining and safranin O staining in the knee joint of high intensity treadmill mice; CM group: blank model control group, M group: treadmill model group, N = 6, **P < 0.01, Mankin's score;##P <0.01, OARSI score)

Figure 3

Effects of moderate intensity treadmill rehabilitation training on mice knee cartilage (A: section-diagram of moderate intensity treadmill running mice knee cartilage with hematoxylin-eosin staining and safranin O-fast green stain under the 200-fold light microscope; B: Mankin's score and OARSI score of hematoxylin-eosin staining and safranin O-fast green stain of the knee cartilage of mice with moderate intensity treadmill running. CK group: blank rehabilitation control group, K group: treadmill model+ rehabilitation training group, and J group: treadmill model + convalescent group. n = 6, *P < 0.01, **P < 0.01, Mankin's score; ##P < 0.01, OARSI score)

Figure 4

Morphological changes of tibial plateau bone in mice of CM group and M group (A: three dimensional (3D) reconstructed images of tibial plateau; B: the image of tibial plateau of CM group was sectioned along the horizontal plane, from the top at 40%; C: the image of tibial plateau of M group was sectioned along the horizontal plane, from the top at 40%. CM group: blank model control group, M group: treadmill model group)

Figure 5

Morphological changes of tibial plateau bone in mice of CK group, K group and J group (A: three-dimensional reconstructed images of tibial plateau; B: the image of tibial plateau of CK group was sectioned along the horizontal plane, from the top at 40%; C: the image of tibial plateau of K group was sectioned along the horizontal plane, from the top at 40%; D: the image of tibial plateau of J group was sectioned along the horizontal plane, from the top at 40%. CK group: rehabilitation blank control group, K group: treadmill model + rehabilitation training group, J group: treadmill model + convalescent group)

Figure 6

Changes in expression of COL2, ACAN, MMP-13, TNF-α mRNA and lncRNA H19 in mice knee cartilage after high intensity treadmill running (CM group: blank model control group M group: treadmill model

group, N = 6, *P < 0.05, **P < 0.01)