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Research Article

Moderate Treadmill Exercise Inhibits the Progression of Osteoarthritis in Rats by Regulating the NLRP3-Mediated Pyroptosis Pathway

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Abstract

Background: Exercise is recognized as fundamental for managing Osteoarthritis (OA), a prevalent joint condition with significant anti-inflammatory effects linked to pyroptosis. However, the precise impact of exercise on OA's pyroptotic processes remains unclear.

Objective: Our study aimed to investigate the impact of moderate treadmill exercise on the NLRP3-mediated pyroptosis pathway as a potential mechanism for inhibiting osteoarthritis progression in rats.

Methods: In this research, 24 Sprague -Dawley rats were randomly divided into three groups: the SHAM group, the DMM (destabilized medial meniscus) group, and the DMM + treadmill exercise (DMM + TE) group. Osteoarthritis (OA) was induced through Anterior Cruciate Ligament (ACL) surgery and DMM. After 4 weeks of OA model establishment, the DMM+TE group engaged in treadmill exercise at a 15 m/min, 30 minutes/day, 5 days/week, for a duration of 8 weeks. The study employed several techniques such as morphology staining, micro-CT, three dimensional, WB, RNA expression, and serum inflammatory factors.

Results: In DMM rats, notable alterations in the cartilage subchondral bone were detected after surgery. These changes encompassed cartilage and subchondral bone damage. Treadmill Exercise (TE) effectively inhibited the pyroptosis factors. As a result, it mitigated the symptoms of OA. Additionally, TE reduced the expression of inflammatory cytokines within the cartilage, thereby demonstrating its beneficial effects on OA progression.

Conclusion: Treadmill exercise holds promise as a treatment for OA through its targeting of the pyroptosis factors, indicating its potential value in the management of OA.

Keywords: Osteoarthritis, Moderate Treadmill Exercise, Pyroptosis, NLRP3

Abbreviations: OA: Osteoarthritis; DMM: Destabilized Medial Meniscus; TE: Treadmill Exercise; ACL: Anterior Cruciate Ligament Transection; SHAM: A Control Group that Underwent an Incised Procedure in the Skin and Medial Capsule; NLRP3: Nod Like Receptor Protein 3; GSDMD: Gasdermin D; MMP13: Matrix Metallo Peptidase 13; COLII: Collagen Type II; Caspase-1: Cysteinyl Aspartate Specific Proteinase 1; IL-1β: Interleukin-1β; IL-18: Interleukin 18; SPF: Specific Pathogen Free; OARSI: Osteoarthritis Research Society International; qRT-PCR: Quantitative Real Time Polymerase Chain Reaction; WB: Western Blot; ELISA: Enzyme -Linked Immunosorbent Assay; CT: Computed Tomography; BV/TV: Bone Volume/Tissue Volume fraction; Tb.Th: Trabecular Thickness; Tb.Nb: Trabecular Number; Tb.Sp: Trabecular Separation; HE: Hematoxylin Eosin; SafO: Safranin O; TB: Toluidine Blue; IHC: Immunohistochemistry; EDTA: Ethylenediaminetetraacetic Acid; SDS PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; BSA: Bovine Serum Albumin; AOVA: Analysis of Variance

Introduction

Osteoarthritis (OA) is a chronic joint degeneration identified by the continuous breakdown of joint structures, including cartilage and subchondral bone compartment [1]. Currently, due to the increasing elderly populations, OA presented as a worldwide substantial clinical issue [2]. During the advancement of OA, various pathological processes take place within the articular cartilage, includes oxidative stress, inflammatory processes, apoptosis, cartilage degradation, and autophagy.

However, the research using genetic mouse models showed the growth factors involvement, such as TGF-β (transforming growth factor-β). Furthermore, its essential to include the molecules signals in the OA progression such as β-catenin, Smad3, and HIF-2α [3-5]. Inflammation and the degeneration of the Extracellular Matrix (ECM) play a critical role in cartilage degeneration in OA [6,7]. Gaining a full understanding of these changes during OA can improve the advancement of effective treatment therapies.

The signals in pyroptosis pathway consist of caspase-1 and the inflammasome, which play a considerable role in the programmed cell death. This includes the release of cytokines, and the macrophage lysis [8]. The NLRP3 and caspase-1 identified by the pro-inflammatory nature and regulate the pyroptosis process. Furthermore, pyroptosis process is facilitated by the NLRP3 and intricate protein complex that activate caspase-1 causing Gasdermin D (GSDMD) fragmentation. The GSDMD has an essential role in making cell membrane pores, cellular swelling, and release of pro-inflammatory cytokines, which lead to tissue damage and inflammation [9-11]. Elevation of NLRP3 in the synovial of SD animals indicate the participation in OA [12]. However, the exact roles of NLRP3, other pyroptosis factors still unclear.

Regular physical exercise has been widely recognized as a beneficial therapeutic method in preventing and treating different chronic diseases, including OA [13-15]. Physical exercise treatment is effective, and appropriate for OA treatment [16-18]. However, moderate training has significant effect on OA by decreasing inflammation, and delay cartilage damage [18,19]. Moreover, moderate strain can alleviate cellular aging and reduce chondrocyte death [20,21]. However, exaggerated or inadequate physical exercise can have adverse effects [22]. This research aims to assess the effect of moderate training on the NLRP3-mediated pyroptosis as a potential mechanism for inhibiting osteoarthritis progression in rats. By elucidating the potential role of exercise in modulating pyroptosis, this research seeks to get insights into the therapeutic of exercise in the management of OA.

Methods

Experiment Animals

24 male Sprague-Dawley (SD) rats, at 8 weeks of age and with an average weight of approximately 220±10 grams, underwent acclimatization training on a treadmill for one week. The training schedule consisted of a daily 10-minute session at a speed of 10 meters per minute, carried out 5 days a week. The SD rats were housed in SPF environment to uphold high level hygiene throughout the long-term experiments. This study obtained ethical approval from the Ethics Committee with the authorization number TJH-202210037. The rats were paired and kept in cages with a controlled environment. The rats had unrestricted access to food and water and could move freely within their enclosures. All SD rats and experimental procedures were strictly in accordance with the relevant rat protection and usage regulations set by the International Association for the Study of Pain.

OA Model and Exercise Protocols

(Figure 1) was shown the study protocols. A total of 24 SD rats underwent a one-week acclimation period of treadmill exercise training. The exercise protocol employed the Zhenghua Biological Instrument Equipment from China and consisted of 10 min/day, 5 days/week with a speed of 10 m/min. After the first week of treadmill exercise training for acclimation. The animals randomly separated into SHAM (n=8) and DMM (n=16). Regarding anaesthesia isoflurane was administered throughout all surgical procedures. In brief, the DMM group underwent surgical interventions on the right knee joint, which included ACLT and DMM. The SHAM group presented as a control group, which underwent an incised procedure in the skin and medial capsule. After 4 weeks' post-surgery, eight animals from each group was randomly chosen and underwent sacrifice to assess the success of the DMM model. Four weeks after surgery, the rats in DMM group allocated randomly into two groups: DMM (n=8) and DMM+TE (n=8) groups, while the SHAM group (n=8) included all of the SHAM animals in the study. The

training exercise protocol was selected based on a lecture study [23,24]. This study showed that engaging in exercise training at a speed of 15 m/min, for 30 min per day, 5 days per week, with a period of 8 weeks, effectively maintained the integrity of both cartilage and subchondral bone. Furthermore, this exercise regimen showed potential in alleviating cartilage degradation, general inflammation, and biomechanical pain [23,25]. The experimental SD rats were euthanized during 48 hours after their final session of treadmill exercise using a 2% isoflurane/oxygen combination. Moreover, collected the blood supernatant by performing a cardiac puncture and obtained tissue samples for further analysis.

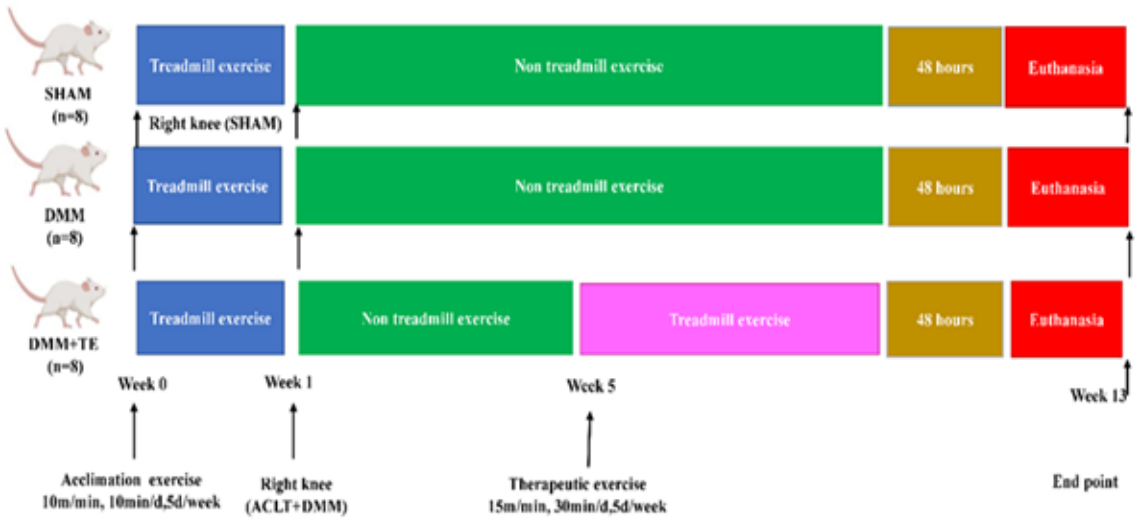

Figure 1: Twenty-four Sprague-Dawley (SD) rats underwent an accumulation exercise protocol on a treadmill for one week at an intensity of 10m/min; 10min/day; and 5days/week. Following the accumulation period; the rats were randomly assigned to three groups: SHAM; DMM; and DMM+TE. The Osteoarthritis (OA) model was induced in the DMM group by transecting the Anterior Cruciate Ligament (ACLT) and Destabilizing the Medial Meniscus (DMM). In contrast, the rats in the SHAM group underwent knee capsule opening and were sutured without any additional surgical procedures. The DMM+TE group engaged in 8 weeks of moderate treadmill exercise at an intensity of 15m/min; 30min/day; and 5 days/week. All SD rats were euthanized within 48 hours of completing the last moderate treadmill exercise session.

Micro-Computed Tomography (Micro-CT) and 3D

After the rats were euthanized, the whole right knee joints were completely removed and scanned with a micro-CT 50 (Scanco Medical, Bassersdorf, Switzerland). The micro-CT scanning parameters were as follows: a voxel size with a resolution of 10.5μm, a voltage of 100kV, and a current of 98μA. The integrated evaluation system the bone volume/tissue volume fraction (BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), and trabecular separation (Tb. Sp).

HE Analysis

After conducting the micro-CT of the sample joint, decalcification was carried out using a 10% EDTA solution. Subsequently, through dehydration, then embedded in paraffin. Sagittal plane slides of the joint were cut, each with a thickness of 4 μm, and these slices were collected for histological analysis. The sample were stained with HE, from Servicebio, SafO, from Servicebio, and Toluidine Blue (TB, from Servicebio) in accordance with the manufacturer's kits. The degree of cartilage injury at the HE level was evaluated using the Osteoarthritis Research Society International (OARSI) scoring system [26] and the modified Mankin scoring system [27]. This scoring system, which ranges from 0 to 6 points, is renowned for its high sensitivity and reliability in assessing the histopathological characteristics of cartilage in DMM rat models.

IHC Analysis

The expression of MMP13, Col2a1 (rabbit, 1:800; A-A14, A-A04), Caspase-1, GSDMD, and NLRP3 (rabbit, 1:200; ER1905-47, ER1901-37, ET1610-93). Digital image analysis with Image-Pro Plus software was applied to determine the positive cell percentage showing positive staining in samples.

qRT-PCR

Total RNA was extracted with Trizol reagent (Powerful Biotechnology, Wuhan; R1100), then reverse transcribed to cDNA using reverse transcriptase (Powerful Biotechnology, Wuhan; AT341). Analyzed via ABI QuantStudio™ 1 System settings. Finally, target gene mRNA expression levels were standardized against GAPDH as a reference gene, primers in (Table 1).

Table1: Primer sequence used in the qRT- PCR experiment of articular cartilage.

Gene	RNA Sequence (5' to 3')
GAPDH F	5'-ACTCTACCCACGGCAAGTTC-3'
GAPDH R	5'-TGGGTTTCCCGTTGATGACC-3'
Caspase-1 F	5'-GACCGAGTGGTTCCCTCAAG-3'
Caspase-1 R	5'-GACGTGTACGAGTGGGTGTT-3'
Gasdermin D F	5'-GATGCCTGCTTGTTGAGTTGG-3'
Gasdermin D R	5'-AGAATTCCGAAGGCAGTCCA-3'
NLRP3 F	5'-CTGCATGCCGTATCTGGTTG-3'
NLRP3 R	5'-GGTACCCCATAGACTGGCAC-3'
MMP13 F	5'-TGCTGCATACGAGCATCCAT-3'
MMP13 R	5'-CCCCGTGTCCTCAAAGTGAA-3'
Coll II F	5'-TCATCGCCACGGTCCTACAA-3'
Coll II R	5'-GGACGAGGGCTTCCATACATC-3'

Table Abbreviations: F: Forward; R: Reverse.

WB

Proteins from articular cartilage tissue were extracted and homogenized in RIPA buffer (P0013B, Biyuntian) with 1% phosphatase and protease inhibitors (Servicebio). After purification and determining the concentration (25μg), protein samples were separated by SDS-PAGE gel electrophoresis (BF0006, Bolfu). Then, proteins were transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% BSA (G5001, Roche), incubated with primary antibodies at 4°C (Supplementary Table 2), and immunoblotted using secondary antibodies (Boster).

ELISA

Before euthanizing the animals, isoflurane was used for anesthesia. After the animals were euthanized, cardiac puncture used to get blood sample, and the supernatant was separated. The serum concentrations of interleukin (IL-18; FY-EH-6593), and IL-1β (FY-EH6675, Feiyue) Biotechnology, Wuhan, China, were measured using ELISA kits in strict accordance with the manufacturer's guidelines.

Statistical Analyses

The data obtained from this study were statistically analyzed using GraphPad Prism 8 software, which was utilized to create statistical graphs. The data are presented in the form of mean ± Standard Deviation (SD). For comparing multiple groups, Analysis of Variance (ANOVA) was carried out, and subsequently, a post-hoc Tukey test was applied for performing multiple comparisons. A difference was regarded as statistically significant when the value of *P set at 0.05.

Results

Moderate Treadmill Exercise Mitigates DMM-Associated Characteristics of Cartilage and Subchondral Bone Unit

At 13 weeks after inducing the DMM model, the knee joint in the DMM group displayed marked histological deformity. The results imply a swifter advancement of cartilage degradation in the DMM. (Figure 2A and B) showed, the subchondral bone in the DMM group underwent substantial remodeling and experienced bone damage. However, in the DMM group, 8 weeks of moderate exercise assisted in mitigating the OA, preserving the cartilage and the structure of the subchondral bone. Following to treadmill exercise treatment, the DMM+TE group demonstrated reduced cartilage degeneration (Figure 1C), lower OARSI (Figure 2D), a decreased number of Caspase -1, GSDMD, NLRP3, and MMP13 -positive cells, and a higher proportion of Coll II -positive cells (Figure 2F). These contrasted with those of the DMM group (Figures 1, 2).

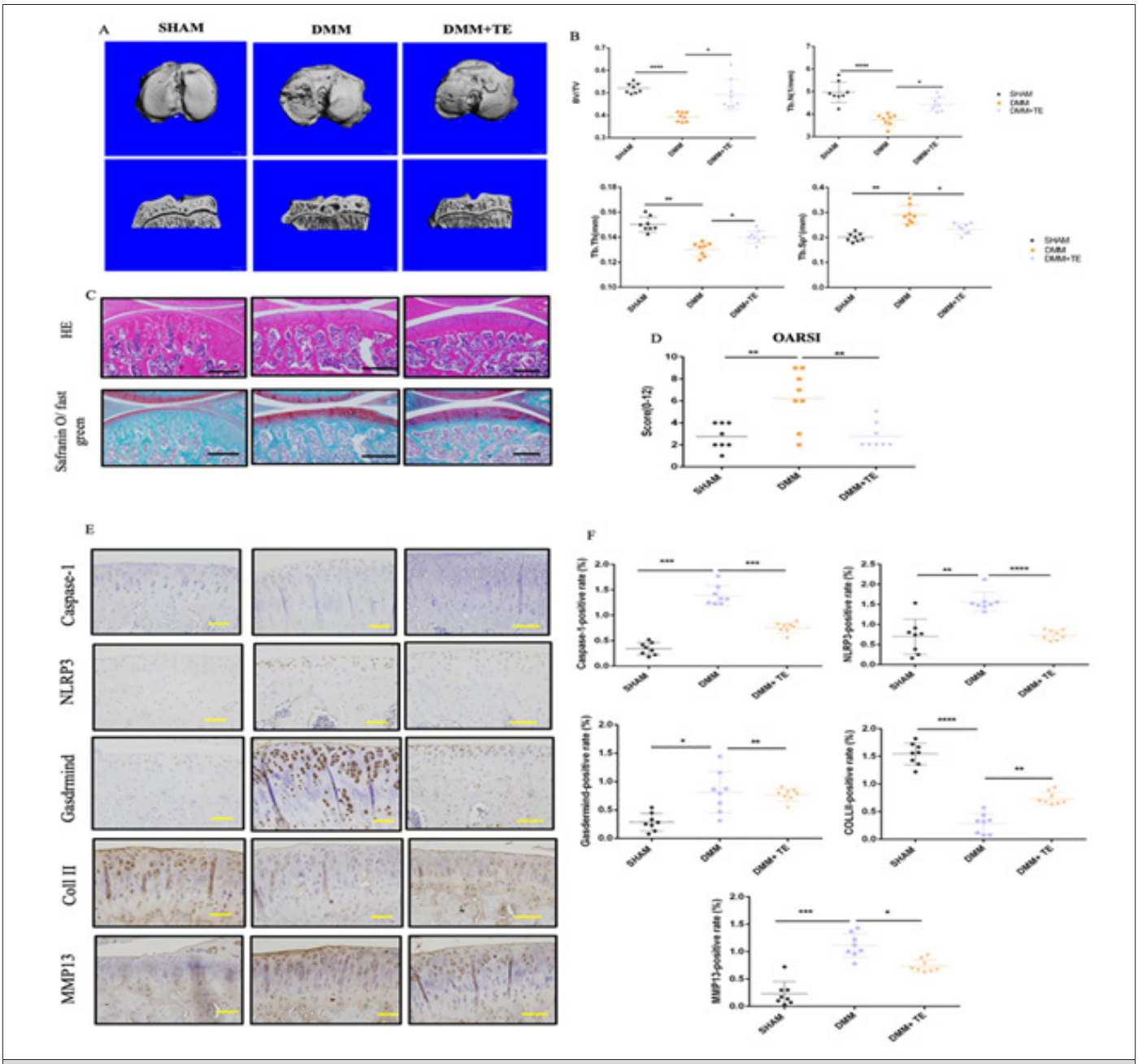

Figure 2: (A) The CT three-dimensional images of SHAM, DMM and DMM+TE groups and (B) Their quantification. (C) The images of Hematoxylin Eosin (HE); safranin O (SafO)/fast green and tolidine blue staining in a sagittal plane. (D) Osteoarthritis Research Society International (OARSI) score of the SHAM; DMM and DMM+TE groups. (E) Immunohistochemical staining of Caspse-1; GSDMD; NLRP3; MMP13; and Coll II positive cells was performed of each group (F) Percentage of Caspse-1; GSDMD; NLRP3; MMP13; and Coll II positive cells in each group. Black scale bar = 1000μm; yellow scale bar=200μm and white scale bar = 1mm. *P < 0.05; **P < 0.01; ***P < 0.001; N=8.

Moderate Exercise Exerted a Suppressive Effect on Cartilage Tissues. Additionally, It Suppressed the Release of Cytokines by The Process of Pyroptosis

qRT -PCR was employed to measure the mRNA expression levels. In comparison with the SHAM group, the DMM group exhibited elevated mRNA levels of Caspase -1, GSDMD, NLRP3, and MMP13, while the mRNA level of Coll II was decreased (Figure 2A). The DMM+TE group had significantly higher Coll II mRNA levels, and the mRNA levels of Caspase -1, GSDMD, NLRP3, and MMP13 were lower than those in the DMM group (Figure 3A). WB analysis was utilized to examine the levels of proteins factors in cartilage tissue. This was done to determine the impact of exercise on these markers and the underlying mechanisms of pyroptosis during DMM development. The results indicated that, compared to the SHAM, and DMM group had significantly higher of these markers, with the exception of Coll II.

Notably, cartilage tissues from the TE group presented lower levels of the proteins, and higher levels of Coll II (Figure 3B-C). These findings suggest that moderate exercise can effectively regulate of these markers related to OA development. Moderate exercise might exert a therapeutic effect on OA by modulating pyroptosis. This modulation, in turn, suppresses the activation of Caspase -1, GSDMD, NLRP3, and MMP13, and restricts the production of inflammatory cytokines. In this study, the concentrations of the pro -inflammatory IL -1β and IL -18 in the serum of DMM affected rats were found to be significantly higher than those in the SHAM group. In contrast, the TE group showed a significant decrease in the levels of IL -1β and IL -18 (Figure 3D) (Figure 3).

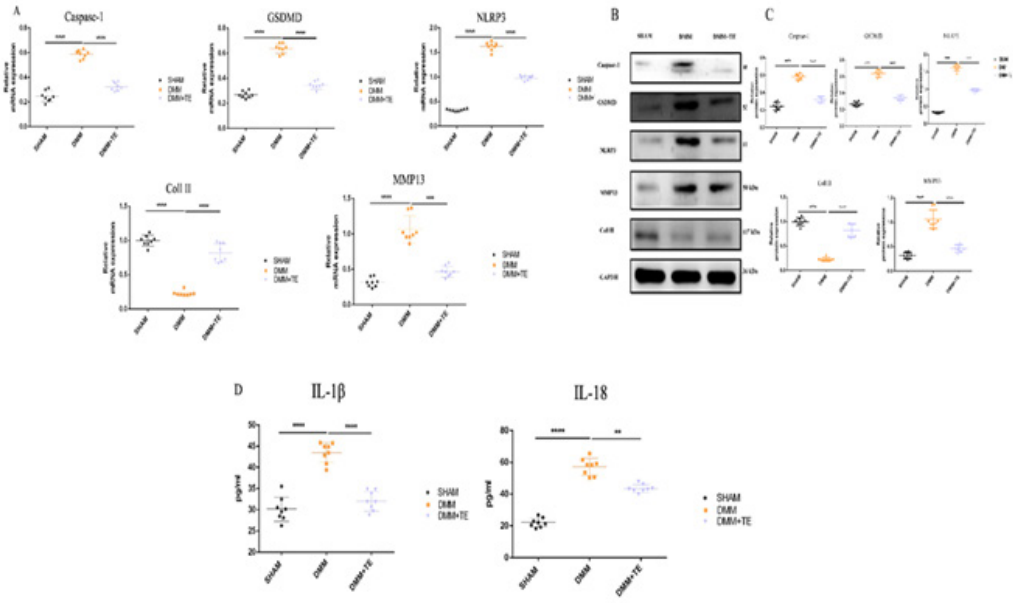

Figure 3: (A) The mRNA levels of Caspase-1; GSDMD; NLRP3; MMP13; and Coll II in each group by q RT PCR. (B) The protein expression of Caspase-1; GSDMD; NLRP3; MMP13; and Coll II in the cartilage of SHAM; DMM; DMM +TE groups. (C) Its quantification. (D) Serum concentration quantification of IL-1β and IL-18. The mean and standard error are used to represent the data; P*<0.05; P**<0.01; P***<0.001; N=8.

Discussion

Osteoarthritis (OA) is the most prevalent joint disease and one of the primary causes of disability among the elderly. With the aging of the population and the increasing prevalence of obesity, the incidence of OA is on the rise. Although mechanical loading and inflammation have been identified as contributing factors to the development of OA, the precise underlying mechanisms remain elusive. This lack of understanding poses a significant challenge in the development of effective preventive and curative treatments for the disease. Currently, pharmacological interventions for OA primarily focus on symptom relief, as there is still no established cure for this condition [28]. Certainly, exercise is widely advocated as the initial nonpharmacological and nonsurgical approach for managing OA. Moreover, exercise has long been regarded as one of the fundamental approaches for managing OA [29]. Our findings corroborated that exercise has the ability to safeguard cartilage from damage and improve the severity of OA significantly. These results consistent with previous investigations [19,30-32]. It has proven efficacy in mitigating symptoms, enhancing joint function, and promoting an improved quality of life.

The exercises have a prominent anti-inflammatory effect linked to pyroptosis [33]. It is widely recognized that exercise can diminish chronic inflammation and effectively suppress the expression of inflammatory factors, consequently enhancing the secretion of anti-inflammatory cytokines. Lecture studies demonstrated that physical exercise can reduce the level of the NLRP3 and significantly suppress the stimulation of ASC, Caspase-1, IL (1β, 18) [33,34]. However, the specific impacts of physical exercise on pyroptosis in OA and the underlying cellular and molecular mechanisms involved in these effects remain to be fully comprehended. This study aimed to show the impact of moderate training on the OA development by inhabiting the pyroptosis. By examining the potential inhibitory impacts of physical exercise on pyroptosis, this research aims to elucidate the underlying mechanisms and therapeutic implications of exercise in managing OA.

In recent years, numerous researches have consistently validated the significance of pyroptosis in the progression of OA [35,36]. In this study, we utilized DMM model in rats and observed that treatment with treadmill exercise had a marked beneficial impact in diminishing inflammation and mitigating cartilage damage in the

DMM knee joint of rats. These outcomes utilized multiple methods, such as micro -CT 3D imaging and morphological staining, to estimate the severity of OA. The results of this study showed that, in comparison to the DMM (D destabilization of the medial meniscus) group, both the SAHM (Stem cell -based Allogeneic Human Mesenchymal Stem Cells) and DMM+TE (DMM plus treadmill exercise) groups had protective and decreased bone loss, indicating reduced damage. Significantly, exercise had impact on articular cartilage. It not only promoted cartilage repair but, in certain instances, also led to cartilage regeneration [37]. Furthermore, we noted that exercise exerted a comparable effect through the inhibition of the levels of NLRP3, Caspase-1, and GSDMD pathways, which are implicated in OA. Nevertheless, the treatment with TE resulted in a remarkable decrease in the levels of IL-(1β, 18). The outcomes from the qPCR analysis revealed that rats in the DMM+TE (destabilization of the medial meniscus plus treadmill exercise) group displayed a notably diminished expression of NLRP3, Caspase-1, and GSDMD, along with a significantly reduced mRNA level of MMP13. As well as, there was an elevated mRNA level of COLL II in the articular cartilage tissue as opposed to the DMM group. These results suggest that moderate exercise possesses potent anti-inflammatory-pyroptotic effects. These outcomes are in accordance with prior research [38,39]. The NLRP3 inflammasome has been linked in the development of various articular conditions by promoting the production of inflammatory cytokines and catabolic enzymes [40]. In recent times investigations, the significant participation of NLRP1 and NLRP3 inflammasomes in the inflammatory response and pyroptosis of fibroblast-like synoviocytes has been emphasized. This finding proposes that NLRP (1 and 3) inflammasomes may play a substantial role in the OA progression [41]. NLRP3 remain as a valuable biomarker for the consideration and administration of OA [40]. The regulation of NLRP3 by curcumin or estradiol reduce the expression level of inflammatory cytokines and block the OA progression [42,43]. Our study presented the rule of TE, which effectively attenuate inflammation and pyroptosis by regulating the NLRP3. These outcomes present a strong evidence of the disease-related function NLRP3 suppressing and highlight the therapeutic possibility of NLRP3 regulation. Pyroptosis is a programmed cell death that depends on activation caspase-1 by inflammasomes and leads to cell rupture and release of cytoplasmic contents [44]. The GSDMD is a part of the Gasdermin (GSDM) and cleavage by caspase-1, which release the N-terminal domain and oligomerizes on the plasma membrane that create a pore and facilitates the secretion of some substrates such as IL (18 and 1β) [45]. The involvement of caspase-1-mediated pyroptosis has been demonstrated in the management of several diseases, including multiple sclerosis [46], retinitis [47], and neurological diseases [48,49].

In addition, involvement of caspase-1-regulated pyroptosis has been demonstrated in the regulation of Brucella joint infection [49]. Furthermore, the nonexistence of caspase-1 has been found to decrease joint pathology in chronic arthritis, indicating its involvement in the disease [50]. Moreover, in line with OA's mechanisms, exercise benefits by reducing inflammation and suppressing pyroptosis. Our study has limitations. The small sample size may limit result generalizability. Its short-term focus on advanced OA means we need to test long -term moderate treadmill exercise in a larger group, especially in early OA, to assess effects on the cartilage subchondral unit. We also need to study long -term impacts on the pyroptosis pathway. More experiments with varying exercise durations and intervention time points in the DMM rat model can provide insights into exercise effects on early and advanced OA stages.

Conclusions

The NLRP3, Caspase-1, GSDMD pathway is implicated in the development of OA. Moderate exercise has the role to mitigate pyroptosis by regulation the (Caspase-1, GSDMD, NLRP3) factors signaling-mediated pyroptosis pathway, consequently Preventing chondrocyte damage and OA onset in rats. These findings suggest that.

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Conflict of Interest

The authors declare no conflict of interest.

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