Receptor-interacting protein 1 inhibition prevents mechanical stress-induced temporomandibular joint osteoarthritis by regulating apoptosis and later-stage necroptosis of chondrocytes

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Research Article

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Abstract

Objectives: Temporomandibular joint osteoarthritis (TMJ OA) is a common degenerative joint disease that has multiple causes. The abnormal stress distribution is known to be an important trigger of TMJ OA. This article explored the pathological changes of the condylar cartilage under 60g mechanical force and whether the inhibition of Receptor-interacting protein 1 (RIP1) can protect stress-induced TMJ OA.

Material and Methods: We used a compressive mechanical force-induced-TMJ OA model and Lenti-virus targeting RIP1 to perform this study. A total of 72 male rats were used in the animal experiment. Each rat was injected with a negative control Lenti-shRNA in the right TMJ and Lenti-siRIP1 in the left TMJ and euthanized after 4 and 7 days, respectively. Quantitative real-time PCR, immunohistochemistry, Tunnel staining and Micro-CT were used to detect cartilage pathological changes and one way ANOVA with LSD analysis was used to determine statistical significance between groups.

Results: The results identified the characteristics of the spatio-temporal changes in stress-induced TMJ OA. Under mechanical force, inflammation and apoptosis, which occur in the whole layer of mandibular cartilage, appear on the 4th day and persist till the 7th day. Necroptosis arises in the later stage of mechanical force and is mainly located in the transition layer. RIP1 inhibition through Lenti-virus could protect stress-induced mandibular cartilage thinning by inhibiting persisted apoptosis and later-stage necroptosis in the transition layer.

Conclusions: RIP1 plays an essential role in the destruction of mandibular cartilage under mechanical force. RIP1 inhibition through Lenti-virus could protect mechanical stress-induced TMJ OA.

Introduction

Temporomandibular joint osteoarthritis (TMJ OA) is a common degenerative joint disease that has multiple causes. The main pathological changes of TMJ OA are progressive cartilage destruction, chondrocyte death and subchondral bone remodeling. It severely affects patients’ daily life by causing joint pain and dysfunction (Kapos et al., 2020). However, the etiology of TMJ OA is unclear and current treatment has limitations. Non-surgical treatment can only relieve the symptoms but cannot prevent the development of cartilage degeneration (Andre et al., 2022). When the disease reaches its final stage, patients are often faced with the only option left for joint surgery (Asquini et al., 2021), which still has a lot of risks (Bach et al., 2022). Therefore, it is necessary to further explore the pathogenesis of OA and seek new potential therapeutic targets.

The pathogenesis of TMJ OA is complex, including inflammation, cartilage degradation and mechanical overload. Among these, the abnormal mechanical stress distribution is known to be an important trigger of TMJ OA (Betti et al., 2018). Many studies proved that compressive mechanical stress could induce TMJ OA-like changes (Huang et al., 2021; Ikeda et al., 2014; Jiang et al., 2018; Li et al., 2021; Wen et al., 2016; Zhang et al., 2015; Zhang et al., 2021) and we found compressive mechanical force could progressively lead to thinning of mandibular cartilage, from proliferation inhibition to chondrocytes death
(Gong et al., 2021; Li et al., 2013; Wen et al., 2016). Using our original rat TMJ OA model, we have previously demonstrated that we can reproduce stress-induced TMJ OA after mechanical force loading, allowing investigation of the pathogenesis and new therapeutic target (Huang et al., 2021; Wen et al., 2016; Zhang et al., 2017; Zhang et al., 2021).

During the development of TMJ OA, many signaling pathways have been reported, including Wnt/β-catenin, TGF-β and BMP signaling, Indian Hedgehog signaling, FGF signaling, NF-κB signaling and Notch signaling (Lu et al., 2022). In addition, apoptosis and necroptosis are also important pathways of chondrocyte death during stress-induced TMJ OA. Receptor-interacting protein 1 (RIP1) is a crucial regulator of cell life and death, including a kinase domain, a death domain and an intermediate domain. In the previous research, we found that RIP1 and TNF-α were increased in the mandibular cartilage under 80 g compressive mechanical stress (Zhang et al., 2017). It indicates that compressive stress can regulate the expression of RIP1, but it is unknown whether RIP1 plays a role in TMJ OA development. Meanwhile, it is reported that RIP1 is a key upstream regulator which mediates many signaling pathways, including RIP1/RIP3-mediated necroptosis (Liu et al., 2019; Zhang et al., 2017), TRADD-dependent apoptosis (Li et al., 2021) and NF-κB-related inflammation (Ofengeim & Yuan, 2013; Patel et al., 2020). Targeting RIP1 might be a novel therapeutic strategy for different diseases including TMJ OA (Ofengeim & Yuan, 2013). Therefore, in the present study, we hypothesized that RIP1 inhibition can block necroptosis, apoptosis and inflammatory signaling pathways in a special spatio-temporal pattern, and protect TMJ from compressive mechanical stress stimulation.

**Materials And Methods**

**Animal Experiments**

All animal experiments were performed with the regulation and approval by the Animal Ethics Committee of Nanjing University Medical School (Protocol Number: IACUC-D2202073) and complied with the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines for preclinical animal studies. Compressive mechanical force loading procedure which could induce TMJ OA was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering (Supplementary Figure 1). A total of 72 7-week-old male Sprague Dawley rats weighing 180-200g were requisitioned in the animal experiment of this study (Figure 1). The experiment was divided into two parts. The part I was used to determine whether Lenti-virus injection in vivo could knock down the expression of RIP1 (24 rats involved), and the part II was used to explore the protective effect of inhibiting RIP1 on TMJ cartilage (48 rats involved). Rats were divided into two groups randomly during part I and part II: Control group (Con group, with none mechanical force) and Force group (F group, with 60 g compressive mechanical force).

For rats in the F group, 60 g compressive mechanical force was applied on the first day of the experiment: Two stainless steel hooks were fixed on the lower incisors of the rats by resin. An anchorage jig was placed around the neck and arms. Between the hooks and jig, two rubber bands were tied on both sides, loading 60 g mechanical force up and backward. The rubbers were changed every 3 days to maintain...
stable force. Meanwhile, at the beginning of the experiment, Lenti-shRNA (negative control of Lenti-virus) was injected locally into the articular capsules of the right TMJ condyle and Lenti-siRIP1 (positive Lenti-virus targeting RIP1) was injected into the left in both Con and F groups. The tailored micro-injector needle was put 5 mm anterior to the external auditory canal and below the zygomatic arch. The orientation of the needle was adjusted so that it could pass along the bone wall. When touching the temporal fossa, the needle was located in the lower compartment of TMJ. Each rat was injected once throughout the entire experiment and according to the results of micro-CT and immunohistochemical staining, the injection of Lenti-shRNA did not affect TMJ or induce TMJ OA. On the 4th day (4 d) and 7th day (7 d) after mechanical force loading, rats in both Con group and F group were euthanized by cervical dislocation with sodium pentobarbital anesthesia for subsequent experiments.

Mandibular chondrocytes culture

The mandibular chondrocytes were isolated from 3-week-old Sprague Dawley male rats to select optimal transfection sequence before the animal experiments (Supplementary Figure 2). In brief, after separating soft tissues of the mandibular condyle, condylar cartilage was carefully dissected and digested with 0.25% trypsin for 30 min and 0.2% collagenase for 3 h. The suspension was filtrated and centrifuged so that chondrocytes were isolated as single cells. Then, primary chondrocytes were seeded at 1×10⁵/cm² density and cultured with DMEM complete medium in a humidified atmosphere at 37°C with 5% CO₂. The medium was replaced every 2 days and the cells were passaged when the fusion reached 90%. The third generation was used for in-vitro transfection. A total of 8 3-week-old rats were used in this section, and 2 disks of primary cells were obtained.

Lenti-virus and transfection

Three pairs of small interfering RNA (siRNA) targeting RIP1 were designed and siRNA3 was chosen to produce Lenti-siRIP1 (77.25% knockdown, Supplementary Figure 2). By in-vitro experiments, Lenti-virus with optimal knockdown siRNA sequence (top strand:5’-CACCAGTTCTTGACTGATAAACGAAATTATGCAGACCAAAGAATCTGC-3’, bottom strand:5’-AAAAGCAGTTCTGACATAAATTATGCAGACCAAAGAATCTGC-3’) was chosen as Lenti-siRIP1 for subsequent in-vivo experiments (Novobio Scientific Co. LTD., Shanghai, China). For each side of TMJ, 20μl hybrid liquid including 5μg/ml Polybrene and 2x10⁸Tu/ml Lenti-virus was injected.

Isolation of total RNA and Analysis

Total RNA was extracted from cartilage by RNA extraction kit (Tianenze, Beijing, China) according to the manufacturer’s instructions and was reversed into cDNA. In brief, after the tissue was cut and
homogenized, 1ml lysate and 0.2ml Ivfnag was added to shake and mix. After centrifugation, supernatant was extracted and isopropyl alcohol was added to shake and mix, and centrifuged again. The supernatant was discarded, washed twice with 75% ethanol, and left at room temperature for 2 minutes. Then, the RNA sediment were dissolved with 30ul DEPC water. After measuring the concentration, 1000ng total RNA was used for reverse transcription. The primers used are listed in Supplementary Table 1. All genes were analyzed by quantitative real-time PCR (Biosystems 7500 real-time PCR machine) and expressions were normalized by GAPDH. Each experiment was performed 3 times and average values were calculated as means.

**Histological observation and histomorphometric measurements**

After 4 or 7 days, 6 rats in each group (Con+shRNA, Con+siRIP1, F+shRNA, F+siRIP1 of 4 d and 7 d) were euthanized and mandibular condyles were isolated with the surrounding tissue. Samples were fixed in paraformaldehyde for 24h and decalcified in a 15% ethylenediaminetetraacetic acid (EDTA) solution for 8 weeks. After paraffin embedding, 5-um-thick sagittal sections were cut from each TMJ block parallel to the lateral surface of the condylar neck of the mandible ramus. Sections were dewaxed with xylene and rehydrated in a graded alcohol series for hematoxylin and eosin staining. Images were captured with an Olympus XI 70 microscope equipped with an Olympus Magna Fire digital camera. The mandibular cartilage thickness was measured on three HE-stained sections per joint by a computer-assisted image analysis system (Image-Pro Plus, 6.0) at the same staining threshold and the average values were taken for statistical analysis.

**Immunohistochemical staining**

Sections from 6 samples in each group (Con+shRNA, Con+siRIP1, F+shRNA, F+siRIP1 of 4 d and 7 d) were treated with 3% hydrogen peroxide to eliminate endogenous peroxidase activity and digested the antigenic sites with Antigen Retrieval Solution (AR0026, Wuhan Boster Biological Technology Ltd. China) for 10 min. Antibodies of RIP1 (1: 100, NB1-77077, Novusbio, UK), TNF-α (1: 500, ab66679, Abcam, UK), IL-1β (1: 100, ab9787, Abcam, UK), RIP3 (1: 100, ab58828, Abcam, UK) and Caspase-8 (1: 100, ab25901, Abcam, UK) were incubated overnight at 4℃. Then, the specimens were incubated with biotin-labeled IgG (GB23404, Servicebio, Wuhan, China) for 30min at 37℃ and an avidin-peroxidase complex for 30min at 37℃. Antibodies in the specimens were stained by a peroxidase/diaminobenzidine (DAB) yellow kit (AR1000, Wuhan Boster Biological Technology Ltd. China). Sections were also stained with hematoxylin, dehydrated in an ethanol series, cleared in xylene and covered slips. A total of 40 rats were used in Immunohistochemical staining. Image acquisition was the same as described above. The color difference marking positive and negative areas was measured with Image-Pro Plus and scores were assessed according to the staining intensity.
TUNEL staining

5-um-thick sections of mandibular cartilages from 6 samples in each group (Con+shRNA, Con+siRIP1, F+shRNA, F+siRIP1 of 4 d and 7 d) were prepared and apoptotic cells were stained using TUNEL cell apoptotic kits (MK1027, Wuhan Boster Biological Technology Ltd. China) according to the manufacturer’s protocol. The percentage of apoptotic cells was measured with Image-Pro Plus (3 sections per specimen, 6 specimens in each group).

Micro-computed tomography

Samples from each group (Con+shRNA, Con+siRIP1, F+shRNA, F+siRIP1 of 4 d and 7 d) were fixed in 4% paraformaldehyde solution. The morphology of condyles was assessed using an animal micro-CT scanner (GE eXplore Locus SP, London). The specimens were scanned with some parameters, including an X-ray tube potential of 80 kV, a tube current of 0.45mA and 9-um voxel resolution. After the micro-CT scan, the visualization of subchondral bone was made with software (Health Care MicroView ABA 2.1.2). Micro-CT measurements included percent bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.sp) were included and eight samples in each group were involved in analysis.

Statistical analysis

All measurements were repeated three times. All statistical calculations were performed using SPSS version 13.0 statistical software. The data were expressed as means±SD and one way ANOVA with LSD (Least Significant Difference, two-sided test) analysis was used to determine statistical significance between groups. P < 0.05 was considered statistically significant. The datasets analysed during the current study are available in the Mendeley Data (DOI 10.17632/hw229v7p2k.1).

Results

1. Lenti-siRIP1 successfully inhibits compressive mechanical force-induced RIP1 expression in vivo

To explore the role of RIP1 in mechanical force-induced TMJ OA, we used our original TMJ OA rat model (Huang et al., 2021; Jiang et al., 2018; Li et al., 2013; Wen et al., 2016; Zhang et al., 2017; Zhang et al., 2015; Zhang et al., 2021) with the intra-articular injection of Lenti-siRIP1 to mediate RIP1 knockdown (Figure 2). After loading 60 g compressive mechanical force for 4 or 7 days, RIP1 mRNA in F+shRNA group was significantly increased and the increase was more obvious in the transition layer on 4 d compared with Con+shRNA group (369.0% on 4 d and 220.0% on 7 d, P < 0.01). With the injection of Lenti-
siRIP1, the mRNA level of RIP1 was significantly decreased in F+siRIP1 group compared to the F+shRNA group on both 4 d and 7 d (50.9% on 4 d and 39.8% on 7 d, P 0.01). Also, the protein level of RIP1 declined (37.1 % on 4 d and 34.9% on 7 d by Immunohistochemistry, P 0.01). On both 4 d and 7 d, the decrease was more obvious in the transition and hypertrophic layers. Considering the low expression of RIP1 in mandibular cartilage, the siRIP1-only group did not reveal a statistical difference compared with Con groups. Thus, the rise of RIP1 in mandibular cartilage under mechanical force could be inhibited by the injection of Lenti-siRIP1.

2. RIP1 inhibition protects mandibular cartilage thinning under compressive mechanical force stimulation

During the progress of compressive mechanical stress-induced TMJ OA, the destruction of mandibular cartilage is an obvious pathological change, including the change in cell morphology, cell number, and cartilage thickness. Therefore, we performed Hematoxylin & Eosin staining to detect the change in cartilage to test whether Lenti-siRIP1 had a protective effect on mandibular cartilage.

In the F+shRNA group, cartilage damage increased time-dependently, showing apparent thinning and chondrocyte loss (Figure 3). Chondrocytes under mechanical force shrunk in size, arranged irregularly and flattened. The number of chondrocytes also decreased compared with Con+shRNA group (20.2% on 4 d and 29.0% on 7 d, P 0.01). Consistent with the cell number, the thickness of mandibular cartilage became thinning (25.7% on 4 d and 39.8% on 7 d, P 0.01). Interestingly, by measuring the thickness of different layers, we found that on 4 d, the thickness of the hypertrophic layer decreased more obviously (32.4% on 4 d’s hypertrophic layer, P 0.01). While on 7 d, the proliferative layer showed more thinning (46.8% on 7 d’s proliferative layer, P 0.01).

In the F+siRIP1 group, the thickness of mandibular cartilage recovered (31.8% on 4 d and 35.0% on 7 d, P 0.01) and the number of chondrocytes raised (22.8% on 4 d and 23.4% on 7 d, P 0.01) compared with the F+shRNA group. On 4 d, hypertrophic layers’ thickness recovered most obviously (56.5% on 4 d and 43.3% on 7 d, P 0.01). This demonstrated that Lenti-siRIP1 significantly relieves mandibular cartilage thinning under compressive mechanical force.

3. RIP1 inhibition decreases the mortality of chondrocytes under compressive mechanical force

To further investigate the protective efficiency of Lenti-siRIP1 among chondrocytes, we used TUNEL staining to observe dead cells. Without mechanical force, there was nearly no dead cell in the mandibular cartilage. After loading force (F+shRNA group), dead chondrocytes occurred and showed a time-dependent increasing trend (Figure 4). The distribution of dead cells also has certain characteristics. In the 4 d F+shRNA group, dead cells were mainly distributed in the transition layer and the hypertrophic
layer, while in the 7 d F+shRNA group, dead chondrocytes were scattered throughout the whole layer of mandibular cartilage.

RIP1 inhibition rescued the death of chondrocytes under compressive mechanical force in both 4 d (30.4%, P 0.01) and 7 d (36.0%, P 0.01) F+siRIP1 groups, strongly demonstrating the therapeutic effectiveness. Chondrocytes' death in proliferative, transition and hypertrophic layers were all alleviated by Lenti-siRIP1, showing that Lenti-virus transfection had effects on the whole layer of mandibular cartilage. Among these, the transition layer recovered most on 4 d (37.3%, P 0.01) while the proliferative layer recovered most on 7 d (50.7%, P 0.01).

4. RIP1 inhibition in mandibular cartilage could not protect subchondral bone under compressive mechanical force

Another pathological change during stress-induced TMJ OA is the destruction and remodeling of subchondral bone. Consistent with our previous study (Figure 5), at the early stage (4 d) in F+shRNA group, subchondral bone showed decreased bone volume fraction (BV/TV), indicating the loss of bone mass. Bone trabecula which support subchondral bone plate also showed damage, including decreased trabecular number (Tb.N), trabecular thickness (Tb.Th) and increased trabecular separation (Tb.Sp). After loading mechanical force for 7 days, the destruction remained but recovered a little, indicating the initiation of bone remodeling under compressive mechanical stress.

Nonetheless, there was no significant difference between the F+siRIP1 group and the F+shRNA group on both 4 d and 7 d. The loss of bone mass remained and bone trabecula still showed diffuse microdamage. Meanwhile, on 7 d, there was no signal of earlier bone remodeling. Thus, RIP1 inhibition in mandibular cartilage by injecting Lenti-siRIP1 into TMJ could not protect subchondral bone under mechanical force.

5. RIP1 inhibition reduces the mechanical stress-induced expression of inflammatory factors in the cartilage

After confirming the protective role of Lenti-siRIP1 in mandibular cartilage under mechanical force, we further determined the underlying mechanism. As for inflammatory markers, TNF-α and IL-1β both increased in the F+shRNA group compared with the Con+shRNA group, indicating the activation of inflammation in the mandibular cartilage during the whole process of force application (Figure 6). The mRNA level of TNF-α rose rapidly on 4 d (589.7%, P 0.01) while the protein level also showed an increase in the F+shRNA group (211.5% on 4 d and 390.0% on 7 d, P 0.01). IL-1β increased obviously on 4 d (196.7% in mRNA and 342.5% in protein, P 0.01). While on 7 d, the mRNA level of IL-1β had no significant difference compared with the Con+shRNA group (122.7%, P 0.05) but the protein level was higher
TNF-α was mainly located in the proliferation and transition layers while IL-1β was more expressed in the transition and hypertrophic layers.

Lenti-siRIP1 prevented the increase of TNF-α and IL-1β in F+siRIP1 group on both 4 d and 7 d, suggesting the inhibition of stress-induced inflammation in the mandibular cartilage. Compared with F+shRNA group, TNF-α decreased both at the mRNA (51.7% on 4 d and 32.0% on 7 d, P 0.01) and protein (28.0% on 4 d and 43.5% on 7 d, P 0.01) levels. The expression of IL-1β also declined both at the mRNA (58.8% on 4 d and 65.8% on 7 d, P 0.01) and protein (55.3% on 4 d and 44.6% on 7 d, P 0.01) levels. The decrease of inflammatory factors appeared in all layers, suggesting the function of Lenti-siRIP1 could penetrate mandibular cartilage and alleviate inflammation induced by compressive mechanical force.

6. RIP1 inhibition reduces the mechanical stress-induced apoptosis and later-stage necroptosis of chondrocytes

TUNEL staining (Figure 4) revealed that a large number of chondrocytes died after loading mechanical force. In order to clarify the form of cell death, we further detected the expression of Caspase-8 and RIP3, which represent apoptosis and necroptosis respectively (Figure 7).

After loading mechanical force for 4 d, the expression level of Caspase-8 in mandibular cartilage of F+shRNA group showed an obvious upward trend compared with the Con+shRNA group (214.9% in mRNA and 640.2% in protein, P 0.01), indicating the activation of apoptosis at the early stage. In the 7 d F+shRNA group, the level of Caspase-8 was still higher than in the Con+shRNA group (148.1% in mRNA and 350.9% in protein, P 0.01), but lower than in the 4 d F+shRNA group, suggesting that apoptosis was reduced but remained under 7 days’ mechanical force. Different from the early high expression of Caspase-8, RIP3 did not show any increase in the 4 d F+shRNA group, suggesting that in the early stage of stress-induced TMJ OA, necroptosis did not appear in mandibular cartilage. On 7 d, the expression of RIP3 increased (152.1% in mRNA and 202.5% in protein, P 0.01). Combined with the decrease of Caspase-8 on 7 d, it represented that necroptosis was activated in chondrocytes at the later stage of stress-induced TMJ OA. Under mechanical stress, Caspase-8 could be found in all layers, especially in the hypertrophic layer, whereas RIP3 was mainly found in the transition layer.

Compared with the F+shRNA group, Caspase-8 in the F+siRIP1 group decreased both at the mRNA (49.3% on 4d and 36.2% on 7d, P 0.01) and protein (62.8% on 4d and 25.4% on 7d, P 0.01) levels, indicating the inhibition of apoptosis during the whole progress. On 7 d, RIP3 decreased obviously (65.5% in mRNA and 25.9% in protein, P 0.01) in the F+siRIP1 group, demonstrating the effectiveness of Lenti-siRIP1 in preventing the necroptosis. Therefore, as an important upstream regulator, RIP1 inhibition could protect mandibular cartilage in stress-induced TMJ OA by alleviating persisted apoptosis and later-stage necroptosis.

Discussion
In our previous study, we found RIP1 increased obviously during chondrocyte death under compressive mechanical force (Zhang et al., 2017). Therefore, the function of RIP1 in stress-mediated TMJ OA awakened our interest. RIP1 is an important upstream kinase, crucial to cell survival and mediating cell death through different pathways (Newton & Manning, 2016). In the present study, we chose Lenti-virus injection to explore the protective effect of RIP1 inhibition on mandibular cartilage under 60 g compressive mechanical stress. The results showed that RIP1 inhibition by injecting Lenti-siRIP1 could effectively protect mandibular cartilage under compressive mechanical stress. The thickness of mandibular cartilage was recovered and chondrocyte death was alleviated. Inflammation, apoptosis and necroptosis induced by compressive mechanical stress were all inhibited. Targeting RIP1 is a feasible therapeutic strategy for stress-induced TMJ OA.

The function of RIP1 is complex. On the one hand, RIP1 could suppress apoptosis and necroptosis through NF-κB pathway (Vanlangenakker et al., 2011). Lacking RIP1 in mice leads to death soon after birth (Kelliher et al., 1998). Mice conditional knockout RIP1 experience inflammation, RIP3/MLKL-dependent necroptosis and Caspase-8-dependent apoptosis and die in early age (Rickard et al., 2014). On the other hand, RIP1 plays an important role in the activation of apoptosis and necroptosis. After TNF-α binding to TNFR1, TNF-receptor-associated death domain (TRADD) recruits RIP1 and then forms the complex I. Meanwhile, complex I can transform into the death-inducing complex Ila/b after Caspase-8 activation and induce apoptosis (Micheau & Tschopp, 2003). When Caspase-8 is inhibited, RIP1 and RIP3 are combined to form complex IIC (the necrosome), which can induce necroptosis (Liu et al., 2019; Wang et al., 2014). During the progress of necroptosis, cellular contents are released to inactivate immune cells and further promote inflammation (Li et al., 2012).

In our study, inflammation and apoptosis occurred at the early stage of mechanical force in F+shRNA group (4 d) and persisted till the end with the increase of TNF-α, IL-1β, RIP1 and Caspase-8, while necroptosis appeared at the later stage (7 d) with the increase of RIP3. Our results confirmed that RIP1 plays different roles during different pathological stages in stress-induced TMJ OA. At the early stage, RIP1 and Caspase-8 increased significantly, indicating the activation of Caspase-8 dependent apoptosis. After loading mechanical force for 7 d, while the expression of RIP3 increased, Caspase-8 went down but still higher than the control, suggesting that necroptosis was activated and apoptosis persisted. When Caspase-8 is inhibited, RIP1 and RIP3 are combined to form complex IIC and induced necroptosis (Liu et al., 2019; Wang et al., 2014). With the activation of necroptosis, cellular contents in dead chondrocytes are released to inactivate immune cells and further promote inflammation by generating TNF-α to stimulate RIP1 (Li et al., 2012), which showed continued existing inflammation. After TNF-α binding to TNFR1, a new circle of apoptosis and necroptosis was activated. Besides, IL-1β increased significantly after loading mechanical force. As a key mediator of the inflammatory response, the release of IL-1β could help rescue and redirect damaged cells (Lopez-Castejon & Brough, 2011).

In summary, inflammation and apoptosis persist in the destruction of mandibular cartilage under 60 g compressive mechanical stress, but necroptosis appears on the later stage. Compared with our previous study (80 g) (Zhang et al., 2017), the lighter force significantly delayed the onset of necroptosis. RIP1
inhibition in F+siRIP1 group could cut down apoptotic and necroptotic signals, reduce the negative circulation of chondrocytes under mechanical force, and avoid creating an inflammatory environment compared with F+shRNA group. In addition, the spatio-temporal change of mandibular cartilage thinning in different layers was not very related to the spatio-temporal change of TUNEL staining and the expression of inflammation and apoptotic markers (TNF-α, IL-1β, Caspase-8). Other mechanisms such as proliferation inhibition may need to explore in the further study.

Unfortunately, we also found that RIP1 inhibition (F+siRIP1 vs F+shRNA) in condylar cartilage could not protect against subchondral bone's destruction. Although it is reported that RIP1/RIP3 pathway plays an essential role in the differentiation of osteoclast and restraining RIP1 could hinder osteoclastogenesis and attenuate bone loss (Liang et al., 2020), our result showed the transfection of Lenti-siRIP1 mainly focused on the cartilage layer, and was hard to transfect and penetrate the subchondral bone.

To sum up, in our rat model of stress-induced TMJ OA, RIP1 was increased significantly in condylar cartilage, accompanied by the elevation of inflammatory, apoptotic and necroptotic factors including TNF-α, IL-1β, Caspase-8 and RIP3. The inhibition of RIP1 showed spatio-temporal protective effects on mandibular cartilage under compressive mechanical force. The RIP1 inhibition could protect chondrocytes by restraining inflammation, apoptosis and later-stage necroptosis. The study demonstrated the therapeutic potential of Lenti-virus targeting RIP1 in stress-induced TMJ OA.

**Conclusion**

Our study suggests that RIP1 plays an essential role in the destruction of mandibular cartilage under 60 g mechanical force. RIP1 inhibition through Lenti-virus could protect mechanical stress-induced mandibular cartilage thinning by inhibiting inflammation, apoptosis and necroptosis spatio-temporal changes.

**Declarations**

**Acknowledgments**

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**Author contributions**

Yiwen Zhou and Shuang Lin contributed to the conception and design of the study, the acquisition of data, analysis and interpretation of data and drafting the article. Ziwei Huang, Caixia Zhang, Huijuan Wang and Baochao Li contributed to acquisition of data and revised the article for important intellectual
content. Huang Li contributed to the conception and design of the study, and revising the article critically for important intellectual content. All authors gave the final approval of the version to be submitted.

**Conflict of interest**

We declare that we have no conflict of interest.

**Competing interests**

The authors declare no competing interests.

**References**


## Figures

### A

#### Objectives

- **Part I:** to confirm the effect of Lenti-siRIP1 (n=24)
- **Part II:** to investigate the protective function of Lenti-siRIP1 (n=48)

#### Groups

- **Control groups**
  - (4&7 days) (n=12)
- **Force groups**
  - (4&7 days) (n=12)

#### Experiments

- Quantitative real-time PCR (n=6)
- Micro-CT scan (n=8)
- Hematoxylin and eosin staining (n=6)
- Immunohistochemical staining (n=6)
- TUNEL staining (n=6)

### B

- Lenti-siRIP1 (Left)
- Lenti-shRNA (Right)

### C

<table>
<thead>
<tr>
<th>The experimental groups are named as follows</th>
</tr>
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<tbody>
<tr>
<td>Control group (None force)</td>
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<tr>
<td>Lenti-shRNA (negative Lentivirus)</td>
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<tr>
<td>Lenti-siRIP1 (positive Lentivirus targeting RIP1)</td>
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</tbody>
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### D

- Day 1: Inject lenti-virus and load mechanical force
- Day 4: Change rubbers every 3 days
- Day 7: Euthanize 4 day group rats
- Day 7: Euthanize 7 day group rats
Figure 1

Detailed description of experimental plan.

(a) Experimental design and schematic diagram of the amount of animal in each experiment. (b) The left and right temporomandibular joints of rats were both injected with Lenti-virus. In the left TMJ, Lenti-siRIP1 was injected. In the right TMJ, Lenti-shRNA was injected. (c) The naming of different groups in this article. (d) The timeline of animal experiments in this article.
Figure 2

RIP1 expression in mandibular cartilage was decreased by Lenti-siRIP1 (one way ANOVA with LSD analysis).

Lenti-virus-constructed short hairpin RNA (Lenti-shRNA) or Lenti-siRIP1 was transfected into mandibular cartilage. Tissues from Con (Lenti-shRNA), siRIP1 (Lenti-siRIP1), F (Force+Lenti-shRNA) and F+siRIP1 (Force+Lenti-siRIP1) groups were collected for analysis of relative RIP1 levels. (a&c) Immunohistochemical analysis of RIP1 in condylar cartilage at 4 d and 7 d (n=6). Scale bar indicates 50 μm. (b) Quantitative real-time PCR analysis of RIP1 in condylar cartilage at 4 d and 7 d (n=6). Data were normalized based on GAPDH and were expressed as mean ± SD. (*P 0.05 vs Con group; **P 0.01 vs Con group; #P 0.05 vs F group; ##P 0.01 vs F group)
Mandibular cartilage destruction under compressive mechanical force was protected by Lenti-siRIP1 (one way ANOVA with LSD analysis).

(a) Haematoxylin and Eosin (H&E) staining of mandibular cartilage from Con, siRIP1, F and F+siRIP1 groups (n=6). The lines represent the measurement of cartilage thickness and figures in the boxes are
amplified to show clear histological change. P indicates proliferative zone, T indicates transition zone, and H indicates hypertrophic zone. Scale bar indicates 50 μm. (b) The measurement of cartilage thickness in each group (n=6). (c) The measurement of chondrocyte number in each group (n=6). Data were expressed as mean ± SD. (*P 0.05 vs Con group; **P 0.01 vs Con group; #P 0.05 vs F group ; ##P 0.01 vs F group)

Figure 4

The death rate of chondrocytes under mechanical force decreases after injecting Lenti-siRIP1 (one way ANOVA with LSD analysis).

(a) Condyle sections from Con, siRIP1, F and F+siRIP1 groups were subjected to TUNEL staining to observe dead cells (n=6). The black arrows indicate representative dead chondrocytes. Scale bar indicates 50 μm. (b) Quantification of positive TUNEL staining chondrocytes in different layers. Proliferation zone, transition zone and hypertrophic zone are the same as described in Figure 2. Data were expressed as mean ± SD. (*P 0.05 vs Con group; **P 0.01 vs Con group; #P 0.05 vs F group ; ##P 0.01 vs F group)
Figure 5

Destruction of subchondral bone was not prevented by Lenti-siRIP1 (one way ANOVA with LSD analysis).

(a) Micro-computed tomography (Micro-CT) reconstructed images of condyles in Con, siRIP1, F and F+siRIP1 groups (n=8). (b-e) Exhibitions of quantitative parameters include percent bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) of subchondral bone (n=8).
trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.sp). Data were expressed as mean ± SD. (**P < 0.01, ns=no significant)

**Immunohistochemistry of TNF-α**

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**Immunohistochemistry of IL-1β**

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**Figure 6**

Inflammation of condylar cartilage under mechanical stress was inhibited by Lenti-siRIP1 (one way ANOVA with LSD analysis).
Condyles from Con, siRIP1, F and F+siRIP1 groups were collected for analysis of relative TNF-α and IL-1β levels. (a&c) Immunohistochemical analysis of TNF-α at 4 d and 7 d (n=6). Fold change diagram represents the expression quantity change and IOD diagram represents the expression distribution in different layers. (b&d) Immunohistochemical analysis of IL-1β at 4 d and 7 d (n=6). Fold change diagram represents the expression quantity change and IOD diagram represents the expression distribution in different layers. (e&f) Relative expression of TNF-α and IL-1β determined by quantitative real-time PCR (n=6). Data were expressed as mean ± SD. (*P 0.05 vs Con group; **P 0.01 vs Con group; #P 0.05 vs F group ; ##P 0.01 vs F group; ns=no significant)

Figure 7

Apoptosis and necroptosis of condylar chondrocytes under mechanical stress were inhibited by Lenti-siRIP1 (one way ANOVA with LSD analysis).

Condyles from Con, siRIP1, F and F+siRIP1 groups were collected for analysis of relative RIP3 and Caspase-8 levels. (a&c) Immunohistochemical analysis of RIP3 at 4 d and 7 d (n=6). Fold change diagram represents the expression quantity change and IOD diagram represents the expression distribution in different layers. (b&d) Immunohistochemical analysis of Caspase-8 at 4 d and 7 d (n=6). Fold change diagram represents the expression quantity change and IOD diagram represents the expression distribution in different layers. (e&f) Relative expression of RIP3 and Caspase-8 determined by quantitative real-time PCR (n=6). Data were expressed as mean ± SD. (*P 0.05 vs Con group; **P 0.01 vs Con group; #P 0.05 vs F group ; ##P 0.01 vs F group; ns=no significant)
Table 1. The spatio-temporal changes in mechanical stress-induced TMJ OA

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As for the comparison:
F+shRNA group was compared with Con+shRNA group
F+siRIP1 group was compared with F+shRNA group

Figure 8

The spatio-temporal changes in mechanical stress-induced TMJ OA.

Summary of rising and decreasing trends of inflammatory, apoptotic, necroptotic and cartilage thickness in different layers and different time. Different colours represent the level of gene expression. Orange represents the rising trend and grey represents the decreasing trend. The darker the colour, the more gene expression increased or decreased.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 2022.11.28.pdf