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TITLE:

Osteoarthritis Pain Model Induced by Intra-Articular Injection of Mono-Iodoacetate in Rats

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SUMMARY:

This study describes the method of intra-articular injection of mono-iodoacetate in rats and discusses the resulting pain-related behaviors and histopathological changes, which provide references for future applications.

ABSTRACT:

The current animal models of osteoarthritis (OA) can be divided into spontaneous models and induced models, both of which aim to simulate the pathophysiological changes of human OA. However, as the main symptom in the late stage of OA, pain affects the patients' daily life, and there are not many available models. The mono-iodoacetate (MIA)-induced model is the most widely used OA pain model, mainly used in rodents. MIA is an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, which causes chondrocyte death, cartilage degeneration, osteophyte, and measurable changes in animal behavior. Besides, expression changes of matrix metalloproteinase (MMP) and pro-inflammatory cytokines (IL1 β and TNF α) can be detected in the MIA-induced model. Those changes are consistent with OA pathophysiological conditions in humans, indicating that MIA can induce a measurable and successful OA pain model. This study aims to describe the methodology of intra-articular injection of MIA in rats and discuss the resulting pain-related behaviors and histopathological changes.

INTRODUCTION:

Osteoarthritis (OA) is the most common joint disease in the world, affecting an estimated 10-12% populations in adults[1]. The most generally involved joint is the knee, and OA has a higher

incidence in older adults, especially women[2]. As a chronic disease, OA develops progressively over decades into joint failure with symptoms such as cartilage loss, synovial inflammation, osteophytosis, decreased function, and chronic pain[3]. According to the World Health Organization (WHO), OA is the fourth most prevalent disease in females and the eighth most prevalent disease in males. By 2020, OA may become the fourth most disabling disease in humans[4]. However, currently available therapies of OA address only symptoms and extend the time until joint replacement surgery[5].

The spontaneous OA in human patients often takes a long time to produce clinical symptoms such as joint related pain[6]. In the early stages of OA, pain is usually intermittent and becomes more frequent and severe as the disease progresses, making it the predominant complaint of patients[7]. Therefore, extensive animal models for OA pain have been developed over the past half century to promote pain relief therapy. OA models have classically been divided into spontaneous and induced models. Spontaneous models include naturally occurring models and genetically modified models, which can more closely simulate the course of primary OA in humans[8]. Induced models can generally be divided into two categories: 1) post-traumatic OA induced by surgery or other trauma; or 2) intra-articular injection of chondrotoxic or pro-inflammatory substances[3]. These models lay a foundation for the pathophysiological study of OA and contribute greatly to the development of drugs to reduce pain and increase function.

Recently, the most widely used inducer for OA modeling is mono-iodoacetate (MIA). MIA, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, can cause changes in cartilage matrix, degradation, loss of cartilage, synovitis and other changes, which are similar to the pathological changes of human osteoarthritis[9]. It has been noted that intra-articular injection of MIA induced ongoing pain at 28 days after MIA administration, indicating that the MIA model may be useful for investigating chronic nociceptive pain[10-12]. In this study, male Sprague-Dawley rats received intra-articular injections with 0.5, 1.5, or 3 mg of MIA in the knee joints. The severity of MIA-induced joint pain was measured by assessment of mechanical and thermal sensitivity at 1, 7, 14, 21, 28 and 35 days after injections. On this basis, 1.5 mg of MIA was selected as the final concentration to evaluate gait patterns and histological changes at 28 days after injections.

PROTOCOL:

Procedures involving animal subjects have been approved by the Medical Norms and Ethics Committee of Zhejiang Chinese Medical University and are in accordance with the China legislation on the use and care of laboratory animals.

1. Intra-articular injection of mono-iodoacetate in the knee

1.1. After one-week adaptive breeding, randomly and equally divide 40 male Sprague-Dawley rats weighing 180−200 g (4−5 weeks old) into four groups (n = 10 rats/group).

NOTE: Rats in the control group will be intra-articularly injected with 50 µL of saline, while rats in the experimental groups will be treated with 0.5, 1.5 or 3 mg of MIA dissolved in 50 µL of saline, respectively.

1.2. On the day of injection, freshly prepare the solution of MIA in sterile saline (0.9% NaCl) at 15, 30 and 60 mg/mL concentration and 10% pentobarbital solution in sterile saline (0.9% NaCl).

CAUTION: MIA has an extremely destructive effect on the mucous membrane, the upper respiratory tract, the eye and the skin and other tissue. Therefore, mask and gloves are recommended when preparing a solution.

1.3. Anesthetize rats by intraperitoneal injection of 10% pentobarbital at 4 mL/kg. Gently clamp the toes of the rat with a tweezer to confirm anesthesia.

1.4. Place the rat with its back facing down. Shave the knee and wipe the area surrounding the knee joint with alcohol.

1.5. Keep the knee at a 90° angle and reveal the white patellar tendon below the patella. Press the patellar tendon with the fingertip to find the gap beneath the patella.

1.6. Choose the junction of the gap and the lateral patellar tendon as the injection site. Then lift a 26 G needle and insert vertically into the site about 5 mm. No resistance should be felt when the needle is in the articular space.

NOTE: It is important to keep the needle perpendicular to the injection site.

1.7. Inject 50 µL of saline or MIA solution into the joint cavity. Slowly pull out the needle and wrap a piece of gauze around the injection site to minimize reflux and leakage.

1.8. Test pain-related behavior at 1, 7, 14, 21, 28 and 35 days after injections as described in section 2.

2. Behavioral assessments

2.1. Mechanical withdrawal threshold (MWT)

NOTE: The MWT was measured by the von Frey test[13] and the observer was blinded to the injections that the animals had received.

2.1.1. Place a rat in an elevated plastic cage (17 cm x 11 cm x 13 cm) with a wire mesh base suspended 50 cm above a table. Ensure the testing environment is quiet and give the rat 30 min to adapt to the environment.

2.1.2. Press the von Frey needle perpendicularly on the plantar surface of each rat's hind paw. Increase the pressure gradually (approximately 20 g/s) and linearly until paw lifting or paw licking occurs.

2.1.3. Use a force lower than the previous threshold to ensure that the threshold is the minimum withdrawal force. Test each rat more than three times, at least 3–5 min apart.

2.1.4. Record the minimal force eliciting a paw withdrawal reflex. Average the data as the MWT of rats.

2.2. Thermal withdrawal latency (TWL)

NOTE: The TWL was measured using the plantar test apparatus (**Table of Materials**) and the observer was blinded to the injections that the animals had received.

2.2.1. Place a plexiglass box (60 cm x 20 cm x 14 cm, divided into 6 compartments) on a 3 mm thick glass plate, and put rats into the box (one in each compartment). Ensure the environment is quiet and the room temperature is constant.

2.2.2. Give rats 30 min to adapt to the testing environment.

2.2.3. Calibrate the thermal stimulus via the infrared radiometer. Set the desired infrared intensity at 70 units.

2.2.4. Place the infrared emitter/detector on the container directly under the center of the paw being tested.

2.2.5. Pressing the **START** button. The timer will start automatically. The controller will automatically turn off the infrared light and stop the timer altogether as soon as movement of the paw occurs.

2.2.6. Record the reaction time when the appearance of paw withdrawal and paw licking.

NOTE: A positive response for the test is regarded as paw withdrawal and paw licking. If only paw withdrawal occurs, it should be regarded as a voluntary movement of the rat rather than a positive response.

2.2.7. Repeat the test more than three times. Average the data as the TWL of rats.

NOTE: Each exposure of radiant heat should not exceed 20 s. Infrared stimulations on the same hind paw should be at least 3–5 min apart.

2.3. Gait pattern analyses

NOTE: The gait pattern analyses were measured using an imaging system (**Table of Materials**) and the observer was blinded to the injections that the animals had received.

2.3.1. Adjust the length of the walking compartment by using the knobs at both ends of the walking compartment to suitable length for rats (e.g., 61 cm).

2.3.2. Place a rat into the walking compartment and train rats to make uninterrupted runs for at least 5 step-cycles at a speed of at 18 cm/s before the formal experiment.

NOTE: When a rat is first placed in the walking compartment, the speed can be set to about 20 cm/s and shut off when running around 2 s. Then set the speed to 18 cm/s. Gently tap the back of the rat with the partition, if the rat pauses or retreats during walking. Try to reduce the speed, if the rat eventually fails to perform the test.

2.3.3. Slowly increase the speed of the treadmill until it reaches the target speed (18 cm/s). Capture at least 5 s video of continuous movement of the rat with the high-speed digital video camera mounted below the transparent treadmill belt.

2.3.4. Test each rat at least 5 min apart to obtain at least three uninterrupted runs.

2.3.5. Draw a “bounding box” to define the boundary of animal walking image in the imaging software and enter the run speed for each video. Select videos as a group and process videos automatically by the software. After processing videos, the software will output several spreadsheets to report gait indices, including stance, swing, braking, propulsion, cadence, step sequence, etc.

2.3.6. Calculate total paw area (cm^2), average stride length (cm), and unite stride length.

NOTE: Total paw area is the mean of the total area of four paws of each group of rats and paw area is defined as the maximal paw area in contact with the treadmill during the stance phase of the step cycle. Stride length is the distance between initial contacts of the same paw in one complete stride. Unit stride length = average stride length (cm)/body length (cm). The protocol can be paused here.

3. Histopathological and immunohistochemical analyses

3.1. Anesthetize rats by intraperitoneal injection of 10% chloral hydrate at 4 mL/kg and sacrifice all rats by taking blood from the heart. Resect their knee joints immediately for the histologic analyses.

3.2. Fix the joints in 10% formalin for 24 h, decalcify with 10% EDTA in phosphate buffered saline (PBS) for 8 weeks, and then embed joints in paraffin.

CAUTION: Formalin can cause eye, skin, and respiratory tract irritation. It should be handled in a hood.

3.3. Section the paraffin embedded joints at 3 mm thickness with a microtome and float in a 40 °C water bath containing distilled water.

3.4. Transfer sections onto glass slides. Dry slides overnight and store slides at room temperature (RT) to continue the following staining.

3.5. Place slides in a 60 °C oven for 4 h to deparaffinize.

3.6. Immerse the slides successively in xylene, xylene, 100% ethanol, 100% ethanol, 95% ethanol, 80% ethanol and 75% ethanol for 5 min, respectively, at RT.

3.7. Stain sections with hematoxylin and eosin (H&E), safranin-O (SO) and Alcian blue hematoxylin (ABH) as well as antibodies against rat type II collagen (Col2), type X collagen (Col10) and matrix metalloproteinase 13 (MMP13), as described in steps 3.8−3.12.

3.8. H&E staining

3.8.1. Rinse slides with deionized H_2O for 3 min. Stain slides with hematoxylin for 3−5 min.

3.8.2. Rinse slides with deionized H_2O 3x, 1 min each, until no hematoxylin remains on the surface.

3.8.3. Stain slides with eosin for 30 s. Then, rinse slides with deionized H_2O 3x, 1 min each, until no eosin remains on the surface.

3.8.4. Immerse slides in 0.1% ammonia for 10−20 s. Then, rinse slides with deionized H_2O 3x, 1 min each.

3.8.5. Immerse slides successively in 95% ethanol, 100% ethanol, xylene, xylene and xylene for 1 min, respectively.

3.8.6. Coverslip the slides by neutral resin.

3.9. SO staining

3.9.1. Rinse slides with deionized H_2O for 3 min. Stain slides with hematoxylin for 3−5 min.

3.9.2. Differentiate quickly in 1% acid alcohol (about 3 s). Then, rinse slides with deionized H_2O 3x, 1 min each, until no hematoxylin remains on the surface.

3.9.3. Stain slides with Fast Green (FCF) solution for 5 min. Then, rinse slides with deionized H_2O 3x, 1 min each.

3.9.4. Stain slides with SO for 1−2 min. Then, rinse slides with deionized H_2O 3x, 1 min each.

3.9.5. Rinse slides with 1% acetic acid for 1−2 min to remove the residual FCF. Rinse slides with deionized H_2O for 1 min.

3.9.6. Immerse slides successively in 95% ethanol, 100% ethanol, xylene, xylene and xylene for 1 min, respectively.

3.9.7. Coverslip the slides by neutral resin.

3.10. ABH staining

3.10.1. Rinse slides with deionized H_2O for 3 min. Immerse slides in 1% acid alcohol for 30 s and drain briefly on a paper towel (do not rinse).

3.10.2. Immerse slides in ABH for 1 h. Then, rinse slides with deionized H_2O 3x, 1 min each, until no ABH remains on the surface.

3.10.3. Differentiate quickly in 1% acid alcohol (about 3 s). Rinse slides with deionized H_2O 3x, 1 min each.

3.10.4. Immerse slides in 0.5% ammonium water for 15 s. Then, rinse slides with deionized H_2O 3x, 1 min each.

3.10.5. Immerse slides in 95% ethanol for 1 min. Immerse slides in eosin/orange G solution for 1.5 min.

3.10.6. Immerse slides successively in 95% ethanol, 100% ethanol, xylene, xylene and xylene for 1 min, respectively.

3.10.7. Coverslip the slides by neutral resin.

3.11. Examine all slides under a microscope and statistically grade on a scale of 0−13 by double-blind observation, according to Mankin's scoring system[14].

3.12. Immunohistochemistry

3.12.1. Rinse slides with deionized H_2O for 3 min.

3.12.2. Immerse slides in 0.1 M sodium citrate and place slides in a 60 °C oven for 4 h to retrieve the antigen.

3.12.3. Immerse slides in 0.3% Triton X-100 in PBS for 10 min. Then, rinse slides with PBS 2x, 3 min each.

3.12.4. Incubate sections in 3% H_2O_2 solution in methanol at RT for 10 min to block endogenous peroxidase activity. Rinse slides with PBS 2x, 3 min each.

3.12.5. Incubate sections with 5% goat serum in PBS for 30 min at RT to block any non-specific binding. Rinse slides with PBS 2x, 3 min each.

3.12.6. Incubate sections overnight at 4 °C with 100 µL of PBS-diluted (1:1,000) primary antibodies against rat type II collagen (Col2), type X collagen (Col10) and matrix metalloproteinase 13 (MMP13). Rinse slides with PBS 2x, 3 min each.

3.12.7. Incubate sections with 100 µL of PBS-diluted (1:1,000) secondary antibody (goat anti-mouse secondary or goat anti-mouse secondary) for 20 min at RT. Rinse slides with PBS 2x, 3 min each.

3.12.8. Incubate sections with 100 µL of 3,3′-diaminobenzidine (DAB) working solution. Monitor the reaction as the chromogenic reaction turns the epitope sites brown.

CAUTION: DAB is a carcinogen. Always wear gloves and work in a hood when working with DAB.

NOTE: The time of color development may vary from a few seconds to 10 min.

3.12.9. As soon as a brown color develops on the sections, rinse slides with deionized H_2O 2x, 3 min each.

3.12.10. Immerse slides in hematoxylin for 1−2 min to counterstain slides. Then, rinse slides with deionized H_2O 3x, 1 min each.

3.12.11. Immerse slides successively in 95% ethanol, 100% ethanol, xylene, xylene and xylene for 1 min, respectively.

3.12.12. Coverslip the slides by neutral resin.

3.12.13. Observe the color of the antibody staining in the sections under a microscope.

REPRESENTATIVE RESULTS:

With this methodology, we established an OA pain model in the rat and detected the resulting changes. MWT and TWL reflected mechanical allodynia and thermal hyperalgesia, respectively. As shown in **Figure 1,** MIA induced mechanical allodynia and thermal hyperalgesia present in a dose-dependent manner. Remarkably, the decrease of MWT reached a peak from 21 days to 28 days, and then rebounded, suggesting that joint repair may occur at this stage, but MWT of 3 mg

MIA group was still at a low level. The change of TWL was roughly consistent with MWT (**Figure 2**).

On this basis, we selected 1.5 mg of MIA as the final dose and assessed gait patterns and histological changes at 28 days after injection. Gait parameters (total paw area and unit stride length) reflected pain related behaviors. Levels of gait parameters including the total paw area (**Figure 3A**) and unit stride length (**Figure 3B**) were significantly reduced in the MIA group after 28 days, suggesting that MIA induced osteoarthritis-related joint pain in rats. With increased Mankin's score on the histopathological slides, degeneration of cartilage, disruption of collagen, and disorganization of matrix were obviously seen in the MIA group (**Figure 4**). As illustrated in **Figure 5**, 1.5 mg of MIA caused a significant upregulation of MMP13, and Col10, and significant downregulation of Col2.

FIGURE LEGENDS:

Figure 1: Development of MTW after MIA injection. Mechanical withdrawal thresholds of hind paws were assessed after injection of MIA (0.5, 1.5, or 3 mg/rat) and saline (0.9% NaCl), n = 10 rats/group. Values are presented as mean ± SD. **P < 0.01 vs. saline-treated group; One-way ANOVA followed by Fisher's least significant difference (LSD) comparison.

Figure 2: Development of TWL after MIA injection. Thermal withdrawal latency of hind paws was assessed after injection of MIA (0.5, 1.5, or 3 mg/rat) and saline (0.9% NaCl), n = 10 rats/group. Values are presented as mean ± SD. **P < 0.01 vs. saline-treated group (NC); One-way ANOVA followed by Fisher's LSD comparison.

Figure 3: Gait analysis at 28 days after MIA injection. (**A**) Total paw area (cm^2). Total paw area: the mean of total area of four paws of each group of rats. (**B**) Unit stride length. Unit stride length = Average stride length (cm)/body length (cm). n = 10 rats/group. Values are presented as mean ± SD. $^{\#\#}P$ < 0.01 vs. saline-treated group (NC) on day 28. This figure has been modified from Yan et al.[15].

Figure 4: Histopathological observation (HE, SO, and AHB staining) and Mankin' s scoring of rat knee joints on day 28 after MIA treatment. n = 10 rats/group. Scale bar = 40 μm. Values are presented as mean ± SD. ##P < 0.01 vs. saline-treated group (NC). One-way ANOVA followed by Fisher's LSD comparison. This figure has been modified from Yan et al.[16].

Figure 5: Immunohistochemical observation of the expressions of MMP13, Col2, and Col10 in rat cartilage on day 28. Scale bar = 50 μm. N = 10 rats/group. This figure has been modified from Yan et al.[16].

DISCUSSION:

The rat model of OA induced by MIA is a well-established, widely used model. Intra-articular injection of MIA initially causes severe and acute inflammation, which gives rise to the longer and degenerative phase of OA[17,18]. In this research, we measured nociceptive sensitivity by MWT and TWL, and assessed gait alterations with an imaging system. Previous reports found that the

injection of MIA could raise the sensitivity of afferent knee joint fibers leading to nociception, which is reflected by thermal hyperalgesia and reduced mechanical threshold[19,20]. It has been proved that gait alterations were related to enhanced nociception, suggesting that gait patterns could be used to evaluate pain models[21]. Accordingly, MIA-induced models are mainly used to assess OA-related pain and screen oral drugs as well as joint injections drugs[3,6].

Although compared with the surgically induced OA model, it is simpler and faster to inject MIA into the joint cavity, there are still critical points in the modeling. First of all, the articular cavity of rats is tiny, and its location should be confirmed before injection. Secondly, MIA is toxic, thus the dose of MIA should be carefully selected. It has been reported that MIA could induce articular cartilage damage in a dose- and time-dependent manner (assessed by the OARSI histological score and the Mankin score), indicating that the progression and severity of articular lesions can be modulated by regulating the concentration of MIA[22,23]. MIA was found to induce pain and oxidative stress markers at high doses[10,18,24]. Previous reports suggested that a 1.5 mg dose of MIA injection in rats produced an inflammatory process that is similar to human knee OA[18,25]. Besides, it is important to use the same experimenter throughout the behavioral test and to familiarize the rats with the environment in advance, to reduce anxiety and avoid affecting the experimental results.

As mentioned above, OA animal models are usually divided into spontaneous and induced models. Intra-articular injection of MIA is widely used due to several advantages: 1) simple operation; 2) ease of induction and reproducibility; 3) controllable dose and severity; 4) short modeling time; and 5) suitability for small animals as well as large animals. However, like other animal models, MIA-induced OA models also have several drawbacks. Extensive cell death and rapid joint destruction after MIA injection are inconsistent with spontaneous or post-traumatic OA in humans[26]. Moreover, residual MIA in the articular cavity may affect the effects of subsequent intra-articular therapies, resulting in an uncertain outcome by using the MIA model. Whether or not to wash the articular cavity before therapeutic injection in this model remains an unanswered question. Overall there is no single animal model that perfectly recapitulates all aspects of human OA, but the wide variety of models available makes it possible to apply multiple models to most relevant questions synthetically.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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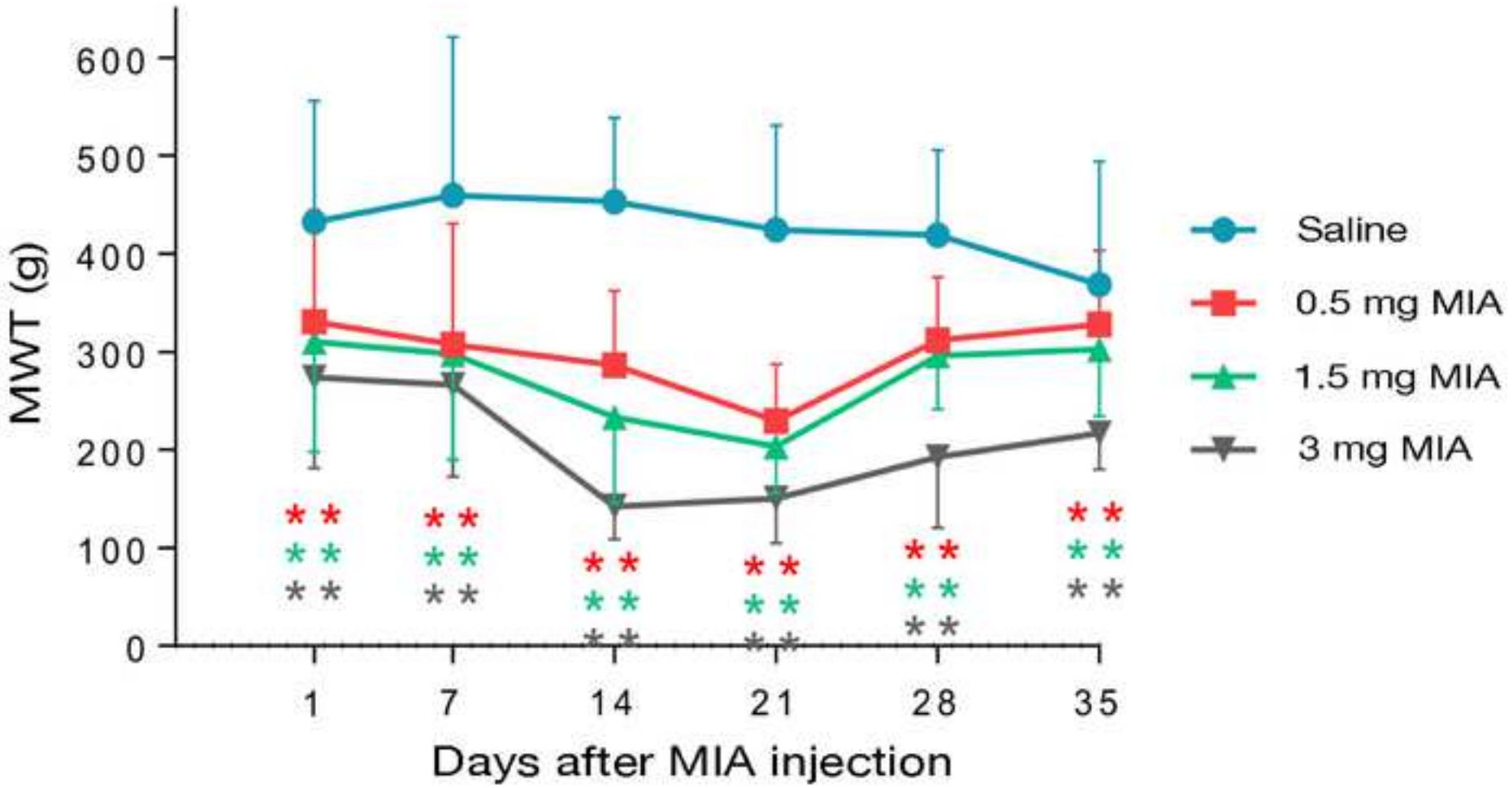

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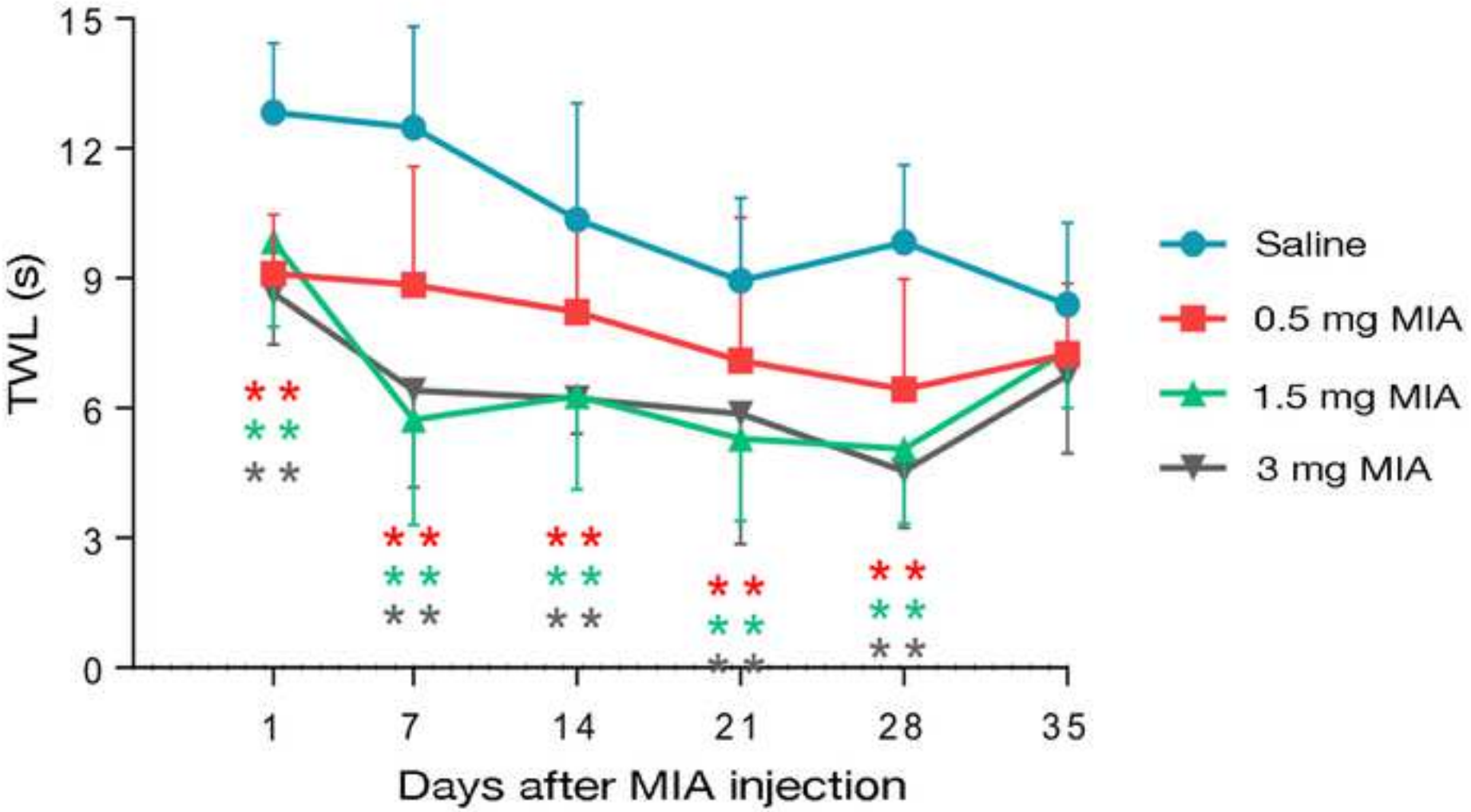

Figure

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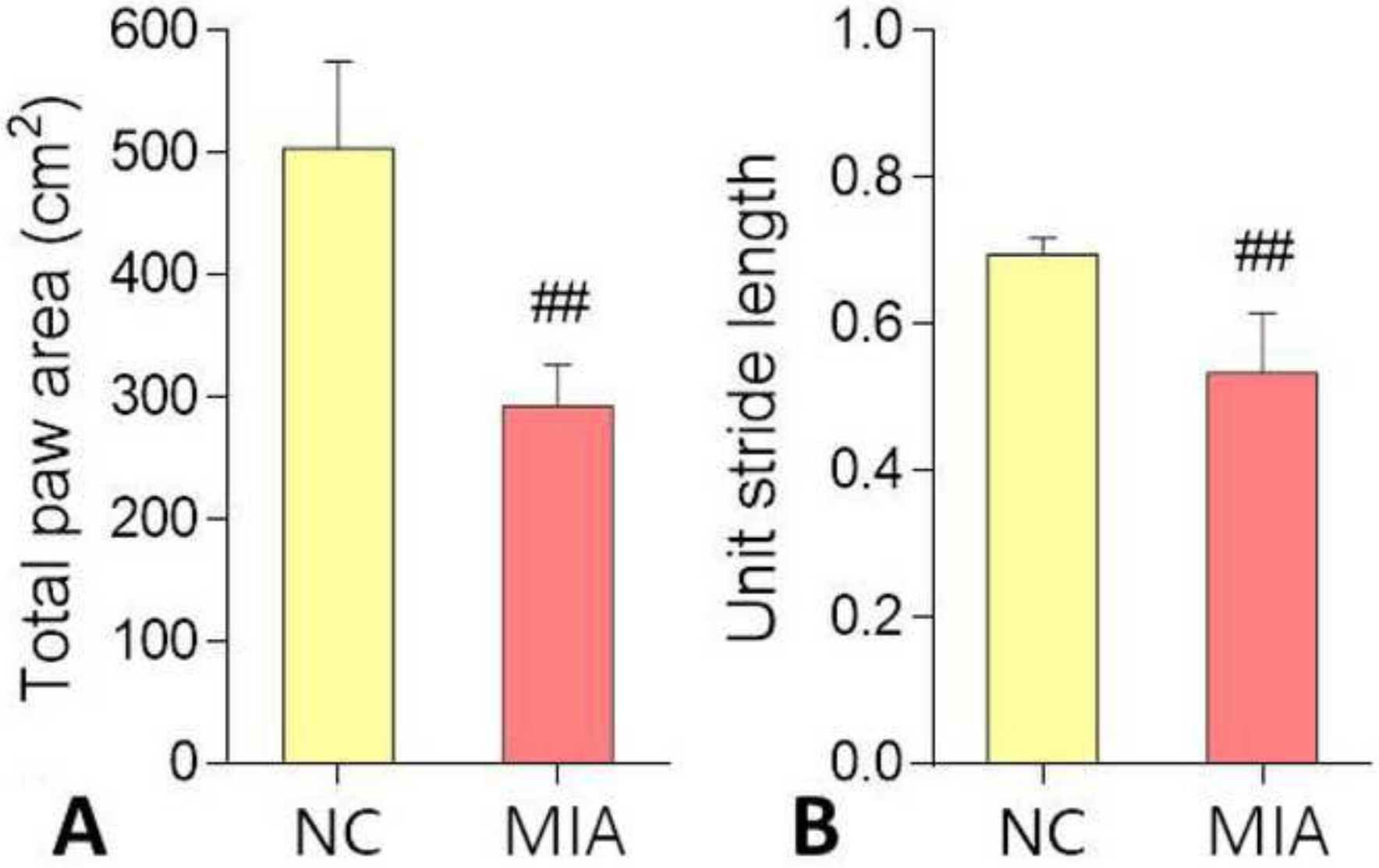
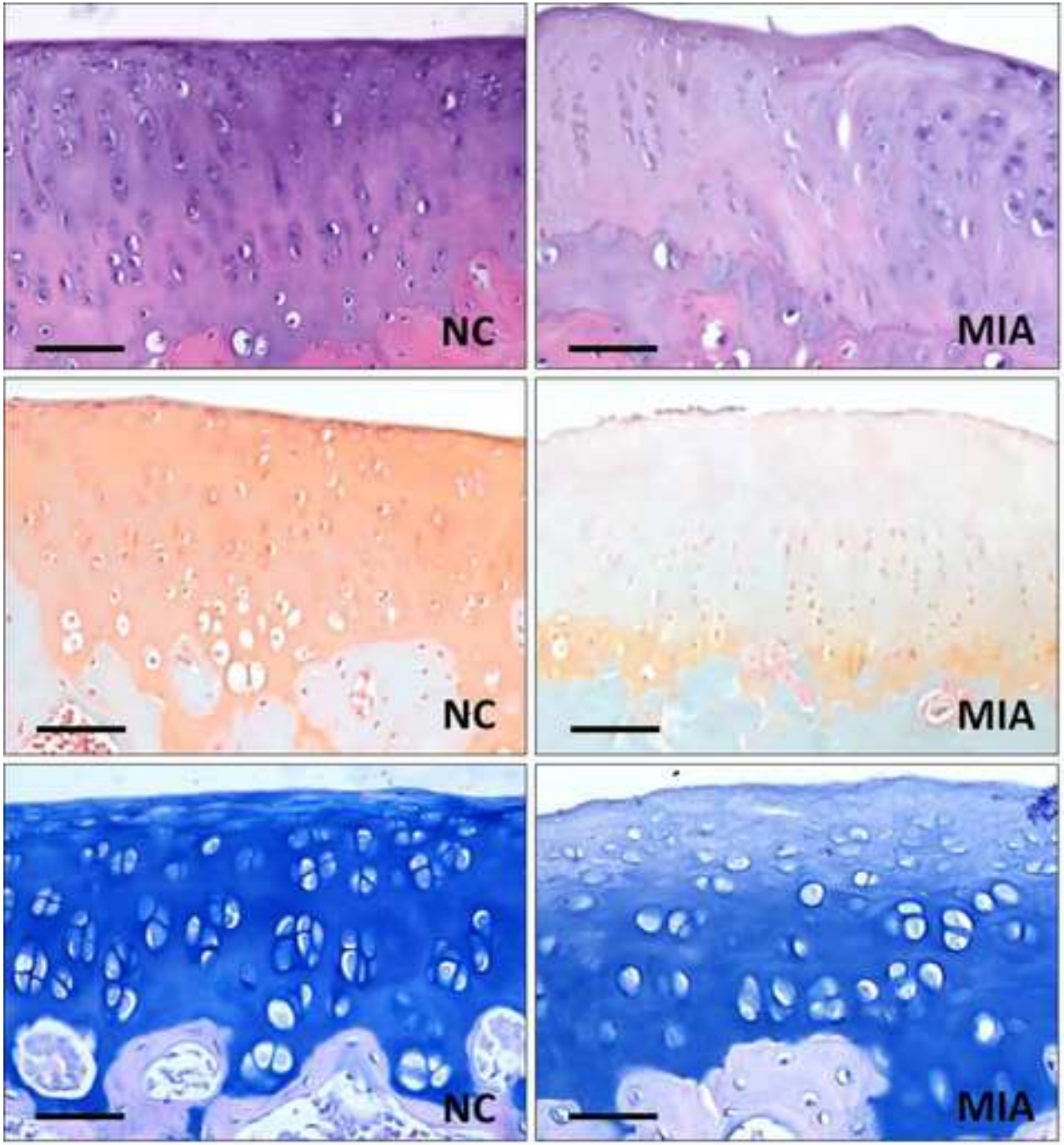
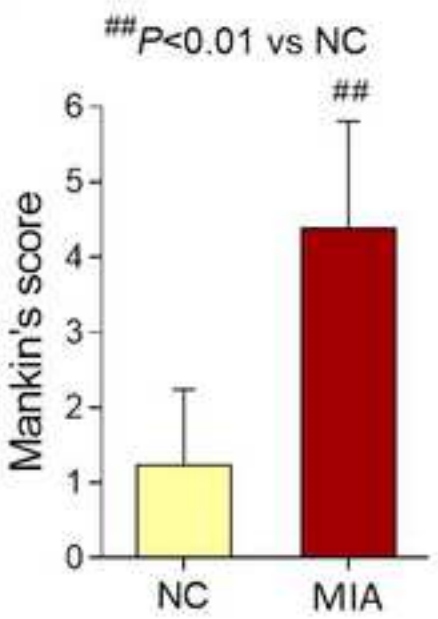


Figure



Scale bar = 40 μm



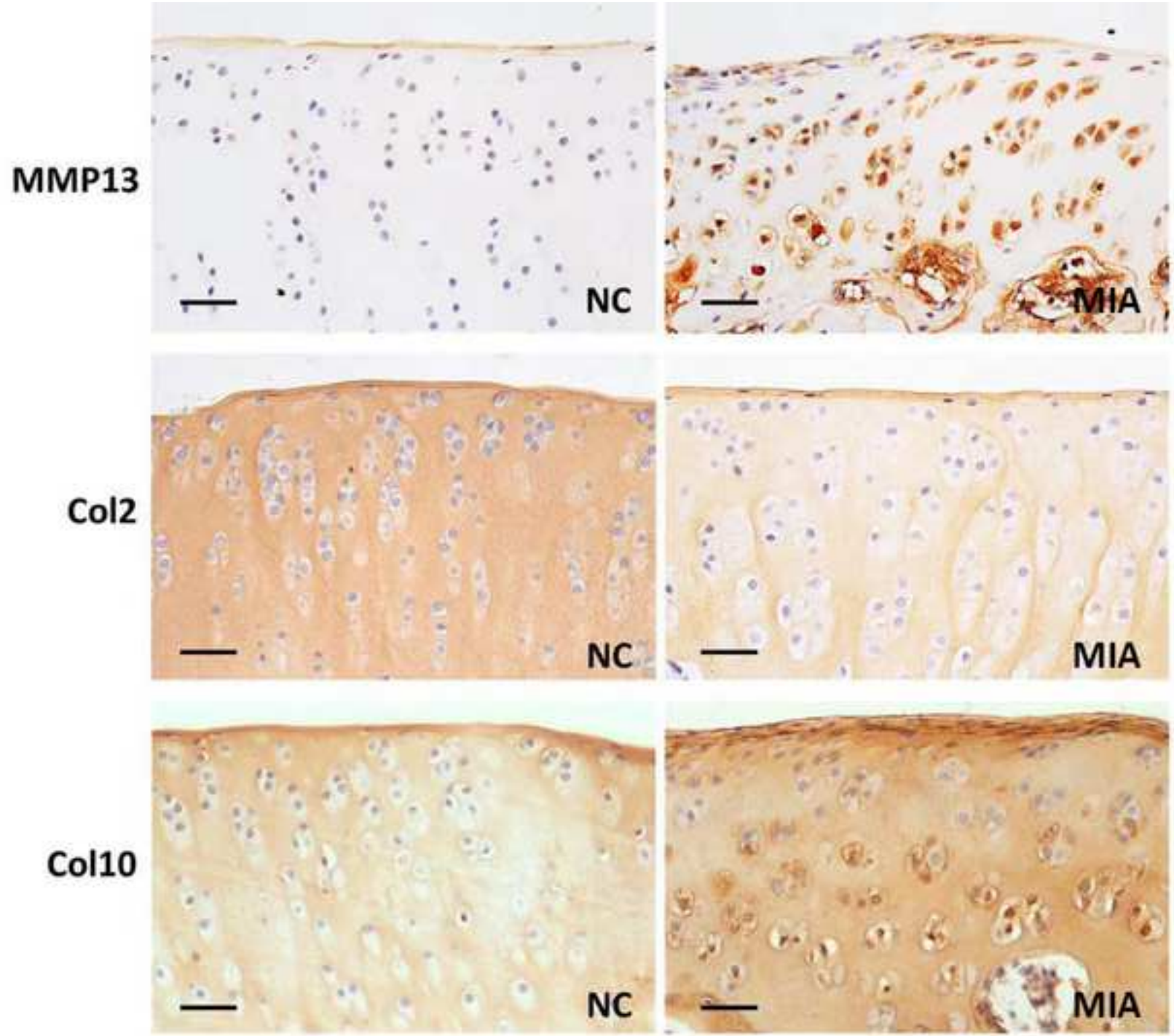
Upper: HE staining

Middle: SO staining

Lower: ABH staining

Figure

Click here to access/download;Figure;Figure 5.jpg



Name of Material/Equipment	Company	Catalog Number	Comments/Description
Anti-Collagen II antibody	Abcam(UK)	34712	Primary antibody for immunohistochemistry (IHC)
Anti-Collagen X (Col10) antibody	Abcam(UK)	49945	Primary antibody for IHC
DigiGait Imaging System	Mouse Specifics (Boston, MA, USA)		Equipment for gait patterns analyses
Eosin	Sigma-Aldrich	861006	The dye for HE staining
Fast Green FCF	Sigma-Aldrich	F7252	The dye for SO staining
Goat anti-mouse antibody	ZSGQ-BIO (Beijing, China)	PV-9002	Secondary antibody for IHC
Goat anti-rabbit antibody	ZSGQ-BIO (Beijing, China)	PV-9001	Secondary antibody for IHC
Hematoxylin	Sigma-Aldrich	H3163	The dye for HE staining
MIA	Sigma-Aldrich	I4386-10G	powder
MMP13	Cell Signaling Technology, Inc. (Danvers, MA, USA)	69926	Primary antibody for IHC
Modular tissue embedding center	Thermo Fisher Scientific (USA)	EC 350	Produce paraffin blocks.
Plantar Test apparatus	UgoBasile (Italy)	37370	Equipment for TWL assay
PrimeScript RT reagent Kit (Perfect Real Time)	TaKaRa Biotechnology Co. Ltd. (Dalian, China)	RR037A	Extracte total RNA from cultured cells
Rotary and Sliding Microtomes	Thermo Fisher Scientific (USA)	HM325	Precise paraffin sections.
Safranin-O	Sigma-Aldrich	S2255	The dye for SO staining
Tissue-Tek VIP 5 Jr	Sakura (Japan)		Vacuum Infiltration Processor

Dear Reviewers,

We wish to thank you for the time and effort you have spent reviewing our paper. We are pleased to note that you have found our research work interesting and also pointed out some problems to help us improve the quality of our work.

We thank the reviewers for acknowledging the strong performance of this work. Motivated by your comments, we have tried to fix the problems you mentioned. And we address the comments as follows.

Editorial comments:

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: According to the comments from you and the reviewers, we polished the ma nuscript to avoid any grammatical error, conscientiously.

Comment 2: 2.3.2: How to train rats to make uninterrupted runs?

Response: When a rat is first placed in the walking compartment, the speed can be set to about 20 cm/s and shut off when running around 2 sec. Then set the speed to 18cm/s. Gently tap the back of the rat with the partition, if the rat pauses or retreats during walking. Try to reduce the speed, if the rat eventually fails to perform the test. And we have added a “Note” in section 2.3.2.

Comment 3: Please define error bars in the figure legend.

Response: We have defined error bars in the figure legend.

Comment 4: References: Please do not abbreviate journal titles; use full journal name.

Response: Thanks for your careful work. We have carefully rechecked and replaced the abbreviate journal titles with full journal name.

Great thanks to you again for the time and effort you expend on this paper.

We look forward to hearing from you regarding our submission. We would be glad to resp ond to any further questions and comments that you may have.

Thank you and best regards.

Yours sincerely,

Corresponding author:

Letian Shan (letian.shan@zcmu.edu.cn)