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New role of calcium-binding fluorescent dye alizarin complexone in detecting permeability from articular cartilage to subchondral bone

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Abstract

Osteoarthritis (OA) is a chronic degenerative joint disorder characterized by the progressive deterioration of articular cartilage and concomitant alterations in subchondral bone architecture. However, the precise mechanisms underlying the initiation and progression of OA remains poorly understood. In the present study, we explored whether the calcification in the articular cartilage occurred in the early stage of mouse OA model, generated by the surgery destabilization of the medial meniscus (DMM), via the intra-articular injection of alizarin complexone due to its anionic nature for binding calcium-containing crystals. Although we did not observe the calcification in the articular cartilage of early stage of DMM mice, we unexpectedly identified alizarin complexone had the diffusion capacity for detecting the permeability from the articular cartilage to subchondral bone. Our data showed that the diffusion of alizarin complexone from the articular cartilage to calcified cartilage was greater in the early stage of DMM mice than that in sham controls. Additionally, we observed enhanced penetration of alizarin complexone through the periosteum in DMM mice compared to sham mice. In summary, we developed a novel imaging method that offers a valuable tool for further exploration of biochemical communication underlying OA development. Our findings provided new evidence that increased molecular interactions between the articular cartilage and subchondral bone is involved in the pathogenesis of OA progression.

KEYWORDS

alizarin complexone, articular cartilage, osteoarthritis, permeability, subchondral bone, tidemark

1 | INTRODUCTION

Osteoarthritis (OA) is one of the leading causes of disability and has been established as an organ disease that affects the whole joint, progressive deterioration of articular cartilage and concurrent alterations in subchondral bone architecture, culminating in impaired joint function.[1–3] In the structure of diarthrodial joints, articular

cartilage, calcified cartilage zone, and subchondral bone are integrated into the osteochondral unit.[4,5] However, their microarchitectures, components, and functions vary. Articular cartilage overlies the calcified cartilage, which exists between the tidemark and cement line. Tidemark is a histological landmark between the noncalcified hyaline cartilage and calcified cartilage, acting as a barrier that interrupts the transfer of interstitial fluid between the articular cartilage and calcified cartilage.[6–8] While articular cartilage provides a cushion for the joints to move smoothly without friction, the subchondral bone offers a mechanical base for articular cartilage and exerts a shock-absorbing function. Although this biomechanical coupling between the articular cartilage and subchondral bone has been well-established, the precise role of direct biochemical communication between these two compartments in the healthy and diseased states remains poorly understood.

Chondrocytes are capable of responding to structural changes in the surrounding extracellular matrix by maintaining a dynamic equilibrium between the production of the extracellular matrix and its enzymatic degradation. However, the articular cartilage has a poor self-healing capacity once damaged due to its avascular and aneural characteristics.[9] Under pathological conditions, such as cartilage injuries and OA, the normal articular chondrocytes enter hypertrophic differentiation, resembling the process of endochondral bone formation, which can cause the upper cartilage to become calcified.[10–12] In contrast to physiological calcification, which describes the deposition of calcium minerals for bone formation, the pathological calcification of articular cartilage is a hallmark of OA.[13,14]

In humans, early stages of OA are rarely symptomatic, making the study of early OA pathogenesis difficult. Calcification of articular structures can be detected radiologically.[15,16] However, radiographically detectable changes in calcification of articular cartilage may be delayed by several months to over a year. Consequently, there is a demand for assessments that respond more swiftly to alterations in cartilage physiology. The destabilization of the medial meniscus (DMM) is one of the most widely used OA models.[17,18] The mouse DMM model develops from mild-to-moderate OA at 4 weeks post-surgery, progressing to moderate-to-severe OA at 8-12 weeks.[17,18] Alizarin complexone is one of the common bone labeling fluorescent dyes and has been employed for detecting active bone formation in animal models due to its anionic nature for binding calcium-containing crystals.[19,20] In the present study, we first assessed whether the calcification occurs in the articular cartilage in the early stage of DMM mouse model via the intra-articular injection of alizarin complexone. Although we failed to find the calcification of articular cartilage in the early stage of DMM mice, we unexpectedly identified a novel role of alizarin complexone, which can penetrate the articular cartilage to form a clear fluorescence signal of the undulating line of tidemark that separates the calcified layer from the noncalcified articular cartilage. It was reported that the calcified cartilage is a penetrable structure, and biochemical interactions exist between the articular cartilage and subchondral bone during OA progression.[6,7] Therefore, we compared the permeability between the articular cartilage and subchondral bone in the early stage of DMM and sham mice.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

All methods including animal care, surgery, intra-articular injection, anesthesia, and euthanasia were performed in accordance with the ARRIVE guidelines and National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 80-23, revised 1996). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University (Protocol # 01830). The intact male C57BL/6J mice aged 11–12 weeks (weighing approximately 25 g) were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in an AAALAC-accredited vivarium (Thomas Jefferson University) and used for this study. All mice were fed with standard laboratory diets (LabDiet 5001 Rodent Diet, Purina, St. Louis, MO, USA). Food and water were provided ad libitum.

2.2 | Generation of the destabilization of the medial meniscus model

The medial meniscotibial ligament (MMTL) anchors the medial meniscus to the tibial plateau. Mouse DMM model of OA was induced by transection of MMTL in the right knee.[17,18] The sham-operated control underwent the same surgery except the meniscotibial ligament was not transected. Mice were anesthetized through a nosecone using a mixture of 2.5% isoflurane with 100% oxygen in an induction chamber of an isoflurane vaporizer system. The mouse was placed in the dorsal recumbent position and electrical hair clippers were used to remove hair from the surgical site. Surgical procedures were performed in a designated surgery room within the animal facility using aseptic procedures. The surgical procedure was conducted

with a 3–5 mm longitudinal incision over the distal patella to the proximal tibial plateau. The joint capsule immediately medial to the patellar tendon was incised with a size 15 blade and the joint capsule opened with micro-iris scissors. Blunt dissection of the fat pad over the intercondylar area was then performed to expose the meniscotibial ligament of the medial meniscus. Mild hemorrhage from the fat pad upon blunt dissection was controlled by pressure from absorption spears. The fat pad over the cranial horn of the medial meniscus was dissected with forceps. The MMTL was identified running from the cranial horn of the medial meniscus laterally onto the anterior tibial plateau. Care was taken to identify and avoid the lateral meniscotibial ligament. Sectioning of MMTL with a size 11 blade directed proximo-laterally destabilized the medial meniscus. The tweezer was used to check if the MMTL was fully transected, and the medial meniscus destabilized. The joint capsule and the subcutaneous layer were then closed with a coated Vicryl suture.

2.3 | Intra-articular injection to knee joint

The injection procedure was performed in a designated surgery room within the animal facility using aseptic procedures. After anesthesia, the mouse was placed in the dorsal recumbent position and an electrical hair clipper was used to remove hair from the surgical site. A 30 G needle attached to a syringe horizontally was used to mark the area by gently pressuring the gap beneath the patella.[21] The injection of 10 μL of different concentrations of sterile alizarin complexone (catalog number A3382, Sigma) was then conducted in the marked area through the patellar tendon. After the injection, the joint was flexed and extended three times to ensure the injected material was evenly distributed throughout the whole articular surface. The intra-articular injection of phosphate-buffered saline (PBS) into mouse knees was used as a negative control.

2.4 | Micro-computed tomography scanning

At 4 weeks post-surgery, the mice were sacrificed, and the right knees were harvested for micro-computed tomography (micro-CT) scanning. The proximal tibias were scanned using high-resolution micro-CT (SkyScan 1275, Bruker, Kontich, Belgium). Micro-CT reconstructions and quantitative analyses were performed using the SkyScan CT Analyzer (Version 2.3.2.0, Bruker, Kontich, Belgium) as described previously.[20] The parameters of calcified thickness and subchondral bone thickness were analyzed.

2.5 | Safranin O/Fast green staining

Knee joint samples were fixed in the 4% cold fresh paraformaldehyde (PFA) for 2 days and washed with PBS, and then were decalcified with 12% ethylenediaminetetraacetic acid (EDTA) for 2 weeks. EDTA was changed twice a week. After the decalcification process was completed, the paraffin embedding was made. The sections were sequentially stained with Safranin O and Fast green solution (0.02% Fast Green for 5 min, 1.0% acetic acid for 10 s, and 0.5% Safranin O for 5 min). Histological changes in DMM and sham mice were examined and the cartilage lesions of medial tibial plateau (MTP) were assessed using OARSI grades and the maximal cartilage damage score is 6.[22]

2.6 | Cryosection processing and image visualization

Both right and left knee joints with alizarin complexone injections were harvested and immediately dropped into 4% PFA to prevent further diffusion of alizarin complexone during the preparation of the samples. The PFA fixation time was for 24 h at 4°C. Specimens were then transferred to a solution of 20% sucrose and 2% polyvinylpyrrolidone for 24 h for preparing embedding, followed by cryoembedding in Tissue-Tek O.C.T. Compound (Sakura Finetek USA Inc, Torrance, CA, USA). The sectioning, staining, and imaging methods were referred to previous publications.[23–25] Ten micrometre thick, un-decalcified coronal plane sections using a Leica RM2155 microtome were collected using cryofilm IIC tape (Section-Lab Co. Ltd., Hiroshima, Japan). Sections were attached to glass microscope slides and mounted with 50% glycerol. Each section in knee joint was subjected to two rounds of imaging on the EVOS™ M7000 Imaging System (Thermo Fisher Scientific Inc, Waltham, MA) including bright field channel and Texas Red emission filter channels to obtain bright field and alizarin complexone fluorescence signal.

2.7 | Detection of alkaline phosphatase enzymatic activity

Ten micrometre thick, undecalcified cryosections of the knee joint were washed completely in PBS. The fresh reaction buffer composed of 1 mL of 100 mM Tris–HCL (pH 8.0) and 25 μL of Vector Blue reagents 1, 2, and 3 (catalog number SK-5300, Vector Laboratories) were mixed well and used immediately according to the manufacturer's instructions. The section was incubated with the reaction buffer for 5 min in the dark. Cy5 emission filter channel was used to get a fluorescent signal showing in teal.

2.8 | Quantification of fluorescence intensity for the diffusion of alizarin complexone

All images were taken by the EVOS microscope in the same conditions. Fluorescence intensity measurement was conducted below the tidemark to calcified cartilage including partial subchondral bone or from the marginal zone (intersection area) between the tidemark and periosteum to the epiphysis of the bone. The quantification was done by Image J software. The areas covered below the tidemark to calcified cartilage or from the marginal zone of the periosteum to the end of epiphysis were hand-drawn in the bright field, and the fluorescence intensity in the same area of the fluorescence signal was then quantified in each sample.[7] The data were expressed as a density mean. The minimum fluorescence intensity with injection of PBS was served as a negative control (Figure S1).

2.9 | Data analysis and statistics

All statistical analyses were performed using GraphPad Prism, version 9. Results in the figures are presented as bar graphs with dot plots, which maximize visualization of the data distribution. For the animal study, n indicates the mouse number in the experiments. Two-tailed Student t-test was performed to determine the effects on mice between the two groups. $p < 0.05$ was considered significant.

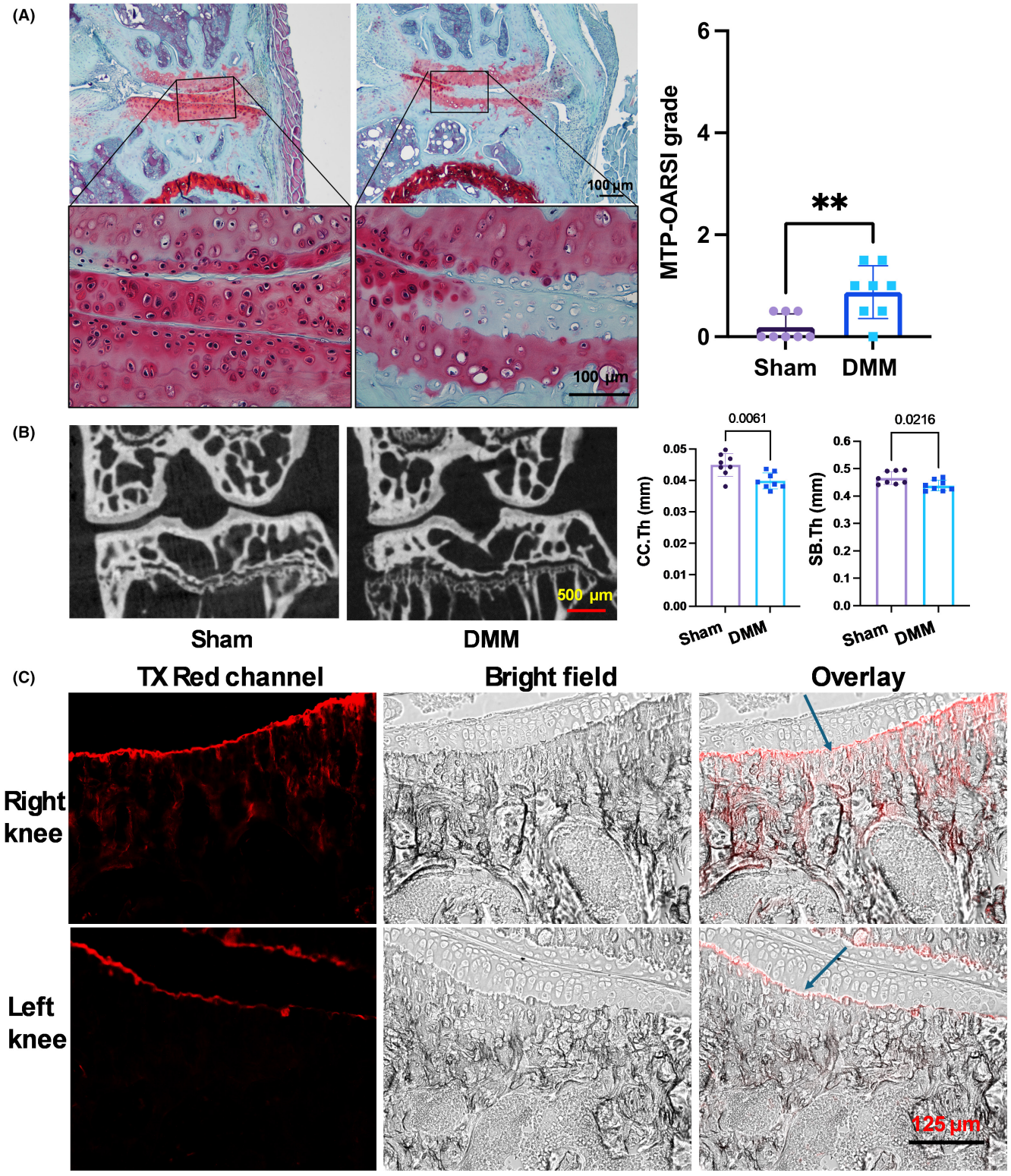
3 | RESULTS

3.1 | No articular cartilage calcification was observed in the early stage of DMM mice

Alizarin complexone is a common bone labeling fluorescent dye for active bone formation in vivo.[19,26] We isolated the mouse right knee joint and then used alizarin complexone to stain knee joint in vitro. Since no calcium minerals were found to deposit in hyaline cartilage in normal mice, no staining occurred in the cartilage surface by alizarin complexone in vitro. However, the calcified cartilage and subchondral bone were stained, which were visualized by bright field and Texas Red emission filter channels (Figure S2). The knee synovial cavity is one of the diarthrodial joints that are suited for intra-articular injection. Thus, we used alizarin complexone by intra-articular injection to assess whether there is articular cartilage calcification in the DMM mice in the following experiments.

Mouse DMM at 4 weeks post-surgery is categorized as the early stage of OA.[17,18] DMM surgery was conducted in the right knee. Since the cartilage lesions occur mostly in the MTP in DMM mice, we therefore focused on the changes of the right tibia. The data showed that the cartilage damage was modest by the Safranin O-Fast green staining (Figure 1A). MicroCT data showed that the thicknesses of calcified cartilage and subchondral bone in DMM mice were significantly reduced compared with those in sham mice (Figure 1B). We then aimed to detect the articular cartilage calcification in DMM mice by a single intra-articular injection of 10 μL of low dose of alizarin complexone (0.75 mg/mL) to both knee joints. After 5 min of post-injection, both right and left knees were collected. No calcification in the cartilage surface was visualized by fluorescence signal in the MTP of both knees. Surprisingly, a clear fluorescence signal was found in the tidemark and the fluorescence intensity declined in the calcified zone and subchondral bone in the right knee (Figure 1C, top panel) and left knee (Figure 1C, bottom panel). We also observed the fluorescence intensity in this region was higher in the right knee with DMM surgery than that in left knee without surgery. Thus, our data confirmed that the tidemark is the barrier that interrupts the substance transfer between the articular cartilage and subchondral bone. Our findings suggest that alizarin complexone has the diffusion capacity from the cartilage surface to calcified cartilage and subchondral bone, and this diffusion capacity may be enhanced in the DMM mice.

FIGURE 1 No articular cartilage calcification was found in the early stage of DMM mice. (A) Four weeks of post-surgery, the right knees with surgeries from DMM and sham mice were collected. The paraffin sections were stained with Safranin O-Fast green. The fluorescence signal of the medial tibial plateau (MTP) at higher magnification in the bottom panel was shown and cartilage lesions of MTP were assessed using OARSI scores (right panel). $n = 8$, **, $p < 0.01$, compared with sham control. Scale bar: 100 μm. (B) Micro-CT analysis of the knee joint 4 weeks after the DMM or sham surgery. The calcified cartilage (CC) and subchondral bone (SB) thickness (Th) of the mice in DMM group were significantly reduced compared to the sham group. Scale bar: 500 μm. (C) DMM surgery was conducted in the right knee and the contralateral non-operated joint was used as a negative control. Alizarin complexone (0.75 mg/mL) was injected into both knee joints of 4-week DMM mice. Five minutes of post-injection, both right and left knees were collected. No calcification in the cartilage surface was visualized by fluorescence signal in the MTP of both knees, but clear fluorescence images in the tidemark of both right (top panel) and left (bottom panel) knees were identified. The representative images of fluorescence, bright field, and overlay are shown. Arrows indicate tidemark. Scale bar: 125 μm.



3.2 | Alizarin complexone via intra-articular injection can move in different directions in the joint

The subchondral bone has blood vessels, which are near the capillaries of the synovial membrane within the joint cavity. The soluble substances can be exchanged between capillaries in the synovial membrane and the articular surface via the synovial fluid.[27,28] The injected substances into knee joints are more readily passed through the capillaries of the synovial membrane than through the articular cartilage due to their structural differences.[28] Thus, the injected substances in the synovial fluid can probably be transported through three

different directions (Figure 2A): (1) diffuse directly into the articular cartilage; (2) enter the capillaries of the synovial membrane subsequently move to the subchondral bone blood vessels (the local blood circulation); and (3) exit the joint and pass through the systemic circulation, especially with higher doses and longer periods post-injection. To test these notions, alizarin complexone (3 mg/mL) was injected into the mouse right knee for 20 min. We then sacrificed the mouse and found fluorescence signal in the tidemark, calcified cartilage, and subchondral bone in the right tibia (Figure 2B, top panel), but again no images were visualized in the articular cartilage of normal mice. These data suggest that alizarin complexone could move via the cartilage surface, diffuse through the tidemark, and then enter the subchondral bone. It is also possible that alizarin complexone partially entered the capillaries of the synovial membrane and then moved into the subchondral bone vial the local blood circulation. We also found that fluorescence images were visualized in the subchondral

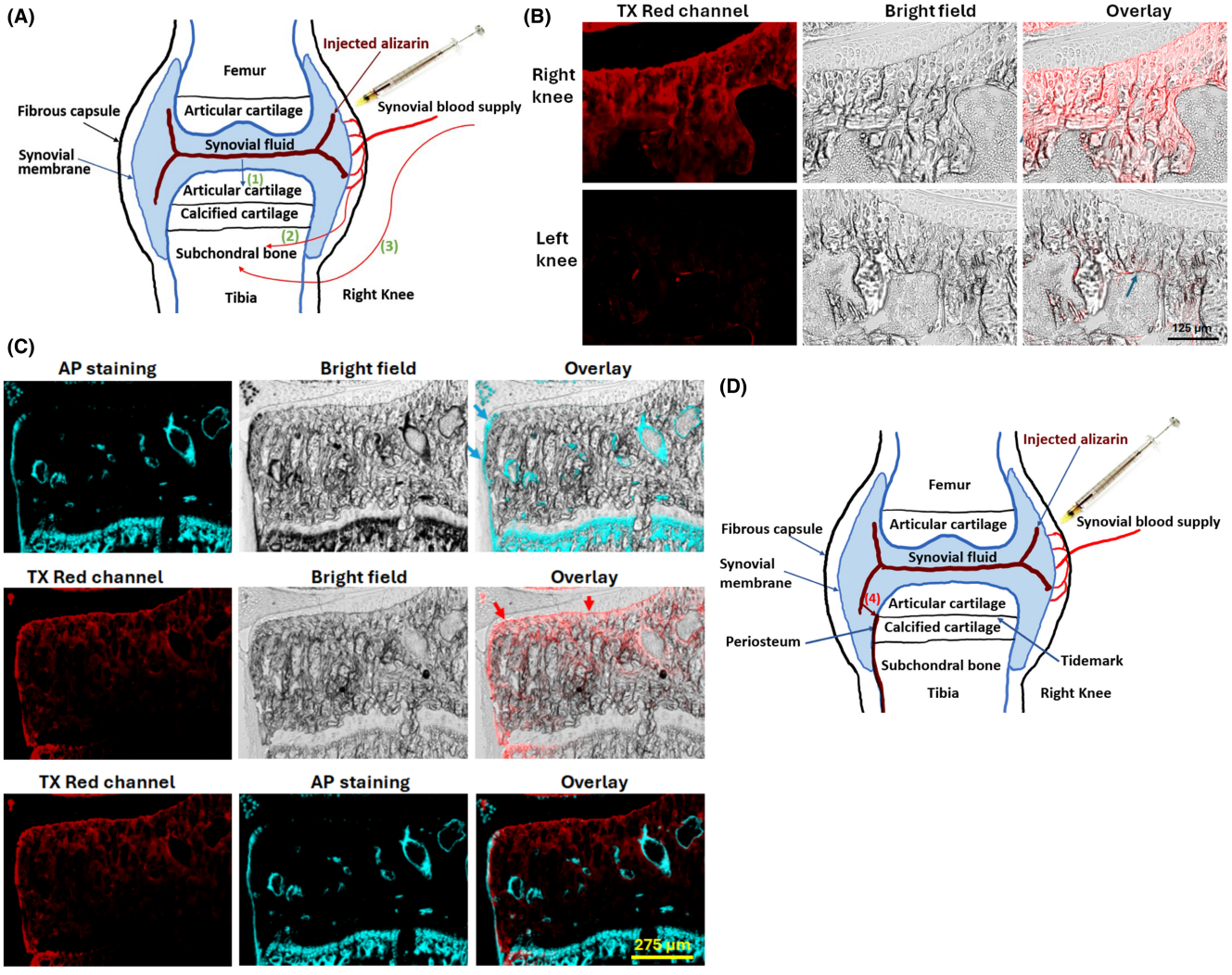


FIGURE 2 Alizarin complexone via the intra-articular injection can enter different directions in the joint. (A) Schematic diagram of alizarin complexone by intra-articular injection to diffuse into the articular cartilage and subchondral bone. Numbers in parentheses indicate the three pathways for the injected alizarin complexone to diffuse into articular cartilage and subchondral bone. To simplify the figure, the alizarin diffusion in femur is not shown. (B) Alizarin complexone (3 mg/mL) was injected into the right knee joint. Twenty minutes of post-injection, both right and left knees were collected. The representative images of fluorescence, bright field, and overlay in the medial tibial plateau of right knee (top panel) and left knee (bottom panel) were shown. Arrow (bottom panel) indicates subchondral bone. Scale bar: 125 μm. (C) The representative images were shown for a positive AP staining in the inner cellular layer of periosteum (top panel), an imaging for alizarin complexone diffused into the periosteum (middle panel), and the partially overlaid imaging for AP staining and diffusion signal (shown in white, bottom panel). Blue arrows indicate the periosteum in the outer surface of right tibia, and red arrows show the tidemark. Scale bar: 275 μm. (D) Schematic diagram of the fourth diffusion direction of alizarin complexone in the knee joint. Alizarin complexone by intra-articular injection is also able to pass into the periosteum in the marginal transitional zone of the tidemark, the fourth entry route (Number four in parentheses). The other three diffusion directions are shown in (A).

bone but not in the tidemark of left tibia (Figure 2B, bottom panel), confirming the direction of movement from the subchondral bone to the calcified cartilage in the contralateral knee joint via the systemic circulation.

The ends of the long bones in the diarthrodial joints are coated by articular cartilage, while the outer surface of the bones is covered by the periosteum. It was reported that tidemark can pass into the periosteum in the marginal transitional zone of the joint.[29] In contrast to the articular cartilage, blood vessels exist in the periosteum. The inner cambium layer of the periosteum contains osteoblasts and other types of cells including progenitor cells.[30–32] After osteoblasts secrete osteoid, calcium-containing salts are deposited in it to form the mineralized bone.[33,34] The alkaline phosphatase (ALP) enzymatic activity represents the function of osteoblast-lineage cells including osteoblasts, osteocytes, and stromal cells in bone marrow.[12] We found a positive AP staining in the inner cellular layer of the periosteum (Figure 2C, top panel), confirming the tidemark passes into the periosteum in the marginal zone of the joint. We also identified that the fluorescence signal passed along the periosteum of bone in the marginal zone due to alizarin complexone binding the calcium crystals in these areas (Figure 2C, middle panel). The overlaid images showed that the diffusion signal of alizarin complexone was located in the inner cellular layer of the periosteum and partially overlaid with the AP signal (Figure 2C, bottom panel). These findings provide evidence that alizarin complexone in the synovial cavity is able to pass into the periosteum in the marginal transitional zone of the tidemark, which is the previously unrecognized pathway, the fourth entry route (Figure 2D).

3.3 | Alizarin complexone is able to detect the permeability from the articular cartilage to the subchondral bone

To limit the transportation of alizarin complexone via the local and systemic blood circulation, we sacrificed mice at different time points before and after the injections of alizarin complexone were conducted into the right knee joints. We found that blood flowed out via the tail vein by cutting the tail before or after mice were immediately sacrificed (Figure S3). Blood flow discontinued from the tail vein after mice were sacrificed between 10 and 20 min, suggesting the reduced local and systemic circulation via the capillaries within the synovial membrane. We then injected alizarin complexone (0.75 mg/mL) into the right knee, waited for 5 min and then sacrificed the mice (Figure 3A). Alternatively, mice were sacrificed and then injections were conducted immediately (Figure 3B), after 5 min (Figure 3C), 10 min (Figure 3D), and 20 min (Figure 3E), respectively. After the injection, the sacrificed mice were placed in the prone recumbent position for 5 min (Figure S4). Both mouse right and left knees in each group were collected after the dwell time of alizarin complexone in the knee joint for 5 min. In order to get a similar position of the cryosections for comparing fluorescence intensity in different mouse samples, we did the serial sections of right knee joints and the sections with similar positions located in the MTP were chosen for the image analyses in the following experiments (Figure S5). Although we found the fluorescence images were visualized in the tidemark and subchondral bone in each group, the fluorescence intensity-dependent increase was clearly observed from the articular cartilage to calcified cartilage when the injections were conducted at 10 or 20 min after the sacrifice of mice (Figure 3D–F), suggesting only the diffusion pathway from the articular cartilage to subchondral bone is present due to the reduced blood circulation in the capillaries of the synovial membrane after mice were sacrificed. No fluorescence signal was found in the left knee because of the reduced blood circulation (Figure S6).

In addition, we did not find any knee joint structure changes by visualizing the bright field of knee joints (Figure 3A–E). Furthermore, no evident differences of AP activity in tibias were found before or after mice were sacrificed within 20 min (Figure S7). Therefore, we intra-articularly injected alizarin complexone 15 min post-scarification of mice to detect the diffusion of alizarin complexone from the articular cartilage to subchondral bone in the following experiments.

3.4 | Time-course of alizarin complexone diffusion from articular cartilage to subchondral bone in normal mice

After we confirmed that alizarin complexone can be applied for the substance diffusion between the articular cartilage and subchondral bone, we first explored the time-course of intra-articular injection of alizarin complexone (0.75 mg/mL) in mice with normal blood circulation. The data in Figure 4A showed that the weak fluorescence intensity in tidemark was visualized at 2.5 min of post-injection of alizarin complexone. The fluorescence intensity in tidemark and calcified cartilage increased at 5 min (Figure 4B), and the diffusion direction from the articular cartilage to the calcified cartilage can also be visualized, evidenced by an intensity-dependent decrease from the tidemark to calcified cartilage (Figure 4A,B). However, the diffusion declined between 10 and 20 min presumably because some alizarin complexone entered the capillaries

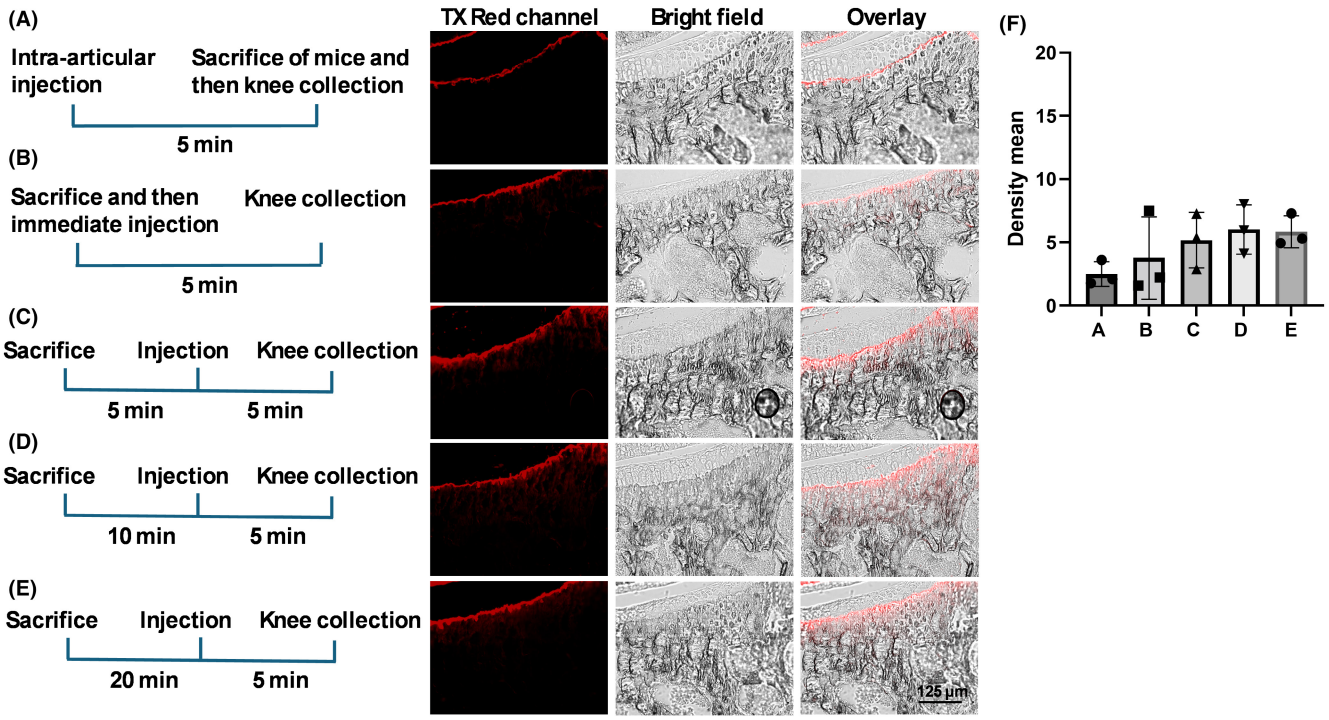
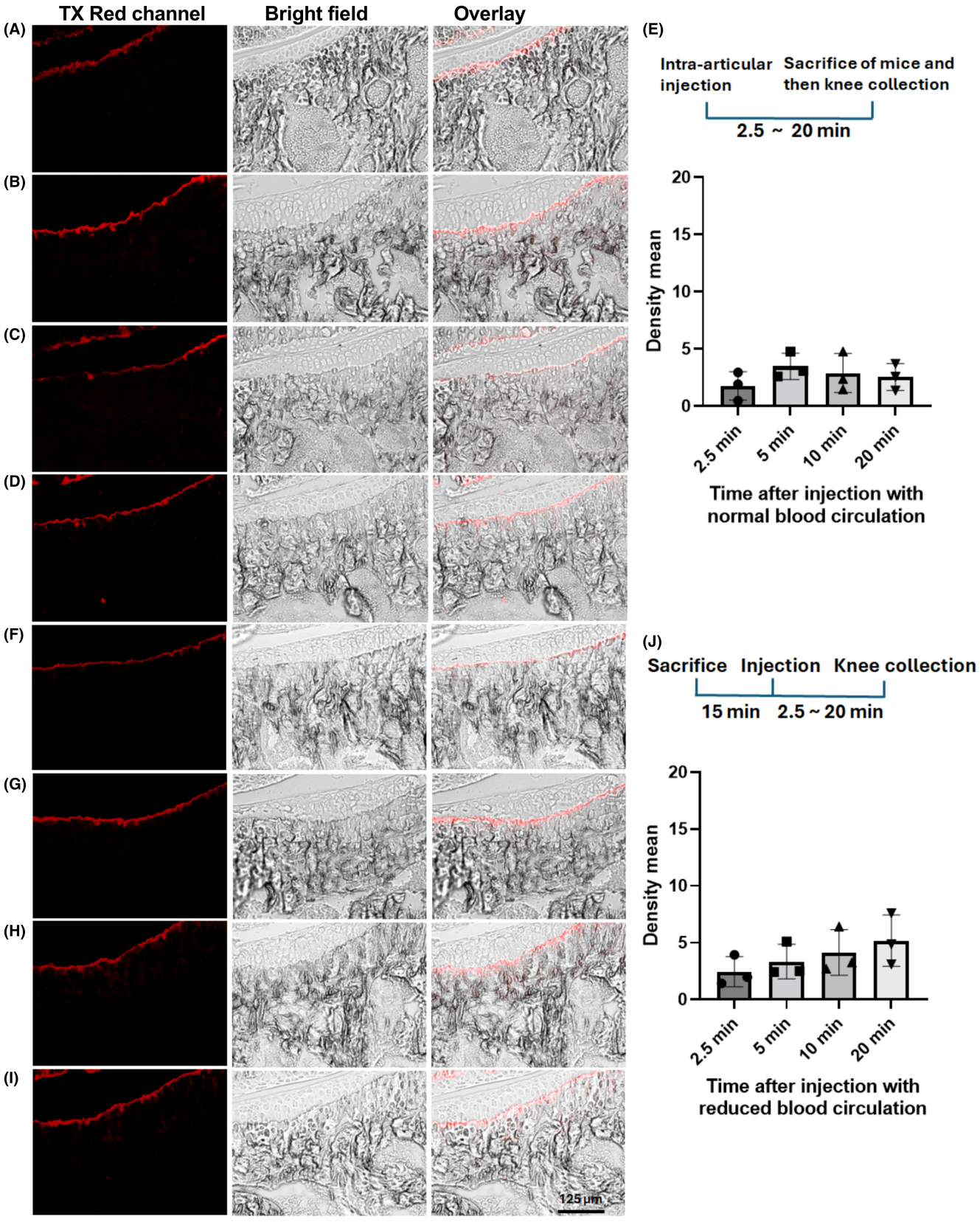


FIGURE 3 Alizarin complexone can be used to detect the permeability from articular cartilage to subchondral bone. Injection of alizarin complexone (0.75 mg/mL) was conducted into right knees before or after mice were sacrificed. The timelines for the sacrifice of mice and the intra-articular injection in each group were shown in the left panels. There were five groups: (A) after injection, waiting for 5 min and then sacrificing the mice; (B) after sacrifice, immediate injection; (C) after sacrificing the mice, waiting for 5 min and then administering injection; (D) after sacrifice of mice, waiting for 10 min and then administering injection; and (E) after sacrifice of mice, waiting for 20 min and then administering injection. The dwell time for the injection in each group was 5 min and both knees were then collected. The representative images for the diffusion of alizarin complexone in each group are shown. The quantification method was described in Materials and Methods, and in Figure S4. The quantification of fluorescence intensity for the diffusion from three independent experiments in each group was shown (F). Scale bar: 125 μm.

of the synovial membrane and exited to the adjacent areas (Figure 4C–E).

To further confirm the diffusion of alizarin complexone from the cartilage surface to calcified cartilage, we also explored the time-course of diffusion in mice with reduced blood circulation when the injections were conducted at 15 min after the sacrifice of mice. Similar results were found between 2.5 and 5 min in mice sacrificed before or after the injection (Figure 4A,B,F,G). However, we found the fluorescence intensity from the tidemark to calcified cartilage increased between 10 and 20 min due to the unidirectional diffusion (Figure 4H–J). Collectively, we chose the 5-min time point as an injection dwell time to investigate the following dose–response of alizarin complexone to restrict the diffusion from the subchondral bone to calcified cartilage.

FIGURE 4 Time-course of alizarin complexone diffusion from articular cartilage to subchondral bone in normal mice. Mice were injected with 0.75 mg/mL of alizarin complexone into right knee joints and the dwell time was 2.5 min (A), 5 min (B), 10 min (C), and 20 min (D), respectively. The mice were then sacrificed, and right knee joints were collected for visualization of fluorescence intensity. Injection of PBS into the right knee was used as a negative control. The summarization of schematic timelines for the injection of alizarin complexone before the mouse sacrifice in each group were shown (E, top panel), and the quantified fluorescence intensity from three independent experiments in each group (A–D) was presented (E, bottom panel). To limit the diffusion of alizarin complexone via the local blood circulation, the injections of alizarin complexone (0.75 mg/mL) were conducted at 15 min after sacrifice of mice. The dwell time of the injection was 2.5 min (F), 5 min (G),10 min (H), and 20 min (I), respectively. The mice were then sacrificed, and right knee joints were collected for visualization of fluorescence intensity. The summarization of schematic timelines for the injection of alizarin complexone after the mouse sacrifice in each group were shown (J, top panel), and the quantified fluorescence intensity from three independent experiments for groups (F–I) was presented (J, bottom panel). The representative images for the diffusion of alizarin complexone in each group are shown. Scale bar: 125 μm.


TX Red channel
Bright field
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(A)
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125 µm
(E)
Intra-articular injection
Sacrifice of mice and then knee collection
2.5 ~ 20 min
Density mean
20
15
10
5
0
2.5 min
5 min
10 min
20 min
Time after injection with normal blood circulation
(J)
Sacrifice
Injection
Knee collection
15 min
2.5 ~ 20 min
Density mean
20
15
10
5
0
2.5 min
5 min
10 min
20 min
Time after injection with reduced blood circulation

3.5 | Dose–response of alizarin complexone diffusion from articular cartilage to subchondral bone in normal mice

At the 5-min time point, we found that the diffusion of alizarin complexone in mice with normal blood circulation was gradually increased over the range of 0.38–0.75 mg/mL (Figure 5A,B). However, when the concentrations of alizarin complexone were increased above 0.75 mg/mL, more fluorescence images were found in the subchondral bone (Figure 5C–E), suggesting that alizarin complexone entered the local blood circulation and exhibited retrograde diffusion from the subchondral bone to the calcified cartilage. We also explored the dose–response of diffusion in mice with reduced blood circulation when the injections were conducted at 15 min after the sacrifice of mice. The diffusion from the articular cartilage to the subchondral bone increased in a dose-dependent manner over the range of 0.38–3.0 mg/mL (Figure 5F–J). Taken together, to avoid the possible diffusion direction from the subchondral bone to articular cartilage, we chose the injection condition of alizarin complexone (0.75 mg/mL and dwell time for 5 min) to investigate the diffusion differences in the early stage of DMM and sham mice.

3.6 | The permeability from the articular cartilage to subchondral bone is increased in DMM mice

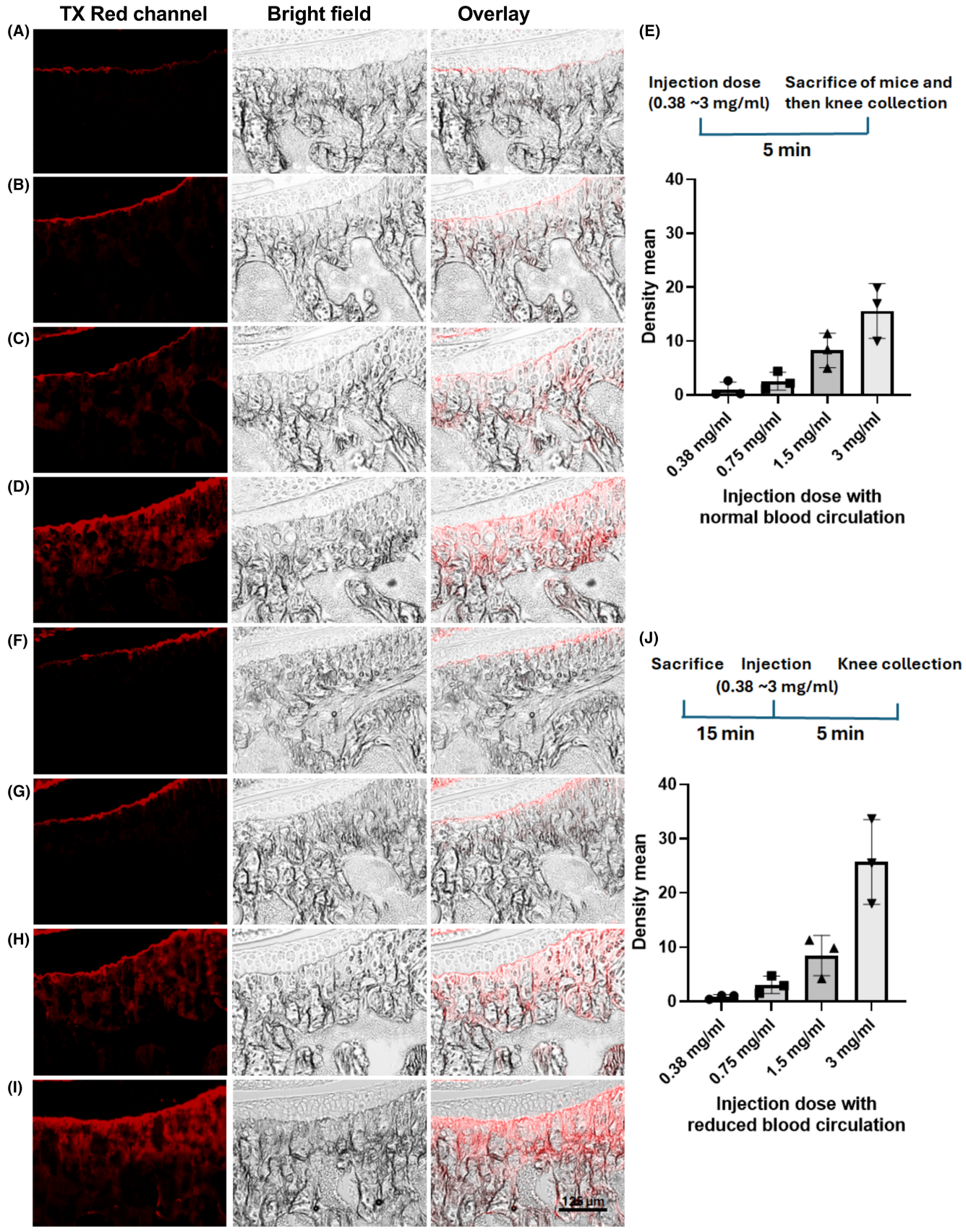
After we found alizarin complexone by intra-articular injection is able to detect the permeability between the articular cartilage and subchondral bone, we conducted DMM and sham surgeries on mouse right knees, then monitored for a period of 4 weeks before intra-articular injection of alizarin complexone (0.75 mg/mL). Five minutes of post-injection, the mice were sacrificed and both right and left knees were then collected. The data in Figure 6A,B,E showed that the enhanced fluorescence intensity between the tidemark and subchondral bone was significant (106% increase) in the DMM mice when compared with sham mice. In addition, we also found a greater fluorescence signal was visualized in the periosteum area of the marginal zone of the tidemark in DMM mice than that in the sham mice at a lower magnification to obtain a broader view of the knee joint (Figure 6G,H,K).

Given the presence of cartilage lesions in DMM mice compared with sham mice (Figure 1A,B), we hypothesized that there would be a greater diffusion from the articular cartilage to subchondral bone in DMM mice than in sham mice if the local blood circulation was limited. Alizarin complexone (0.75 mg/mL) was injected into the right knees of the sham and DMM mice at 15 min after these mice were sacrificed. After 5 min of post-injection, both right and left knees were collected. The data (Figure 6C,D,F) showed that the diffusion of alizarin complexone from the articular cartilage to the calcified cartilage was significantly augmented, evidenced by a 233% increase in fluorescence intensity in DMM mice compared to sham mice. However, similar fluorescence intensity was detected in the periosteum region before and after scarification of mice (Figure 6G–L). Because we limited the diffusion time to 5 min, we did not find that alizarin complexone entered the systemic circulation in both DMM and sham mice by visualizing the fluorescence images in the left knees (Figure S8). In addition, we further confirmed no calcification occurred at the upper cartilage in the early stage of DMM mice (Figure 6B,D).

4 | DISCUSSION

In the present study, we did not find articular cartilage calcification in the early stage of DMM mice, but we serendipitously discovered that alizarin complexone had a novel role in detecting permeability between the articular cartilage and subchondral bone. To the best of our knowledge, this study is the first to report the development of an imaging method based on alizarin complexone binding to

FIGURE 5 Dose–response of alizarin complexone diffusion from articular cartilage to subchondral bone in normal mice. Mice were injected with different doses of alizarin complexone into right knee joints and the injection dwell time was 5 min with concentrations of 0.38 mg/mL (A), 0.75 mg/mL (B),1.5 mg/mL (C), and 3 mg/mL (D). The right knee joints were collected for visualization of fluorescence intensity. Injection of PBS into the right knee was used as a negative control. The summarization of schematic dose regimen for the injection of alizarin complexone before the mouse sacrifice in each group were shown (E, top panel), and the quantified fluorescence intensity from three independent experiments in each group (A–D) was presented (E, bottom panel). To limit the diffusion of alizarin complexone via the local blood circulation, the injections of different doses of alizarin complexone into right knee joints were conducted at 15 min after sacrifice of mice. The injection dwell time was 5 min with concentrations of 0.38 mg/mL (F), 0.75 mg/mL (G),1.5 mg/mL (H), and 3 mg/mL (I). Injection of PBS into the right knee was used as a negative control. The summarization of schematic dose regimen for the injection of alizarin complexone after the mouse sacrifice in each group were shown (J, top panel), and the quantified fluorescence intensity from three independent experiments in each group (F–I) was presented (J, bottom panel). The representative images for the diffusion of alizarin complexone in each group are shown. Scale bar: 125 μm.



calcium-containing crystals located in the tidemark, calcified cartilage, and subchondral bone in both osteoarthritic and sham mice.

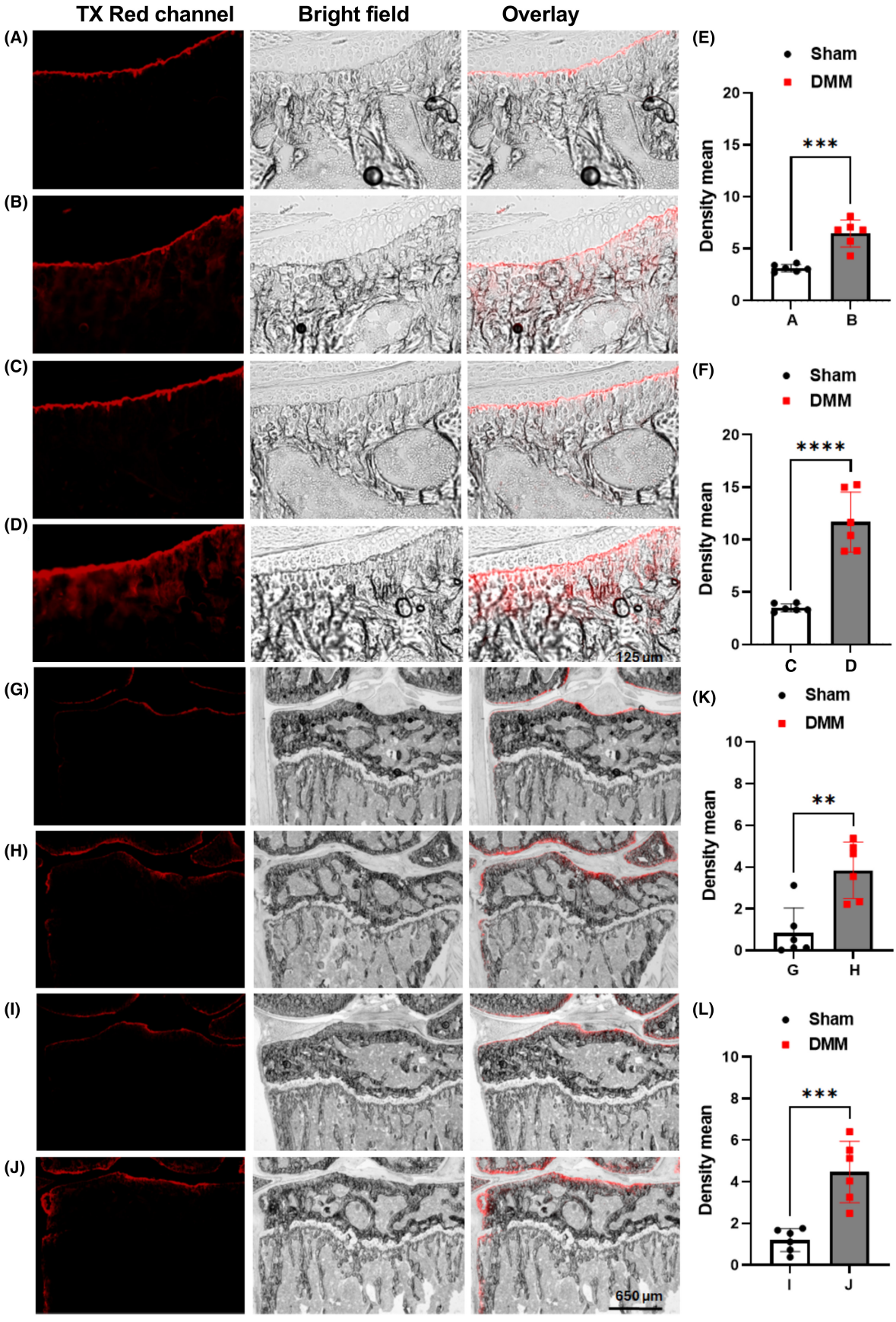
Due to the avascular and aneural nature of cartilage, significant attention has been directed on the intracellular communication of signal molecules, delivery of

essential nutrients to chondrocytes, and removal of waste products in both physiological and pathological conditions. Some progress has already been made in experimental methodologies to probe solute transport in detecting such conditions either in vitro or in vivo. For example, data form Arkill's group demonstrated that the tidemark and calcified cartilage are permeable to low molecular weight solutes.[27] They used the cartilage and subchondral bone from the metacarpophalangeal joints of mature horses to form the membrane in the diffusion chamber. They showed that nutrition supplements for articular cartilage were provided via the diffusion from both superficial synovial fluid and the subchondral area. Wang et al. quantified the diffusivity of sodium fluorescein within calcified joint matrix using the fluorescence loss induced by a photobleaching method.[6] They found that measurable transport of solute across the mineralized calcified cartilage in normal joints and elevated biochemical communication between the subchondral bone and cartilage in the late stage of osteoarthritic joints. Recently, Huang et al. developed a novel fixing device combined with an undecalcified fluorescence observation method to address the permeability of calcified cartilage zone in live 1 and 6-month mice.[7] They found the calcified cartilage zone of the knee joint in 1-month mouse was immature and the calcified cartilage zone blocked the solute diffusion from the articular cartilage to subchondral bone in 1 and 6-month mice. However, the permeability between the articular cartilage and subchondral bone in the early stage of osteoarthritic mice has not yet been reported.

In the present study, we first examined whether the calcification occurred in the early stage of osteoarthritic mice. Four weeks following DMM surgery, we administered alizarin complexone injections into knee joints intra-articularly. The calcification in the hyaline cartilage was not found. However, we unexpectedly identified a clear fluorescence signal of tidemark between the articular cartilage and calcified cartilage, and the fluorescence intensity decreased as it approached the subchondral bone. This finding provided us an incentive to explore whether alizarin complexone by intra-articular injection could be employed for revealing the permeability between the articular cartilage and subchondral bone.

Due to the different structures of the synovial membrane and cartilage surface, alizarin complexone has a greater permeability to enter the capillaries of the synovial membrane than to diffuse into the articular cartilage.[27,28] Our data showed that a low dose of alizarin complexone was not only able to diffuse into the articular cartilage but also enter the local blood circulation, the latter of which probably conducted the opposite direction for alizarin complexone diffusion from the subchondral bone to articular cartilage. Our data also indicated that higher doses and extended post-injection time of alizarin complexone could also enter the systemic circulation, which was confirmed by visualizing the fluorescence signal in the subchondral bone of the contralateral non-injected joint. These results made it challenging for utilizing alizarin complexone to detect the exclusive permeability from the articular cartilage to subchondral bone. To circumvent this difficulty, we injected alizarin complexone within a shorter time after the mice were sacrificed. Our data suggested that mouse knee joint structure and the ALP activity in the subchondral bone were not evidently altered after mice were sacrificed within 20 min. To further confirm the reduced blood circulation within the synovial membrane of knee joint, a 20 min interval after the sacrifice of mice was observed until cessation of blood flow. In this condition, we injected alizarin complexone (0.75 mg/mL and dwell time for 5 min) in these sacrificed mice and found exclusively unidirectional diffusion from the articular cartilage to subchondral bone. We then compared the permeability between the articular cartilage and subchondral bone in DMM and sham mice 4 weeks after the surgery. The results indicated that the diffusion of alizarin complexone to the calcified cartilage was significantly enhanced in DMM mice than in sham mice, confirming the increased biochemical communication between the articular cartilage and subchondral bone during the osteoarthritic progression.

FIGURE 6 The alizarin complexone diffusion to subchondral bone and periosteum was increased in DMM. Four weeks of post-surgery in the right knees, the intra-articular injection of alizarin complexone (0.75 mg/mL) was conducted in the right knees. Five minutes after injection, both right and left knee joints were then collected. The images were visualized for alizarin complexone diffusion into the subchondral bone in sham (A) and DMM (B) mice, and into the periosteum in sham (G) and DMM (H) mice. To limit the diffusion of alizarin complexone via the local blood circulation, alizarin complexone (0.75 mg/mL) was injected into the right knees of the sham and DMM mice at 15 min after these mice were sacrificed. Five minutes of post-injection, both right and left knees were collected, and the images were visualized for alizarin complexone diffusion into the subchondral bone in sham (C) and DMM (D) mice, and into the periosteum in sham (I) and DMM (J) mice. The quantified fluorescence intensity for alizarin complexone diffusion into the subchondral bone (E, F) and the periosteum (K, L) was presented, respectively. The representative images for the diffusion of alizarin complexone in each group were shown. $n = 6$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, compared with sham control. Scale bar in (A–D) is 125 μm, and scale bar in (G–J) is 650 μm.



It is established that the injected substances in the synovial fluid can be transported through three different directions. We not only confirmed these notions but also identified alizarin complexone in the synovial cavity is able to pass into the periosteum in the marginal transitional zone of the tidemark, which is previously unrecognized

pathway. These four pathways for the substance transport in knee joints summarized in Table 1. Additionally, our data also disclosed that alizarin complexone in the joint cavity exhibited more diffusion via the periosteum area of the bone in DMM mice than that in the sham mice. Osteophyte formation is a form of endochondral ossification and located along the edges of bones. Osteophytes can form at 2 weeks post-DMM surgery.[35] The fluorescence intensity in the periosteum area may also be involved in the osteophyte formation.

The local delivery of therapeutics by intra-articular injections has several advantages over systemic administration.[28,36] These benefits include increased bioavailability, fewer adverse events, and lower total drug costs. The data in the present study support these advantages of intra-articular injection. Corticosteroids and hyaluronate preparations were FDA-approved for intra-articular therapeutics. Recombinant proteins, autologous blood products and analgesics have also found clinical use via intra-articular delivery.[28,36] However, the rapid egression of injected substances from the joint was also reported.[28,36] High dose of alizarin complexone by intra-articular injection can also be found in the contralateral non-injected joint via the systemic blood circulation. In addition, frequent injections of therapeutic agents will likely induce an inflammatory response and potential fibrosis of the knee joint. Therefore, the potential systemic side-effect also needs to be considered when intra-articular therapy is employed.

Despite the advancements achieved from this work in the discovery of the new role of alizarin complexone used in detecting the permeability between the articular cartilage and subchondral bone, there are some limitations in the present study. First, alizarin complexone is a fluorescent dye with relatively small molecule weight (385 Da). However, inflammatory cytokines and the receptor activator of nuclear factor κB ligand (RANKL), which are large molecular weight molecules, participate in the initiation and progression of OA activation. Several reports have demonstrated that RANKL is expressed in articular chondrocytes of normal and OA cartilage and contributes to the subchondral bone resorption.[37–39] It is unknown whether RANKL can diffuse from the articular cartilage to subchondral bone during the OA progression. Thus, the large molecular weight calcium-binding fluorescent dyes should be considered for the detection of diffusion alterations between the articular cartilage and subchondral bone. Second, the changes in permeability between the articular cartilage and subchondral bone were not assessed at earlier time point (such as 2 weeks of post-surgery) and in the middle and late stages of DMM mice. In addition, it is noted that the presence of multiple tidemarks, a characteristic observation in human OA,[29,40,41] was not identified in the early-stage DMM mice but could be potentially manifested in the later stages of DMM progression. These shortcomings, therefore, need to be overcome in the future studies.

In conclusion, alizarin complexone has the capacity to detect the diffusion from the articular cartilage to calcified cartilage. Thus, the new role of alizarin complexone warrants further investigation of other calcium-binding fluorescent dyes including the large molecular weight molecules for detecting the diffusion in healthy and pathological conditions. Although we did not find the calcification in the hyaline cartilage in 4-week DMM mice, we identified that the diffusion of alizarin complexone from the articular cartilage to subchondral bone was significantly increased in DMM mice. We also found that a greater amount of alizarin complexone passed through the periosteum in DMM mice compared with sham mice. These findings provided new evidence that the increased biochemical communication between the articular cartilage and subchondral bone is involved in the pathogenesis

TABLE 1 Alizarin complexone diffusion in the keen joints by intra-articular injection.

Mouse with normal blood circulation	Mouse with reduced blood circulation
Four pathways: 1. Via articular cartilage 2. Via local bloodstream 3. Via systemic bloodstream 4. Via the periosteum in the marginal transitional zone of the tidemark	Two pathways: 1. Via articular cartilage 2. Via the periosteum in the marginal transitional zone of the tidemark
Two diffusion directions: 1. From articular cartilage to the calcified cartilage or periosteum 2. From the subchondral bone to articular cartilage	One diffusion direction: Intensity-dependently decrease from the articular cartilage to the calcified cartilage or periosteum
Diffusion is reduced from the articular cartilage to subchondral bone	Diffusion is not reduced from the articular cartilage to subchondral bone

of OA progression. The periosteum is the major contributor to cartilage and bone repair during bone injury.[30–32] Further studies are needed to investigate the role of the periosteum in OA pathogenesis and the relationship between bone injury and OA progression, and to test whether targeting biochemical crosstalk between the articular cartilage and subchondral bone can be served for the treatment of OA.

AUTHOR CONTRIBUTIONS

Bin Wang conceived and designed the study and wrote the paper. Mingshu Cui, Mengcun Chen, Yanmei Yang and Hamza Akel performed and analyzed the experiments. All authors edited the paper and approved the final version of the manuscript.

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DISCLOSURES

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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