A Less Invasive Approach of Medial Meniscectomy in Rat: A Model to Target Early or Less Severe Human Osteoarthritis

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Abstract

Objectives: The existing medial meniscectomy (MMx) procedure in rodents involves transection of MCL and wide opening of the knee capsule followed by meniscus transection that results into articular cartilage degeneration and causes significant changes in subchondral bone at specific post-MMx period. These animals serve as experimental osteoarthritis (OA) models.

Structurally, knee is very complex. There may be a need to uncouple articular cartilage degenerative changes from subchondral bone degenerative changes in experimental OA models. Therapeutic drugs/agents, designed to treat articular cartilage degenerative changes of OA knee, may not be effective to treat subchondral bone degenerative changes or vice versa.

The purpose of the study was to modify the existing MMx procedure to a less invasive procedure so that it causes only articular cartilage degenerative OA changes and no subchondral bone changes. This will serve as an early OA model to target degenerative changes in articular cartilage in human.

Methods: Ten 8-9 weeks old male athymic nude rats underwent less invasive MMx on right knee; the left knee served as unoperated control. The surgery involved no transection of MCL and opening of knee capsule wide. The medial meniscus was pulled out from a slit made on the medial aspect of the knee capsule, and was transected. At 10 weeks post-MMx, animals were sacrificed and knees were assessed.

Results: There was no significant alteration in bone strength parameters (BV/TV, Tb.Th, Tb.Sp, Tb.N, Tb.Pf, cortical wall thickness) in subchondral bone in the less invasive MMx knee as analyzed by μ CT. X-ray radiography did not indicate the presence of any osteophyte at the knee margins. On the other hand, the less invasive MMx caused degenerative changes in articular cartilage as follows: fibrillation and thinning in the medial tibia; narrowing of medial knee joint; reduced proteoglycans shown by safranin-O staining; decreased metachromasia by PSR staining; alteration in collagen fibril thickness under cross-polarization light. Immunohistochemical expression was reduced (COL-II, aggrecan), increased (NITEGE, Col2-3/4m, COL-X, and MMP13) or imbalanced (lubricin) in articular cartilage thickness of less invasive MMx knee.

Conclusion: The less invasive MMx procedure, in rat, can be a model for early or less severe human OA due to articular cartilage degenerative changes only and with no subchondral bone degenerative changes in the knee synovial joint. The therapeutic drugs/agents designed to repair articular cartilage may be suitable for this model and may lead to treatment of early or less severe human OA.

Keywords: Osteoarthritis; Medial meniscectomy; Rats; μ CT; Immunohistochemistry; Articular cartilage; Subchondral bone

Introduction

Osteoarthritis (OA) is one of the leading causes of disability in the elderly. OA is defined by the American College of Rheumatology as a “heterogeneous group of conditions that lead to joint symptoms and signs which are associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone at the joint margins”. The OA progresses with age, 30 to 50% of adults over the age of 65 years suffer from OA. Radiography of multiple joints (knee, hip, spine, hand) shows OA in at least one joint in over 80% of older adults. However, only about half of people with radiographic OA experience significant symptoms. And also not all the older adults with joint pain have radiographic evidence of OA in the affected joint [1]. Diagnosis is usually confirmed by using X-ray with joint space narrowing, osteophytes formation and subchondral sclerosis [2]. Osteophytes generally appear at joint margins, initially as outgrowths of cartilage and those subsequently undergo endochondral ossification supported by growth factors including TGF β [3].

Medial meniscus is attached to the tibial plateau via anterior and posterior horn by insertions that hold the meniscus in place during weight bearing [4]. The menisci of the knee are important load distributors and shock absorbers in the joint. Meniscectomy causes instability to the knee joint with an increased risk of OA [5]. Partial meniscectomy has been reported to increase the risk for the onset of OA by increasing stress and strain in the articular cartilage [6]. The

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external medial tibial cartilage is vulnerable to thinning once the meniscus extrudes and its surface is in direct, non-physiological, cartilage-cartilage contact with femur [7]. Papalia et al. summarized that meniscectomy is a risk factor of OA though the extent of cartilage degeneration depends upon the 'minimal invasion to open total meniscectomy', gender, and other factors [8]. There is no disease modifying OA drug on the market that slows down OA progression or induces neo-formation of the articular cartilage. In search of therapeutical solutions, surgical joint instability models are of great interests. Several animal models of OA, spontaneously mutated, genetically modified or created by multiple manipulations, have been described in different species. Among them are: dog partial MMx [9]; dog anterior cruciate ligament transection (ACLT; [10]); rat medial meniscus tear [11]; rat ACLT [12]; gene mutation model in mice, e.g., mutation in COL-II [13]; spontaneous development of OA in male STR/ort mice [14]; surgically or chemically-induced OA in mice, e.g., DMM (destabilization of knee by MMx) model [15], or collagenase injection (CIA) in knee capsule [16]; spontaneous OA in Hartley albino guinea pigs [17]; meniscus tear-induction in guinea pig [18]; meniscus-tear in rabbit [19]; and spontaneous OA in non-human primates [20].

In rat and mouse, MMx surgery is usually conducted first by transecting the medial collateral ligament (MCL) fully or partially followed by wide opening of the knee capsule, and finally excising the medial meniscus [15,21,22]. Following the MMx procedure, a substantial subchondral bone changes occurred in rat knee at 8-weeks post-MMx as determined by μ CT along with articular cartilage degeneration [23]. For analyzing subchondral bone damages, McErlain and team used MRI and μ CT and showed that 75% of 4-weeks post-ACLT rats had at least 1 subchondral bone cyst [24]. The uncoupling of cartilage damage and subchondral trabecular bone changes in OA is suggested [25], a separate set of markers need to be considered in experimental OA. A model that causes articular damage and less or no subchondral bone damage can serve better choice for experimental OA for cartilage repair.

The purpose of the current investigation was to modify the existing MMx procedure into less invasive procedure that causes predominantly articular cartilage degeneration and not the subchondral bone changes in rat. The procedure was conducted in which MCL of the knee joint was not transected, knee capsule was not opened wide, and the MMx was accomplished successfully with degenerative changes in the articular cartilage of medial knee joint but with non-significant changes in the subchondral bone strength parameters. By using this modified procedure, we assumingly avoided most of the additional synovial inflammation as compared to wide-opened knee capsule surgery. Synovium inflammation has been directly linked to enhanced OA [26]. Macrophages from the synovial lining have been shown to cause inflammation and cartilage damage in experimental arthritis model [27]. The less invasive MMx in rat may reflect post-traumatic OA model and not post-inflammatory OA model serve as an early OA model to target degenerative changes in articular cartilage in human. The purpose of the study is to use less invasive MMx procedure in rat in search of an experimental model for early or less severe human OA. We used athymic nude rats instead of normal rats for this study for the reason that this rat is becoming an experimental animal of choice for human cells transplantation studies to avoid rejection, and will also serve as human stem cells transplantations in our future experiments.

Methods

Medial meniscectomy (MMx)

Animals care: Male homozygous athymic nude rats, also called

NIH nude rats (CrI:NIH-Foxn1^{nu}; rnu/rnu), were purchased from Charles Rivers, USA (1.800.LABRATS). Animal use protocol (AUP) was approved by animal resources center (ARC) of University Health Network, Toronto. Upon arrival, rats were allowed to acclimatize for three days to new housing. Animals were provided with sterile housing, food and drinking water with restricted entry.

Surgeries: Surgeries were conducted on rats at the age of 8-9 weeks using eye loupes (4.9x, Surgical Acuity). Rats were anesthetized using 5% isoflurane in oxygen (inhalation anesthetic) and maintained at 2% isoflurane during surgery. Pre- and post-operative care was provided to animals. Tear gel (Alcon Tear-Gel Liquid Eye Gel) was applied to rat's eyes immediately after anesthetization prior to surgery. Rats received one injection of analgesia (buprenorphine, 0.03 mg/kg) just before the beginning of surgery and two injections daily (one in morning and one in the evening) for three days post-surgery. Animals were kept hydrated by injecting sc saline ($\approx$ 2-3 ml) at the back area before beginning of the surgery. Surgeries were conducted on a clean bench covered with sterile drape sheets cushioned and warmed underneath with a pad equipped with T/Pump Warm Water Recirculator (STRYKER). Knees and surrounding area was shaved, cleaned with iodine surgical scrub (7.5% iodine), 70% isopropanol and 10% Providone iodine (equivalent to 1% iodine) in sequence. MMx was conducted on the right knee (N=10) of the rat with less invasive approach as compared to the methods used by earlier researchers in rat or mouse [15,21,22]. Left knees served as unoperated controls (N=10). A longitudinal incision was made on the anterior aspect of the right knee with a blade no. 15 (M90-15, Almedic)/scalpel handle no. 3. The skin, underneath and around the knee, was gently dislodged from the subcutaneous layer to make a small pocket flap by using few backward strokes of scissors. A medial peripatellar incision was made to expose MCL along with the outer surface of knee capsule. The later was cleaned with cotton gauze to locate the position of medial meniscus that was visible through capsule's translucent wall under high-powered eye loupes (Figure 1A). The meniscus was gently dislodged from medial femur and medial tibia with the help of a micro knife (10316-14, Micro knife 22.5°, Fine Scientific Tools; Figure 1A). The meniscus was pulled out with a pair of fine-toothed forceps (Figure 1B), cut first at one end and than pulled out, and cut at the second end (Figures 1C1, 1C2 and 1D). The slit in the medial knee capsule was washed with 0.5 ml PBS (D8662, Dulbecco's phosphate-buffered saline, Sigma-Aldrich) and closed with 2 interrupted stitches using 6-0 PolysorbTM suture (Polysorb, UL-201, Covidien). The subcutaneous layer was sutured using 5-0 PolysorbTM (UL-202, Covidien) using three interrupted stitches. Skin was closed by uninterrupted intradermal suturing system using Monocryl suture (Y303, Ethicon). Finally the sutured skin was glued with a thin layer of tissue adhesive (Vetbond, 3M). Animals were placed under warm lamp until they moved freely in the cage ($\approx$ 5-10 min). Animals were monitored twice daily for first 3 days; daily for next 4 days, and later twice a week till the end of animal experiment.

X-ray radiography and μ CT imaging of rat knee joints

At 10-week post-MMx, rats were euthanized using CO₂ gas anesthesia. Whole body and separated individual hind limbs were radiographed using VXvue digital imaging system following the instruction manual. Specimens were placed on the detector plate at a distance of 30 inches from the X-ray emitter. X-ray dose for the generator was set at kV (kVP=70) and mAs (1.000). X-ray machine was handled and operated in a designated area approved by Radiation Hazards and Protection division, Ministry of Labor, Ontario, Canada. The digital images were downloaded. The representative images are

shown in Figure 2. Knee joints were cut separated with a dremel, fixed in 10% neutral buffered formalin (HT-5011, Sigma-Aldrich) with two changes during 72h, washed in PBS (2x, 10 min), transferred to 70% ethanol and processed for μ CT imaging. The knee joints were scanned using a small-animal Inveon CT scanner (Siemens Preclinical Solutions, Knoxville, TN), with the parameters shown in Table 1. The reconstructed datasets were analyzed with the Inveon Research Workplace software (v4.0). Histomorphometric analysis of subchondral bone (medial tibia and femur) was performed by 3-D volume of interest (VOI). The VOI included the subchondral trabecular bone starting below the subchondral plate extending (0.5 mm in depth) towards the growth plate, while excluding both the cortical bone and growth plate interface. For analysis of medial tibia and femur cortical bone, VOI regions included the load-bearing region of the medial cortical plates. ROIs were designed throughout the full width and thickness of the cortical layer, and measured 2.5 mm in length from the anterior-posterior side (Figure 3 and Table 1). Scanned images of MMx and control knee are shown in Figure 4. The following bone strength parameters were calculated: BV/TV, Tb.Th, Tb.Sp, Tb.N, Tb.Pf, cortical wall thickness (Figure 5).

Histological analysis

After μ CT imaging, knee joints were rinsed in PBS, decalcified in a very mild formic acid-based decalcifier (15x vols; 1414-1, Immunocal™, StatLab Medical Products) for 10 days with 4 changes, equilibrated with PBS for 2 hours, and embedded in paraffin blocks. Coronal sections (4 μ m thickness) of knee joints were cut. For IHC, slides with paraffin tissue sections were heated at 55-58°C for 4 hours to avoid falling during multiple steps of the staining procedure. Paraffin sections, for all the histological and IHC stainings, were deparaffinized, cleared and hydrated by passing the slides thorough Histo-Clear II® (3x, 3 min, 64111-01, EM Sciences), and decreasing concentration of ethanol [(100%, 95%, 70%, 0% (dH₂O), 3x, 3 min each], and finally immersed in fresh dH₂O. Deparaffinized hydrated knee sections were used in the following procedures.

H&E staining: Coronal sections of rat knee were stained using H&E protocol. For that, the deparaffinized hydrated sections were stained with Mayer’s hematoxylin solution (MHS-16, Sigma-Aldrich)

for 10 min, washed in warm tap water for 10 min, rinsed once in distilled water, dipped in 95% ethanol (10x, 1 sec), and stained with eosin (HT110316, Eosin Y solution with phloxine, Sigmaaldrich) for 45 secs. The slides were dehydrated in 95% ethanol (3x, 3 min), 100% ethanol (3x, 3 min), cleared in Histo-Clear II® (3x, 3 min) and mounted in Omnimount™ mounting medium (17997, EM sciences). Mounted slides were scanned at 20x magnification using Aperio ScanScope XT. The images were opened in a PC computer using the image analysis software Aperio ImageScope (v10). The images were saved as tif files. Nuclei presented blue and cytoplasm as pink coloration.

Safranin O staining: Safranin O binds to glycosaminoglycan and the staining represents the presence of proteoglycans in the cartilage. The deparaffinized hydrated knee sections were stained in freshly reconstituted Weigert’s iron hematoxylin (1x, 5 min), rinsed in changes of dH₂O till leaching of blue coloration stopped. Sections were differentiated in 1% acid-alcohol (2 secs), rinsed in dH₂O (3x), treated with 0.02% Fast Green (1x, 15 min), cleared in 1% acetic acid (10 secs), and finally treated with 1% safranin O (20800, EM Sciences) for 10 min. The sections were dehydrated in 95% ethanol (4x, 15 secs) and 100% ethanol (4x, 15secs) and cleared in Histo-Clear II® (3x, 3min) and mounted in Omnimount™ mounting medium (17997, EM sciences). Proteoglycans stains orangered, cytoplasm as gray green and nuclei as black. Mounted slides were scanned, and saved as tif files as described earlier.

CT scan parameters	
Total Rotations	360°
Rotation Steps	180
Number of Calibrations	20
Settle Time	200 ms
FOV	256 × 3072
Binning	2
Exposure Time	1500 s
Average Frames	1
Effective Pixels size	18 μ m.40
System magnification	High
Voltage	80 KV
Current	500 μ A
Thresholds	
-368.664 – 350.793	Trabecular
-839.079 – 350.793	Bone marrow

Table 1: μ CT scan parameters used in the study.

Picrosirius red (PSR) staining, and Polarization light microscopy: The deparaffinized hydrated knee sections were stained with PSR stain kit following the manufacturer's instructions (24901,

Polysciences, Inc.). PSR-stained sections were dehydrated by passing through increasing concentration of ethanol (70%, 95%, 100%, 3x, 3 min each) and cleared through Histo-Clear II® (3x, 3 min). The sections were mounted in Omnimount™ mounting medium. Mounted slides

were viewed under upright brightfield and polarization Olympus BX51 microscope. The collagen stains bright red color under light microscope, whereas, under polarization light, collagen shows different colors based on thickness of fibers: thick (red/orange/yellow), thin (green), and thick/thin mix (lime green-blue).

Immunohistochemistry (IHC): The deparaffinized and hydrated paraffin sections of knee joints were processed for antigen retrieval by enzymatic treatment as described in Table 2. The sections were washed in dH₂O (3x, 3 min), treated with freshly diluted 3% H₂O₂ (H1009, Hydrogen peroxide solution, Sigma- Aldrich), and rinsed in dH₂O (4x). The sections were incubated with blocking buffer (Table 2) for 30 min, replaced with primary antibody (Table 2) and incubated at 4°C for overnight. The sections were washed with PBS-T (Table 2; 4x, 5 min), treated with blocking buffer for 30 min, replaced with secondary antibody (Table 2) and incubated for 30 min at room temperature. The sections were washed with PBS-T (4x, 5 min) and incubated with ABC reagent [ABC reagent prepared 30 min before incubation (PK-6100, Elite ABC-HRP kit, Vector)] for 30 min. The sections were washed with PBS-T (2x, 1 min) and then with dH₂O (4x, 1 min). The light to dark brown color (i.e., peroxidase reaction) was developed using 0.4 ml freshly diluted peroxidase substrate, diaminobenzidine (DAB; Sk-4105, ImmPACT™ DAB Substrate, Vector) on sections for 2-3 min while watching under the stereomicroscope depending upon the peroxidase activity. Immersing the slides in dH₂O stopped the reaction. The slides, once rinsed in dH₂O, were stained in Meyer's hematoxylin (MHS-16, Sigma) for 4 min, rinsed in changes of dH₂O till leaching of blue coloration stopped. The sections were dehydrated in the increasing concentrations of ethanol (70%, 95% and 100% (3x, 3 min, each), cleared in Histo-Clear II® (3x, 3 min) and mounted in Omnimount™ mounting medium. Mounted slides were scanned and saved as tif files as described earlier. Human OA knee cartilage or human normal tracheal cartilage or post-natal day 1 (P1) mouse spine, were used as positive control during various histological or IHC procedures. The human OA knee specimens were obtained from two OA patients (age, 58, 64 year) undergoing knee joint replacement surgery at Toronto Western Hospital, Toronto. The protocol, to collect human specimens from patients, was approved by research ethics board (REB) committee of University Health Network, Toronto. Mouse tissue was obtained

from a recently died P1 pup obtained from Animal Resources Center of University Health Network, Toronto. Paraffin sections of normal human (age, 58 year) tracheal cartilage were purchased (CAR01, Pantomics, Inc. Richmond, CA).

Statistical analysis

The μ CT scan data was analyzed by one-way ANOVA using Tukey HSD all pair comparison ($\alpha=0.05$). N=10.

Results

Medial meniscectomy (MMx)

The medial meniscectomy was achieved successfully in rat by using a less invasive approach. The procedure did not require transection of MCL and opening of the knee capsule wide during the surgery, rather a small slit was made on the medial side of knee capsule to pull out the majority of medial meniscus (Figure 1). Rats recovered within one week post-surgery without complication, and remained healthy throughout the experimental period of 10 weeks post-surgery.

X-ray radiography, and μ CT scan analysis of knee joints

Images of X-ray radiographs showed that MMx was successful in rat using the less invasive procedure. There was no noticeable bone sclerosis or subchondral bone cyst in MMx knee (Figures 2A-2D). There was a gap visible between medial femur and medial tibial plateau in MMx knee as compared to control counterpart indicating the absence of medial meniscus (Figures 2C and 2D). There was no noticeable osteophyte in MMx knee joint compartment. Cortical and subchondral ROIs of medial femur and medial tibia were chosen for μ CT analysis and are shown as 3-D representation images (Figures 3A and 3B). The μ CT scan of MMx knee showed a gap between medial femoral condyle and medial tibia confirming the absence of medial meniscus and success of the less invasive procedure (Figures 4A and 4B). The analysis of the subchondral bone strength parameters is shown in Figures 5A-5F. BV/TV ratio, in MMx knee, decreased at all the 4 ROIs (tibial cortical, tibial subchondral, femoral cortical, femoral subchondral) as compared to control knee, though the change was not statistically significant (Figure 5A). Of all, the most affected ROI was femoral subchondral (p, 0.09). Tb.Th, measured at 2 ROIs (tibial

Antigen	Primary antibody	Secondary antibody	Antigen retrieval
Type II collagen (COL-II)	Mouse monoclonal, anti-COL II, II-II6B3, DSHB	Biotinylated horse anti- mouse IgG, rat adsorbed, BA-2001, Vector	*
Denatured COL II ¾ fragment (Col2-3/4m)	Mouse monoclonal, Col2-3/4m antibody, 50-1011, IBEX Pharmaceuticals Inc.	Same as above	**
Type-X collagen (COL-X)	Mouse monoclonal, anti-COL X 2031501001, Quartett, GE	Same as above	*
Lubricin (PRG4)	Mouse monoclonal, Anti- lubricin (9G3).	Gregory Jay's lab	*
NITEGE	Rabbit polyclonal antibody to aggrecan N-terminal neopeptide NITEGE (BC13), NB100- 74350, NOVUS	Biotinylated horse anti- rabbit IgG, BA-1100, Vector	***
Aggrecan	Rabbit polyclonal, aggrecan antibody, Ab36861, abcam	Same as above	***
MMP13	Rabbit Polyclonal, MMP13 antibody, ab39012, abcam	Same as above	****

Negative controls contained no primary antibody and contained normal IgG isotype control;

Each antibody was diluted in blocking buffer that constituted of PBS-T with 2% BSA (ALB-001, Albumin bovine serum fraction V, Bioshop) and 2% horse serum (16050122, GIBCO) or Donkey serum (D9663, Sigma-Aldrich); PBS-T was composed of D-PBS containing 0.05% Tween-20 (TWN510, Bioshop). The antigen retrieval for knee sections and controls used were as follows:

*The hydrated tissue sections were treated with pepsin (P-7000, Sigma-Aldrich; 4 mg/ml in 0.01N HCl) for 10 min at 37°C, washed with dH₂O (4x, 1 min), treated with hyaluronidase (H-3506, Sigma-Aldrich) solution at 1 mg/ml (in 0.1M phosphate buffer, pH 5.0) for 30 min at 37°C, and rinsed in PBS-T (3x, 2min);

**The hydrated tissue sections were treated with hyaluronidase at 10 mg/ml (in 0.1M phosphate buffer, pH 5.0) for 30 min at 37°C, and rinsed in PBS-T (3x, 2min);

***The hydrated tissue sections were treated with hyaluronidase at 25 mg/ml (in 0.1M phosphate buffer, pH 5.0) for 35 min at 37°C, rinsed in PBS-T (4x, 1 min), treated with chondroitinase ABC (C3667, Sigma-Aldrich; 0.125 Unit/ml in 0.01M citrate buffer, pH 6.0) for 45 min at 37°C, and rinsed in PBS-T (3x, 2min);

****The hydrated tissue sections were treated with Proteinase K [(25530-049, Invitrogen; 20 μ g/ml in TE buffer (50mM TRIS with 1mM EDTA and 0.5% Triton X-100, pH 8.0)] for 15 min at 37° followed by slow cooling for 5 min, and rinsed in PBS-T (3x, 2min).

Table 2: List of antibodies used for immunohistochemistry.

subchondral, femoral subchondral), did not differ between MMx and control knee (Figure 5B). Tb.Sp was higher at all the 4 ROIs (tibial cortical, tibial subchondral, femoral cortical, femoral subchondral) in the MMx knee as compared to control knee, indicating more porosity at all the regions, however the values were not statistically different from control knee (Figure 5C). The most affected ROI was femoral cortical ($p, 0.07$). Tb.Pf, measured at all the 4 ROIs (tibial cortical, tibial subchondral, femoral cortical, femoral subchondral) showed alterations in their values in MMx knee joint as compared to control counterparts, indicating a 4-D distortion in all the regions, however, these changes were not significantly different (Figure 5D). Tb.N, measured at 2 ROIs (tibial subchondral, femoral subchondral), were not different between MMx and control knee (Figure 5E). Cortical wall thickness was measured at all the 4 ROIs (tibial cortical, tibial subchondral, femoral cortical, femoral subchondral) in MMx knee joint, and their values did not differ significantly from the control knee. The most affected ROI was tibial cortical with p value of 0.14 (Figure 5F).

Histological assessment of knee sections: H&E, Safranin O, and PSR staining: Collagen fibers evaluation

H&E staining of coronal sections from medial knee joint showed thinning and fibrillation of articular cartilage on the medial aspect of tibia in MMx knee (Figures 6B and 6B1), whereas, control knee showed no fibrillation (Figures 6A and 6A1). There was narrowing of space between femur and tibia in MMx group as compared to control group (Figure 6B vs. 6A). Chondrocytes were arranged in parallel rows in the deep zone of tibial articular cartilage in control knee (Figure 6A1), whereas, the parallel cellular arrangement was disrupted in the tibial

articular cartilage of MMx knee (Figure 6B1). There was an increase in the number of hypertrophic chondrocytes in the calcified cartilage zone of articular cartilage in MMx knee (Figure 6B1) as compared to control knee (Figure 6A1). A non-osseous fibrotic tissue was present at the medial end of tibia in the MMx knee (Figure 6B1), however there was no osteophyte. Human OA knee cartilage, serving as positive control, showed H&E staining (Figures 6C and 6C1). Decreased metachromasia and fibrillation is shown in human OA knee cartilage (Figures 6C and 6C1). In summary, H&E staining showed that the less invasive MMx causes degenerative changes in articular cartilage of medial joint in rat. Articular cartilage from MMx and control knee stained positive with Safranin O for proteoglycans showing red coloration (Figures 7A and 7B). However, there was substantial loss of staining, dominantly in the medial aspect of tibia including fibrillation area, and in the femur of MMx knee (Figure 7B). Articular cartilage from human OA knee, serving as positive control, showed positive staining with safranin O. However, there was a loss of staining in the region of longitudinal separation of collagen bundles (Figure 7C) and fibrillation (Figure 7D) in the superficial layers of cartilage. Picrosirius red (PSR) staining exhibited red coloration in the articular cartilage of tibia and femur in medial control (Figure 8A) as well as in MMx (Figure 8C) knee joint under light microscopy. However, there was a reduction in the intensity of red coloration (i.e., decreased metachromasia), more in tibial cartilage and less in femoral cartilage in MMx knee (Figure 8C1) as compared to control knee (Figure 8A1). Articular cartilage from human OA knee, serving as positive control, stained positive for PSR (Figure 8E) and also showed decreased metachromasia (Figure 8F) under light microscopy. In the control knee, the polarization light

Figure 6: H&E staining of articular cartilage. Control (A), and MMx (B) knee joint. Human OA knee articular cartilage, served as positive control (C). A1, B1, and C1 are higher magnification of boxed area in A, B, and C, respectively. Note fibrillation in the articular cartilage (white arrowheads) and thinning of articular cartilage at the medial aspect of tibia, and the presence of a non-osseous fibrotic tissue (green arrowhead) at the medial end of tibia in MMx knee (B1). Chondrocytes are arranged in parallel rows (green arrows) in the tibial articular cartilage in control knee (A1), the parallel cellular arrangement disrupted in MMx knee (B1). Note an increased number of hypertrophic chondrocytes (yellow arrows) in the calcified cartilage zone in MMx knee (B1) as compared to control knee (A1). There is narrowing of space between tibia and femur in MMx knee joint as compared to control knee joint (B vs. A). Human OA knee cartilage, serving as a positive control, shows H&E staining (C, C1). Fibrillation (white arrowheads), and decreased metachromasia, is shown in human OA knee articular cartilage (C1). M, meniscus; S, synovium; CL, cruciate ligament; AC, articular cartilage.

Figure 7: Safranin O staining in the articular cartilage. Control (A), and MMx (B) knee joint. Human OA knee articular cartilage, served as positive control (C, D). Articular cartilage from MMx knee as well as from control knee stained with Safranin O showing red coloration (8A, 8B). However, note the substantial loss of staining, dominantly in the medial aspect of tibia including fibrillation area (white arrowheads), and in the femur of MMx knee (8B). Articular cartilage from human OA knee shows positive staining with safranin O. However, there was a loss of staining in the region of longitudinal separation of collagen bundles (C) and fibrillation (D). (Safranin O staining)

microscopy of mounted PSR-stained sections showed green coloration in superficial zone (indication of thin fibers) and the mixture of colors 'orange to red' (thick fibers) and green (thin fibers) indicating more thick and less thin 9 fibers in other part of the articular cartilage (Figures 8B-8B2a). In MMx knee, a large part of tibial and also a small part of femoral articular cartilage exhibited green coloration indicating the conversion of thick fibers into thin fibers in MMx knee articular cartilage (Figures 8D-D2a). Collagen fibers orientation, in fibrillated cartilage, was disrupted in MMx knee as compared to parallel collagen fibers arrangement in control knee (Figure 8D2a vs. 8B2a). Articular cartilage from human OA knee, serving as a positive control, showed mixture of colors but dominated by green color, indicating OA cartilage is rich in thin fibers (Figure 8F). Collagen fibers, in the surface area of human OA articular cartilage containing longitudinal collagen bundles, were disorganized and were not parallel to surface (Figures 8F and 8F1). In brief, the less invasive MMx caused alteration in collagen network and disruption of collagen fibers orientation in articular cartilage.

Immunohistochemistry (IHC)

Aggrecan expressed in the articular cartilage of control and MMx knee (Figures 9A and 9B). However, aggrecan staining was decreased in the articular cartilage of MMx knee as compared to control knee (Figure 9B vs. 9A). Higher magnification of tibia shows that reduction or loss of aggrecan staining was dominant in the fibrillation area (red arrowheads) as compared to other part of the tibia in MMx knee (Figure 9B1). Human OA knee articular cartilage, serving as positive control, showed areas where aggrecan was expressed (Figures 9C and 9C1) but also showed damaged areas where aggrecan was absent (Figure 9C, red arrows). Note that aggrecan showed territorial and inter-territorial expression in control articular cartilage (Figure 9A1), whereas, expression was more territorial in MMx knee articular cartilage (Figure 9B1), and human OA knee articular cartilage as well (Figure 9C1). Cleaved aggrecan neopeptide, G1 NITEGE374 (NITEGE) expressed in the articular cartilage of control knee (Figures

9D-9D2) and MMx knee (Figures 9E-9E2). However, the intensity of NITEGE expression was higher in all areas of articular cartilage except the fibrillated area where there was less aggrecan available (Figures 9E-9E2). Human OA knee articular cartilage, serving as a positive control, showed NITEGE staining (Figures 9F and 9F1). COL-II showed expression in the articular cartilage of control and MMx knee (Figures 10A and 10B). The staining in MMx knee was reduced (Figures 10B and 10B1) as compared to control knee (Figures 10A and 10A1). Articular cartilage, in human OA knee (Figures 10C-10C2) and in human normal trachea (Figure 10D) serving as positive controls, showed COL-II expression. Denatured COL-II fragment $\frac{3}{4}$ (-2-3/4 m) expressed at higher level in the articular cartilage of MMx knee as compared to control knee (Figures 10F1 and 10F2 vs. 10E1 and 10E2). As a positive control, human OA knee articular cartilage showed intense staining for Col2-3/4m (Figures 10G-10G2), the intensity varied from fibrillated to non-fibrillated region, and from superficial zone to deep zone (Figures 10G-10G2). COL-X expression was low, and restricted to hypertrophic chondrocytes near the tidemark in the articular cartilage, in control rat knee (Figures 11A-11A2). However, the 10 intensity of COL-X expression increased around tidemark and the in calcified cartilage zone; the expression further extended to non-calcified deep zone (Figures 11B1 and 11B2). P1 mouse spinal vertebrae, serving as positive control, showed COL-X expression in the hypertrophic chondrocytes (Figures 11C and 11C1). MMP13 expressed in the tangential, transitional, radial, and calcified cartilage zone of the articular cartilage with variation in intensities among the zones in the control knee (Figures 12A-12A2), as well as in the MMx knee (Figures 12B-12B2). The expression level was higher in the articular cartilage of MMx knee as compared to control knee (Figures 12B1 and 12B2 vs. 12A1 and 12A2). Human OA knee articular cartilage, serving as positive control, showed MMP13 expression, in all the zones but with variation in the intensity among the zones (Figures 12C-12C3). Lubricin (PRG4) expressed in the superficial zone of articular cartilage in the control knee (Figures 13A-13A2). The expression was also present in the articular cartilage in MMx knee (Figures 13B-13B2) except in

(Picrosirius red staining)

Figure 8: Picrosirius red (PSR) staining. Light microscopy and polarization light microscopy of control and MMx rat knee joint (A-D). Light microscopy of PSR-stained coronal sections of control (A), and MMx (C) knee joint. Polarization light microscopy of PSR-stained sections of control (B) and MMx (D) knee joint. PSR-stained human OA knee articular cartilage, served as positive control for light microscopy (E) and for polarization light microscopy (F). A1, B1/B2, C1 and D1/D2 are higher magnification of boxed area in A, B, C and D, respectively. B1a, B2a, D1a, D2a and F1 are higher magnification of boxed area in B1, B2, D1, D2 and F, respectively. Key observations noted are described as below: §♣ Decreased intensity of PSR staining (i.e., decreased metachromasia) in the articular cartilage of MMx knee as compared to control knee (C1 vs. A1) is shown; §♣ More thick collagen fibers (orange-red colored) and less thin collagen fibers (green colored) present in the articular cartilage of control knee (B), on the other hand, more thin collagen fibers (green colored) and less thick collagen fibers (orange-red colored) present in the articular cartilage of MMx Knee (D); §♣ At higher magnification, it is more clear that more thin collagen fibers (green colored) in MMx knee articular cartilage (D1a) as compared to control knee articular cartilage (B1a, B2a) are present; §♣ D2a (MMx) being fibrillated, most of collagen fibers are missing, cannot be compared with non-fibrillated region of B2a (control knee); §♣ Collagen fiber orientation of surface zone of D2a (MMx) is impaired as compared to that of B2a (control); Fibrillation area (D3a) showing under light microscopy §♣ Decreased metachromasia in PSR-stained human OA articular cartilage (E); Human OA knee articular cartilage (F1) contains mainly thin collagen fibers (green coloration) under polarization light; §♣ Surface of human OA articular cartilage contains longitudinal separation of collagen bundles. Surface collagen fibers are not aligned parallel; rather collagen fibers are disoriented (F1).

Figure 9: IHC of aggrecan the articular cartilage. Control (A), and MMx (B) knee joint. Human OA knee articular cartilage, served as positive control for aggrecan. A1, B1, and C1 are higher magnification of boxed area in A, B and C, respectively. Aggrecan expressed in the articular cartilage of control and MMx knee (A and B). However, aggrecan staining was decreased in the articular cartilage of MMx knee as compared to control knee (B vs. A). Higher magnification of tibia shows that reduction or loss of aggrecan staining was dominant in the fibrillation area (red arrowheads) as compared to other part of the tibia in MMx knee (B1). Human OA knee articular cartilage, serving as positive control, showed areas where aggrecan was expressed (C. C1) but also shows damaged areas where aggrecan was absent (C, red arrows). Note that aggrecan showed territorial and inter-territorial expression in control articular cartilage (A1), whereas, expression was more territorial in MMx knee articular cartilage (B1) and human OA articular cartilage (C1). IHC of NITEGE in the articular cartilage. Cleaved aggrecan neopeptide, G1 NITEGE374 (NITEGE) IHC in control (D), and MMx (E) knee joint. Human OA knee articular cartilage, served as positive control for NITEGE (F). Rat femoral articular cartilage, served as negative control (G). D1/D2, E1/E2, F1 and G1 are higher magnification of boxed area in D, E, F and G, respectively. NITEGE expressed in the articular cartilage of control knee (D, D1, D2) and MMx knee (E, E1, E2). However, the intensity of NITEGE expression was higher in all areas of articular cartilage except the fibrillated area where there was no aggrecan available (E, E1, E2). Human OA knee articular cartilage, serving as a positive control, showed NITEGE staining (F, F1). Rat femoral articular cartilage, serving as negative control for aggrecan and NITEGE, showed no staining in the articular cartilage (G, G1).

Figure 10: IHC of COL-II in the articular cartilage. Control (A), and MMx (B) knee joint. Human OA knee articular cartilage, and human normal tracheal cartilage, served as positive control (C), and rat femur articular cartilage, as negative control (H). A1, B1, C1/C2 are higher magnification of boxed area in A, B, and C, respectively. Articular cartilage showed reduced immunostaining for COL-II in MMx as compared to control knee (B1 vs. A1). Human OA knee articular cartilage, serving as positive control, show reduced positive staining for COL-II (C2) and contained area where staining was lacking (red arrowheads, C1). Human normal tracheal cartilage showed intense COL-II immunostaining (D). IHC of Col2-3/4 in the articular cartilage. Denatured COL-II fragment ¼ (Col2-3/4 m) in the articular cartilage of control (E), and MMx (F) knee joint. Human OA knee articular cartilage, served as positive control (G), and rat femur articular cartilage, as negative control (H). E1/E2, F1/F2, G1/G2, H1 are higher magnification of boxed area in E, F, G and H, respectively. Col2- 3/4m expression was higher in the articular cartilage of MMx knee as compared to control knee (red arrowheads; F1, F2 vs. E1, E2). As a positive control, human OA knee cartilage showed intense staining for Col2-3/4 m (G, G1, G2), the intensity varied from fibrillated to non-fibrillated region, from superficial zone to deep zone (G, G1, G2). Rat femur, serving as a negative control for COL-II and Col-2/3m exhibited no staining (H, H1).

Figure 11: IHC of COL-X in the articular cartilage. Control (A), and MMx (B) knee joint. Mouse P1 spine, served as positive control (C). Rat femoral articular cartilage, served as negative control (D). A1/A2, B1/B2, C1 and D1 are higher magnification of boxed area in A, B, C and D, respectively. COL-X expressed weakly near tidemark (white arrowheads) dominantly in the tibial articular cartilage (A1, A2). However, the intensity of COL-X expression increased around tidemark and in calcified cartilage zone (white arrowheads); and also the expression extended to non-calcified deep zone (green arrowheads, B1, B2). P1 mouse spinal vertebrae, serving as positive control, showed COL-X expression in hypertrophic chondrocytes (C, C1). Rat femur articular cartilage, serving as negative control, exhibited no staining (D, D1).

Figure 12: IHC of matrix metalloproteinase 13 (MMP13) in the articular cartilage. Control (A), and MMx (B) knee joint. Human OA knee articular cartilage, served as positive control (C) and as negative control (D). A1/A2, B1/B2, C1/C2/C3 and D1 are higher magnification of boxed area in A, B, C and D, respectively. MMP13 expressed in the tangential zone (white arrowhead), transitional zone (yellow arrowhead), radial zone (green arrowhead), and calcified cartilage zone (red arrowhead) of articular cartilage in the control knee (A, A1, A2) as well as in the MMx knee (B, B1, B2). The expression level was higher in the articular cartilage of MMx knee as compared to control knee (B1, B2 vs. A1, A2). Human OA knee articular cartilage, serving as positive control, showed MMP13 expression, in all the zones with variation in the intensity among the zones (C, C1, C2, C3). Human OA knee articular cartilage, serving as negative control, did not show staining (D, D1).

the fibrillated region at the medial aspect of tibia where superficial/transitional zone and part of radial zone was damaged in the articular cartilage (Figure 13B2). Human OA knee articular cartilage, serving as positive control, showed lubricin expression in the superficial zone and transitional zone (Figures 13C-13C2).

Discussion

Less invasive MMx causes non-significant changes in subchondral bone in medial knee joint

Animal models of OA are tools for investigating the development of the disease more quickly than human develops OA in real life. Mice or rats are particularly of great interest because of their small size, short life span, breeding efficiency and commercial availability. A number of genetically altered mice are also available at an increasing rate and are being used as surgically-induced OA models that provides a great deal of information on the genes playing role in OA [28]. MMx-induced knee OA is a common experimental procedure in mice and rats [15,21,22] that is to get the first-hand information on the effectiveness of therapeutic agent before testing it on large animals and or in human trials. MMx surgery conducted, on medial knee capsule, involved the following invasive steps, i.e., beginning with transecting

MCL followed by dislodging the patella from the trochlear groove and diverting it laterally and exposing the knee capsule wide-open, and finally transecting the medial meniscus at entheses attachments [15,21,22]. Following the procedure that involved wide opening of knee capsule for MMx, substantial subchondral bone degeneration has been reported in MMx rat knee at 8-weeks post-MMx, as determined by μ CT analysis [23]. Using a joint injury to initiate joint degeneration represents post-traumatic OA. 'Less invasion' to 'more invasion' to cause post-traumatic OA may have significant effects due to the surgical/invasive injury procedure itself, rather than the targeted joint injury [29]. We took a less invasive approach on MMx surgery in the rat. By using eye loupes (4.9x), it was easy to see the location of medial meniscus on medial aspect of the intact knee capsule. Therefore it was easy to avoid transection of MCL, and MMx surgery was conducted with minimum damage to knee capsule without exposing the knee capsule wide-open (Figure 1) and thus causing minimum synovial inflammation in the knee capsule. Synovial inflammation has directly been linked to enhance OA [26]. The major part of meniscus including wide portion was cut removed in our procedure that was responsible for degenerative changes in articular cartilage and also that was equivalent to standard MMx or DMM procedure that causes articular cartilage degeneration as well as subchondral bone degenerative

Figure 13: IHC of lubricin (PRG4) in the articular cartilage. Control (A), and MMx (B) knee joint. Human OA knee articular cartilage, served as positive control (C) and as negative control (D). A1/A2, B1/B2, C1/C2 and D1 are higher magnification of boxed area in A, B, C and D, respectively. Lubricin showed expression mainly in the superficial zone of articular cartilage in the control knee (A, A1, A2). However, in MMx knee, lubricin expression was present in the articular cartilage except in the fibrillation area (arrowheads) at the medial aspect of tibia where superficial/transitional zone and part of deep zone of articular cartilage is damaged. Human OA knee articular cartilage, serving as positive control, shows lubricin expression in the superficial zone and transitional zone (C, C1, C2). Human OA knee articular cartilage, serving as negative control, did not show staining (D, D1).

changes [23]. Osteophytes and subchondral sclerotic changes were reported in mice, at 8 weeks post-surgery for knee instability, imaged at subluxation position by X-ray radiography [30]. We imaged knee joints at different views (PA view of whole body; ML, AP and PA view of knee joint; Figures 2A-2D). We detected no noticeable sized osteophyte at the medial margin of MMx tibia. And also there was no bone sclerosis in subchondral bone region (Figures 2A-2D). X-ray imaging showed that the current less invasive MMx procedure did not cause any subchondral bone degeneration in rat. As shown by μ CT images of knee joint, the MMx procedure was successful in this investigation (Figures 4A and 4B). Bone strength parameters (i.e., BV/TV, Tb.Th, Tb.Sp and Tb.N etc.) have been shown to measure bone strength alterations with age, pharmaceutical intervention or gene mutation [31-34]. Subchondral bone strength parameters changed after OA induction [35]. In an elegant study of collagenase-injected-induced arthritis (CIA) model of mice, subchondral bone damage was shown by significant changes in bone strength parameters (BV/TV, Tb.Sp and Tb.Th) that occurred upto 14 weeks post-treatment [16]. Using similar bone parameters, to measure the subchondral bone strength in a less invasive MMx rat, we discovered that there were no significant changes in the values of bone parameters (BV/TV, Tb.Th, Tb.N, Tb.Sp, Tb.Pf, cortical wall thickness) in tibial and femoral cortical or tibial and femoral subchondral ROIs (Figure 5). In another study of monosodium iodoacetate (MIA)-induced OA model of mice, MIA injection in knee resulted in a significant decrease in BV/TV and an increase in Tb.Sp but with no changes in Tb.N in rats after 5-days of post-treatment [36]. Panahifar and colleagues showed substantial degenerative changes in subchondral bone in rat knee at 8 weeks post-MMx, as determined by μ CT, along with articular cartilage degenerative changes [23]. The current study demonstrated that the less invasive MMx procedure caused no changes in subchondral bone.

Less invasive MMx causes articular cartilage degeneration at moderate OA level

Histological evaluation by H&E staining, proteoglycans, and collagen localization: Fibrillation and thinning of articular cartilage, in the knee joint, has been shown in experimentally induced OA in several species including rat and mouse [15,21,22], and human OA. The narrowing of space is a common feature in OA [2]. Our study showed that the less invasive MMx procedure, in medial rat knee joint, causes thinning and fibrillation of articular cartilage at the medial aspect of medial tibial articular cartilage, and narrowing of medial knee joint (Figures 6B and 6B1). The parallel arrangement of chondrocyte rows has been shown in the knee articular cartilage, and the disruption of the parallel arrangement has been linked to OA [37]. Our study showed that less invasive MMx procedure in rat, caused disruption of parallel arrangement of chondrocyte rows (Figure 6A1 vs. 6B1). We also showed the presence of non-osseous fibrotic tissue at the medial end of tibia (Figure 6B1). In OA joints, periarticular fibrotic deposits have been shown near erosion sites in OA in mice [38]. Our study showed that the less invasive MMx causes an increase in the hypertrophic chondrocytes in calcified cartilage zone in medial tibia (6B1) similar to report by Hayami and team in ACLT, and ACLT with MMx rats [12]. In brief, the less invasive MMx procedure, in rat knee joint, caused a number of degenerative 12 changes in the articular cartilage. Safranin O staining detects proteoglycans (PGs) in the articular cartilage [39]. Earlier studies indicated that the human knee articular cartilage from OA patients exhibited reduction in PGs as shown by reduced safranin O staining [40]. And similar findings are available in animal models. PGs showed reduction in articular cartilage of OA knee joints in 8 weeks post-MMx rats [41]. In the current study, by using safranin

O staining, we showed that there was substantial loss of PGs in the articular cartilage dominantly in the medial aspect of knee joint (Figure 7B). Mild and severe OA cartilage, in human patients, showed the absence of PGs in the area closer to superficial zone [40]. In the current study, we demonstrated that the surgically induced less invasive MMx procedure that was effective in causing degenerative changes in the articular cartilage of rat knee.

Picrosirius red (F3BA) is a linear anionic dye comprising six sulfonate groups that can associate with cationic collagen fibers, and enhance their birefringence under cross-polarized light [42-44]. The collagen fibers in the ECM can be classified as thin fibers ($\leq 0.8 \mu\text{m}$) or as thick fibers (1.6 to $2.4 \mu\text{m}$) [44]. Integrity and organization of the collagen fibers represents the repair activity of articular cartilage [45,46]. Biomechanical models have shown that the changes in collagen network influence the biomechanical behavior of the cartilage [47]. The current study showed decrease in PSR staining or metachromasia in the articular cartilage of MMx knee, indicating alteration in collagen network (Figures 8C and 8C1). Polarization light microscopy of PSRstained cartilage revealed decrease in birefringence intensity in the superficial-middle zones of patellar cartilage of laterally meniscectomized joints in rat [48]. In the current study, MMx caused thinning of collagen fibers in articular cartilage in rat knee as shown by dominance of green coloration under polarization light (Figures 8D-8D2). In brief, the study indicates that the less invasive MMx was effective in causing alteration in collagen fibril network in the articular cartilage. In human OA cartilage, green colored thin fibers were dominating (Figure 8F). Collagen fiber orientation was altered in fibrillated surface region of articular cartilage in MMx knee as compared to control non-fibrillated cartilage, the later being parallel to surface (Figure 8D2a vs. 8B2a). Human OA knee cartilage showed disoriented collagen fibers orientation (Figure 8F1).

Less invasive MMx causes alteration in articular cartilage markers

Aggrecan, and cleaved aggrecan neopeptide (NITEGE): Aggrecan is the major proteoglycan of hyaline cartilage and it associates with hyaluronic acid (HA) and link protein, to form aggregates [49]. The multi-molecular structures can contain >100 molecules of aggrecan and link protein associated with a single HA chain, that give rise to aggregates. The aggregates are hydrated due to their negative charge resulting from the large number of poly-anionic GAG chains on aggrecan, providing cartilage with a high water content about 70% wet weight of the tissue [50]. They act as a space-filling gel and are responsible for the compression and resilience of articular cartilage during joint loading. A mutation in the variable repeat region of the aggrecan gene causes a form of spondyloepiphyseal dysplasia (SEDC) associated with severe premature OA [51]. In knee joint articular cartilage, aggrecan expression was shown to be present in calcified cartilage and non-calcified cartilage zone. Aggrecan contributes to territorial and inter-territorial matrix in mice [52]. In the current study, MMx causes reduced expression of aggrecan in the knee tibial articular cartilage, indicating that the less invasive MMx procedure was effective in causing degenerative changes in the articular cartilage in the medial compartment of the knee (Figure 9B vs. 9A). Suppression of anabolic activity of chondrocytes in the upper zones contributes to the metabolic imbalance observed in OA cartilage [53]. Using ISH, most chondrocytes in the deeper zones of OA cartilage showed an increase in aggrecan mRNA expression, as compared with normal specimens. However, chondrocytes of the upper zone were largely negative for aggrecan mRNA [53]. More loss of aggrecan is shown in medial aspect

of tibial cartilage since that region represents non-physiological contact zone between femur and tibia and that causes fibrillation and some loss of chondrocytes and matrix (Figure 9B1). Aggrecan is composed of three major globular domains: G1, G2, and G3. Between G1 and G2 domains, there is an interlobular domain (IGD) that is the major site of cleavage by specific proteases like MMPs and aggrecanase (ADAMTS) [54]. Aggrecanase cleaves aggrecan in the IGD at Glu373-Ala374, generating a G1 fragment with a COOH terminus of NITEGE373. The cleaved G1 fragment NITEGE373 can be recognized by an antibody called NITEGE [55], the expression of which increased with age in articular cartilage [56]. In a CIA rat model of cartilage degeneration, NITEGE neopeptide increased on day 21 post-injection [57]. The current study showed that the less invasive MMx procedure, in rat the knee, causes degradation of aggrecan as shown by an increase in NITEGE immunostaining in knee articular cartilage (Figures 9E1 and 9E2) except fibrillated region, the later being the damaged area lacked aggrecan to begin with (Figure 9B1).

Type II collagen (COL-II), and Denatured COL-II 3/4 fragment (Col2-3/4m): COL-II is specific for cartilaginous tissues. It is essential for normal embryonic development of the skeleton, linear growth, and for the ability of cartilage to resist compressive forces. The major reason of OA is the degradation of hyaline articular cartilage [58]. Mutation in COL2A1 gene results in abnormal cartilage morphology and bone development. With a single point substitution mutation in COL2A1 in families (Arg75Cys), the kindred displayed early onset OA and mild spondyloepiphyseal dysplasia [59]. MMx caused reduced COL-II expression in rat knee articular cartilage (Figures 10B and 10B1) indicating that the less invasive MMx procedure was effective in causing degenerative changes in the articular cartilage. Kaufman and team reported that ACLT caused significant loss of COL-II immunostaining in articular cartilage surface in rat [60]. In one study, Xu and colleagues showed reduction in COL-II immunostaining in the articular cartilage by more than 60% at 6 weeks post-surgery involving ACLT and MMx [61]. Interstitial collagenases (collagenase-1, -2, and -3) catalyze specific cleavage site of collagen at the intra-helical 775Gly-776Ile bond [62]. This generates characteristic three-quarter (3/4) and one-quarter (1/4) collagen fragments. These fragments are spontaneously denatured and then rapidly degraded by collagenases and gelatinases [63]. Col2-3/4c antibody can detect native form of cleaved Col2-3/4 fragment, whereas, Col2-3/4m antibody can detect degraded form of the same fragment, by immunoassays. We demonstrated the expression of denatured Col2-3/4 fragment in less invasive MMx knee articular cartilage in the medial knee joint by using Col2-3/4m antibody, and that indicated increased COL-II degradation in the articular cartilage (Figures 10F-10F2). On the other hand, control rat knee showed lower immunostaining for Col2-3/4m (Figures 10E-10E2). The immunostaining clearly demonstrates that less invasive MMx causes degenerative changes in articular cartilage.

Type X collagen (COL-X): The synthesis of COL-X is mostly restricted to hypertrophic chondrocytes, regardless of whether these cells are found in growth plate or OA cartilage [64,65]. Mutations in COL10A1 gene has been shown to lead to Schmid metaphyseal chondrodysplasia, a rare autosomal dominant disorder of the skeleton that is manifested in early childhood by short stature, coxa vara and a waddling gait [66]. COL-X mutant mice develop coxa vara, exhibit reduction in thickness of growth plate resting zone cartilage and articular cartilage, and altered bone content [67]. In the current study, the less invasive MMx caused an increase in COL-X expression near tidemark, and in the calcified cartilage zone, and also caused induction of expression in the deep zone of articular cartilage (Figures 11B1 and

11B2). Similar findings have been reported in a study in which ACL was injured and COL-X was assessed at 10 weeks post-injury [68]. Investigation by von der Mark and colleagues demonstrated an irregular distribution of COL-X that was localized around chondrocyte clusters in fibrillated OA cartilage and that was absent from the non-calcified cartilage region of normal articular cartilage [65]. COL-X localization and transient expression at sites of calcification suggest that it is associated with events in early stages of endochondral bone formation. COL-X gene expression was detected in chondrocytes present in OA tissue in that area where there appeared to be a re-initiation of the endochondral bone formation process including osteophytes and in the areas of subchondral bone sclerosis [69]. COL-X was present in hypertrophic chondrocytes in the vertebrae of P1 spine (Figure 11C).

Matrix metalloproteinase 13 (MMP13; Collagenase 3): Matrix metalloproteinase 13 (MMP13) or collagenase 3 is a member of the MMP family, and the MMPs belong to a larger family of proteases known as the metzincin superfamily. During embryonic development, MMP13 is expressed in the skeleton as required for restructuring the collagen matrix for bone mineralization. In pathological situations such as carcinomas, rheumatoid arthritis and OA, it is highly overexpressed [70]. MMP13 is a major enzyme that targets cartilage for degradation. Compared to other MMPs, the expression of MMP13 is more restricted to connective tissue [71]. It not only targets COL-II in cartilage for degradation, but also degrades aggrecan, COL-IV and -IX, osteonectin and pelican in cartilage [72]. Patients with articular cartilage damage have high MMP13 expression suggesting that increased MMP13 is associated with cartilage degradation [73]. In the current study, MMP13 expression was higher in the MMx knee articular cartilage (Figures 12B-12B2) as compared to control knee articular cartilage (Figures 12A-12A2), indicating the success of our less invasive MMx procedure in causing damage to articular cartilage in rat.

Lubricin (PRG4): Lubricin (or proteoglycan 4, PRG4, superficial zone protein, SZP) is a mucinous glycoprotein and is secreted from synovial fibroblasts, and from chondrocytes in the superficial zone of articular 15 cartilage [74]. Lubricin lubricates the apposing and pressurized cartilage surfaces of the joint and provides chondroprotection [75]. In Prg4 knockout mice, surface cartilage of knee joint deteriorates, and the intimal cells become hyperplastic in the joint synovium [76]. Prg4 expressing cells, located at the joint surface at embryonic stage, serve as progenitors for all the deeper layers of mature articular cartilage. Prg4 expressing superficial chondrocytes, in young mice, expands into deeper regions of the articular cartilage as the animal age [77]. Alteration in joint mechanics or joint loading leads to altered lubricin expression in the superficial layer. Lubricin coating was reduced on the articular cartilage surface in meniscectomized sheep [78]. The intensive mechanical loading of knee joint, like running, induce Prg4 expression in the superficial zone of articular cartilage in mice [79]. The maximum Prg4-expressing articular cartilage progenitors occurred in the region of knee joints that experience the highest levels of mechanical loading, and thus lie in the central domains of the condyles [79]. In the current study, less invasive MMx may have caused more expression of lubricin in lateral articular cartilage of femur and tibia (Figure 13B1) and in medial articular cartilage of femur only (Figure 13B2). On the other hand, expression was minimum in the medial aspect of tibial cartilage, the region where the damage to articular cartilage was substantial due to fibrillation (Figure 13B2). The diffused PRG4 staining was found in the region from superficial tangential zone (200µm deep) in OA articular cartilage in human patients [80]. The increased lubricin expression in MMx

knee could be due the resistance of chondrocytes to additional tension due to MMx for the protection mechanism. In the fibrillated area, the tension had gone beyond limit, the addition of lubricin expression may not have helped, rather surface chondrocytes secreting lubricin, were sloughed off (Figure 13B2).

Summary and Conclusion

We applied less invasive approach of MMx to right knee of athymic rats and the left knee served as an unoperated control. At 10 weeks post-MMx, MMx knee exhibited degenerative changes in articular cartilage and caused no significant subchondral bone damage. The less invasive MMx procedure, in rat, can be a model for early or less severe human OA due to articular cartilage degenerative changes only and with no subchondral bone degenerative changes in the knee synovial joint. The therapeutical drugs/agents designed to repair articular cartilage may be suitable for this model and may lead to treatment of early or less severe human OA.

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Conflict of Interest

All the authors have nothing to declare.

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