Bone & Joint Research

Supplementary Material

10.1302/2046-3758.118.BJR-2021-0575.R1

Methods and materials

Outcome measures

Static weightbearing distribution

Knee osteoarthritis (OA) in mice produces asymmetrical changes in downward paw-force distributed between the two hind paws.[1] Static weightbearing (SWB) distribution was evaluated using a SWB apparatus (model #BIO-SWB-TOUCH-M; Bioseb, France) at eight, ten, 12, 14, and 16 weeks post-DMM surgery.

A mouse was gently placed into an acrylic chamber in a quiet, temperature-controlled room. The mouse would naturally rear and make weight adjustments between the hind paws, according to the degree of pain experienced. The mean downward force on the apparatus was recorded via two separate sensor plates under the hind paws. Percentage weightbearing asymmetry was presented as relative values of Right/Left Hind (R/L) in which “100” represented equal weight distributions. Each mouse was tested three times and the mean percentage weightbearing asymmetry was taken into calculation.

von Frey test

Hypersensitivity to pain was assessed by electronic von Frey test at the same intervals as the SWB tests. Mice were individually placed in a wire mesh grid (12 × 10 × 17 cm) in a quiet room for at least 30 mins before testing. A handheld force transducer (model BIO-EVF4; Bioseb) with a 0.5 mm^2 polypropylene pressure tip was used. The tip was applied orthogonally onto the central area of the plantar hind paw, with the investigator (WH) gradually applying more pressure. The minimum force intensity was automatically recorded when the mouse withdrew its hind paw. Hind-paw withdrawal threshold values were taken as the mean value of three trials repeated with an interval of 30 minutes. Percentage withdrawal threshold of R/L was calculated to reflect the relative mechanical allodynia.

Gait analysis

Gait parameters of freely moving mice were measured by a computerized video-based CatWalk gait analysis system[2,3] at the same intervals as the SWB tests. With this system, it was possible to objectively and rapidly quantify gait parameters of locomotion, including duration of different phases of the step cycle and pressure applied during locomotion. Each mouse experienced three trials repeated with an interval of at least 30 minutes.

Briefly, a mouse was placed on an elevated glass platform located in a dark room. It was allowed to move freely on the surface after three days of adaptation training. A light beam from a fluorescent lamp below the platform illuminated the surface (Shanghai Mobile Datum Information Technology, China). This illumination made an image of every footprint, which was recorded by a camera. Walk Analysator software (Shanghai Mobile Datum Information Technology) calculated gait parameters for statistical analysis. Percentage of paw print area, mean placement intensity, leg swing speed, and duty cycle were recorded and analyzed independently as R/L limb. Duty Cycle was defined as follows:

$$Duty\ Cycle = (Stance\ Phase)/(Stance\ Phase + Swing\ Phase)$$

Histological evaluation

Mice were euthanized at 16 weeks after surgery for histological evaluation. Knee joints were dissected free of skin and excess muscle. The joints were immersion-fixed in 4% paraformaldehyde for 24 hours. Whole joints were decalcified for two weeks in 15% ethylenediaminetetraacetic acid (EDTA) on a shaker at 4°C. The decalcified joints were embedded in paraffin and sectioned in the frontal plane, which allowed for concurrent evaluation of the medial and lateral tibiofemoral joints.

Next, the paraffin-embedded block of tissue was sectioned (5 μm) on a microtome for evaluation of the entire articular cartilage. The landmarks we employed for the posterior aspect of the joint were the appearance of the flattened tibial plateau. For the anterior margin, the landmarks we employed were the entry of significant amounts of synovial tissue in the joint space with flattening of the femur and loss of cartilage on the tibia.[4] Two 5 mm sections were placed on each slide in case that if histological artifact happened in one section, we could still retain the other section for further processing. Three consecutive slides were harvested at approximately 80 mm intervals for additional stains or immunohistochemistry. A total of 18 to 21 slides for every knee were mounted to slice and were stored for histological evaluation. At least three representative sections of each knee were stained with Safranin O/Fast green, which stains the proteoglycans in cartilage for assessing OA damage.

The mounted articular cartilage sections were then heated at 60°C overnight. The sections were deparaffinized with xylene, hydrated with decreasing concentrations of ethanol, and treated with a pepsin kit (Solarbio, China) for antigen retrieval. The sections were incubated with a peroxidase blocking kit and 5% bovine serum albumin (Solarbio) before application of primary antibodies. Mounted sections were incubated at 4°C overnight with collagen type II antibody (1:200; Proteintech, China). Excess antibody was then washed off with 1% Tween 20/phosphate-buffered saline, and incubated with horseradish peroxidase (HRP)-polymer-conjugated secondary antibodies. The slide was placed in diaminobenzidine (DAB) substrate (Abcam, UK) for five minutes. Finally, the slide was dipped in haematoxylin for one minute to counterstain the sections. The number of immunopositive cells and the average optical density (AOD) of each section were determined in the software program Image-Pro Plus (Media Cybernetics, USA).

In order to assess the patency of articular cartilage, three slides containing articular sections from each knee were chosen randomly, immunostained for collagen type II and counterstained with Safranin O/Fast green, then photographed and analyzed with a Leica DM6 B microscope imaging system (Leica, Germany). The recommended Osteoarthritis Research Society International (OARSI)[4] semi-quantitative scoring system was used to analyze four quadrants of the joint: medial femoral condyle (MFC); medial tibial plateau (MTP); lateral femoral condyle (LFC); and lateral tibial plateau (LTP). Three slides each were scored by two experienced scorers (YY, KF), and the mean score of each was taken into calculation. Total score of three slides was regarded as the 'Summed OARSI score', and the maximal score of three slides was chosen to be the 'Maximal OARSI score'. We also assessed the sections for inflammatory infiltrate of the articular synovium according to a semi-quantitative synovitis scoring system.[5]

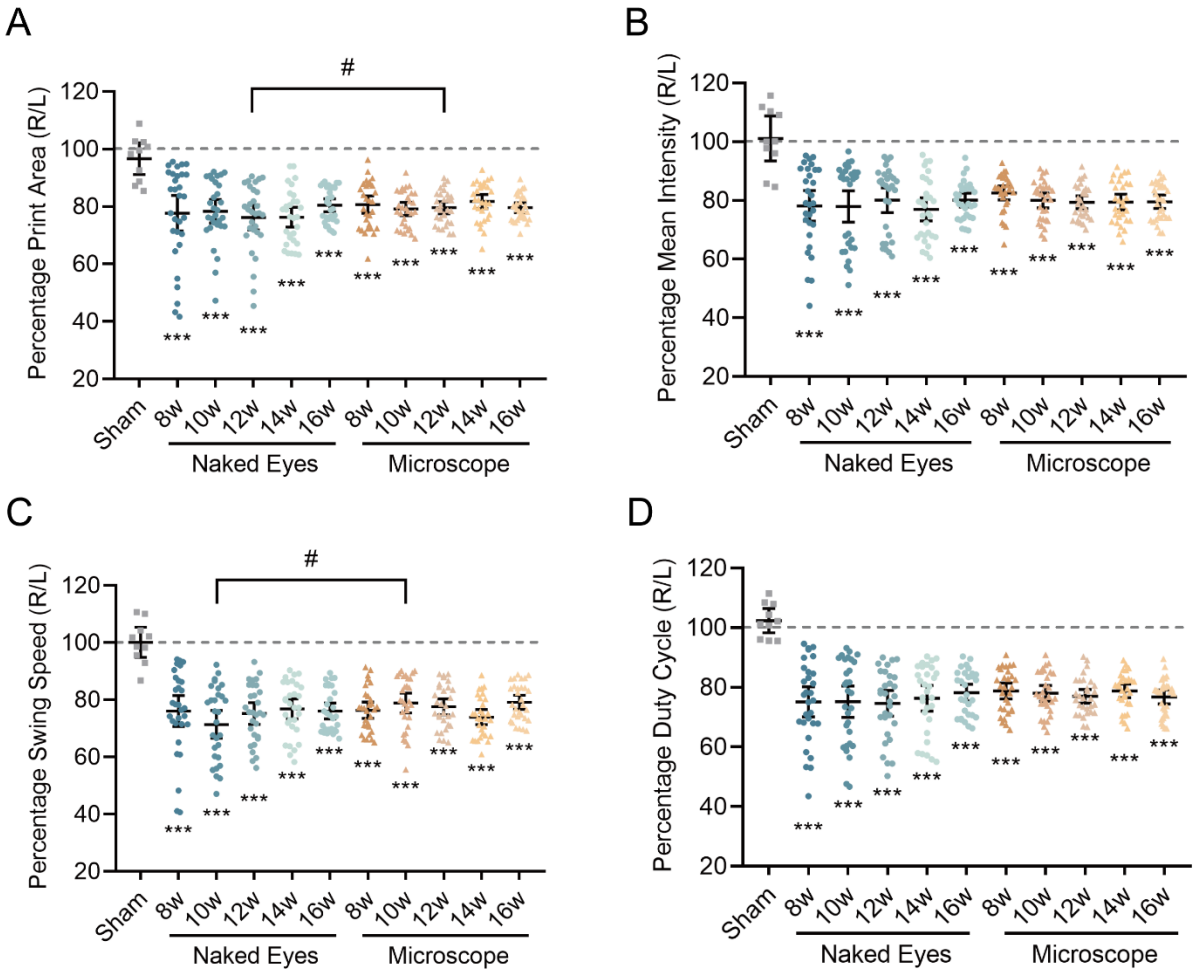

Fig a. a) to d) Quantitative data of gait analysis parameters at 16 weeks after surgery: percentage paw print area, mean intensity, swing speed, and duty cycle for right hind foot/left hind foot. Data are expressed as means with 95% confidence intervals. ***p < 0.001 in comparison to the sham-operated group; #p < 0.05 in comparison to the naked-eye group.

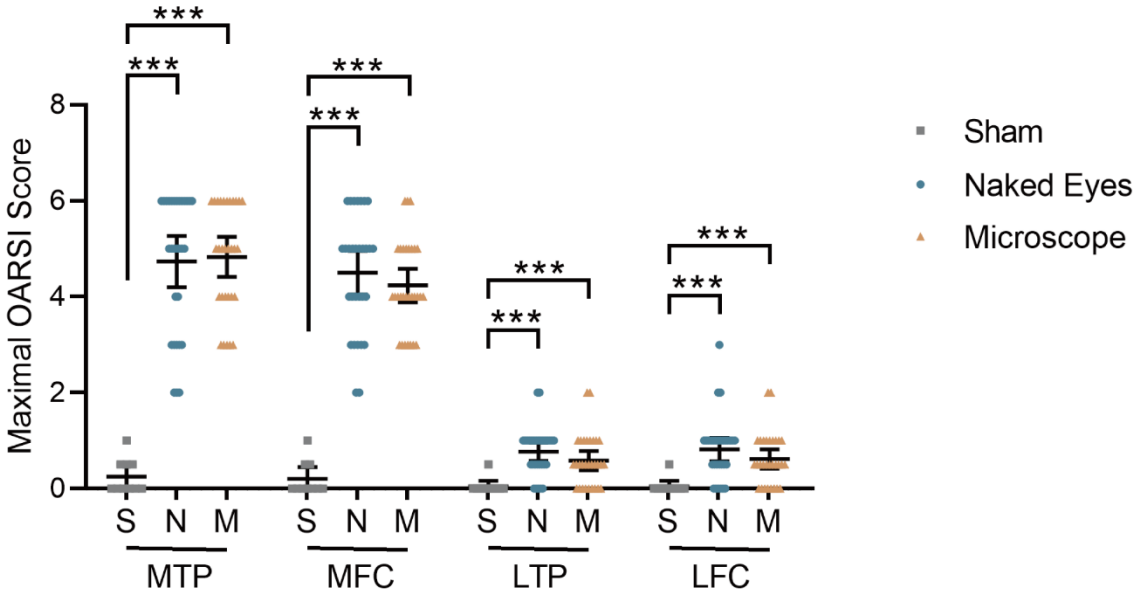

Fig b. The maximal Osteoarthritis Research Society International (OARSI) scores for the four knee joint quadrants. S, Sham group (n = 10); N, naked-eye group (n = 30); M, microscope group (n = 30). Data are expressed as means with 95% confidence intervals. ***p < 0.001 in comparison to the sham-operated group. LFC, lateral femoral condyle; LTP, lateral tibial plateau; MFC, medial femoral condyle; MTP, medial tibial plateau.

References

1. **Haase T, Sunkara V, Kohl B, et al.** Discerning the spatio-temporal disease patterns of surgically induced OA mouse models. *PLoS One*. 2019;14(4):e0213734.

2. **Vrinten DH, Hamers FF.** 'CatWalk' automated quantitative gait analysis as a novel method to assess mechanical allodynia in the rat; a comparison with von Frey testing. *Pain*. 2003;102(1-2):203-209.

3. **Aceves M, Dietz VA, Dulin JN, Jeffery U, Jeffery ND.** An analysis of variability in "CatWalk" locomotor measurements to aid experimental design and interpretation. *eNeuro*. 2020;7(4):ENEURO.0092-20.2020.

4. **Glasson SS, Chambers MG, Van Den Berg WB, Little CB.** The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis Cartilage*. 2010;18 Suppl 3:S17-23.

5. **Jackson MT, Moradi B, Zaki S, et al.** Depletion of protease-activated receptor 2 but not protease-activated receptor 1 may confer protection against osteoarthritis in mice through extracartilaginous mechanisms. *Arthritis Rheumatol*. 2014;66(12):3337-3348.

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The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	Method-Study design-Randomization Method-Study design-Randomization
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	Method-Study design-Randomization Method-Study design-Sample size calculation
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori.* If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of *n* in each experimental group.	Methods-Ethical approval, experimental animals, and maintenance no exclusions Figures 2&4
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	Method-Study design-Randomization Method-Study design-Randomization
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Method-Study design-Blinding
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	Method-Outcome measures Method-Outcome measures
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	Methods-Statistical analysis Methods-Statistical analysis
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	Methods-Ethical approval, experimental animals, and maintenance Methods-Ethical approval, experimental animals, and maintenance
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	Methods-Surgical DMM procedures Methods-Surgical DMM procedures Methods-Surgical DMM procedures Introduction
Results	10	For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	Results Results

The Recommended Set

These items complement the Essential 10 and add important context to the study. Reporting the items in both sets represents best practice.

Item		Recommendation	Section/line number, or reason for not reporting
Abstract	11	Provide an accurate summary of the research objectives, animal species, strain and sex, key methods, principal findings, and study conclusions.	Abstract
Background	12	a. Include sufficient scientific background to understand the rationale and context for the study, and explain the experimental approach. b. Explain how the animal species and model used address the scientific objectives and, where appropriate, the relevance to human biology.	Introduction Introduction
Objectives	13	Clearly describe the research question, research objectives and, where appropriate, specific hypotheses being tested.	Introduction
Ethical statement	14	Provide the name of the ethical review committee or equivalent that has approved the use of animals in this study, and any relevant licence or protocol numbers (if applicable). If ethical approval was not sought or granted, provide a justification.	Methods-Ethical approval, experimental animals, and maintenance
Housing and husbandry	15	Provide details of housing and husbandry conditions, including any environmental enrichment.	Methods-Ethical approval, experimental animal
Animal care and monitoring	16	a. Describe any interventions or steps taken in the experimental protocols to reduce pain, suffering and distress. b. Report any expected or unexpected adverse events. c. Describe the humane endpoints established for the study, the signs that were monitored and the frequency of monitoring. If the study did not have humane endpoints, state this.	Methods-Surgical DMM procedures N/A Methods-Surgical DMM procedures
Interpretation/ scientific implications	17	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including potential sources of bias, limitations of the animal model, and imprecision associated with the results.	Discussion Discussion
Generalisability/ translation	18	Comment on whether, and how, the findings of this study are likely to generalise to other species or experimental conditions, including any relevance to human biology (where appropriate).	N/A
Protocol registration	19	Provide a statement indicating whether a protocol (including the research question, key design features, and analysis plan) was prepared before the study, and if and where this protocol was registered.	N/A
Data access	20	Provide a statement describing if and where study data are available.	N/A
Declaration of interests	21	a. Declare any potential conflicts of interest, including financial and non-financial. If none exist, this should be stated. b. List all funding sources (including grant identifier) and the role of the funder(s) in the design, analysis and reporting of the study.	ICMJE COI statement Funding statement