Bone & Joint Research

Supplementary Material

10.1302/2046-3758.104.BJR-2019-0359.R2

Implantation of minced meniscus embedded in atelocollagen gel into the medial meniscus defect

Japanese white rabbits weighing between 2.5 kg and 3.2 kg were used. Surgery was performed under general anaesthesia by intramuscular injection of xylazine (3 mg/kg) and ketamine (10 mg/kg). Through a medial parapatellar approach, the patella was dislocated laterally, and the medial meniscus was exposed. The anterior half of the medial meniscus was then excised to produce the massive meniscus defect model. Resected meniscus tissue was manually minced to pieces < 1 mm^3 in a Petri dish using a scalpel. In the minced meniscus group (n = 5), 25 mg of the minced meniscus was mixed with 100 µl of atelocollagen, placed in a culture dish to form a firm gel for 30 minutes at 37˚C, and then implanted in the meniscus defect (Supplementary Figure a).

Evaluation of cell migration and proliferation from one fragment of the meniscus in vitro

To evaluate the outgrowth of meniscus cells from one fragment of minced meniscus, 2D and 3D cultures of meniscus fragments were performed. The weight of one meniscus fragment was measured. Then, one fragment was embedded in 100 µl atelocollagen gel and cultured for two weeks for the 3D culture. For the 2D culture, each fragment was placed in the well of a six-well plate and cultured for two weeks. At two weeks, the 3D gel composites were digested by type 1 collagenase (Worthington Biochemical, USA). Then, the cells were isolated and counted. For 2D cultures, cells were treated by trypsin/EDTA and isolated cells were counted. From these counts, the outgrowth cell number per 10 mg of the fragment was obtained.

Three lineage differentiation assay

Medial and lateral menisci of rabbits were harvested from the knee joints. The harvested menisci were minced to pieces < 1 mm^3, washed in phosphate-buffered saline (PBS) three times, and digested with 0.05% (w/v) type I collagenase (Worthington) for one hour at 37°C. The digestion suspension was washed, and the cells were then plated on 10 cm standard tissue culture plates in 10 ml of culture medium and cultured in a 5% CO_2 and 90% humidity incubator at 37°C. To examine the pluripotency of the meniscus cells, three-lineage differentiation assays were conducted using cells after three passages. In order to induce osteogenesis, the cells were cultured in an osteogenesis medium (StemPro osteogenesis differentiation kit, Life Technologies, USA) and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B, Nacalai Tesque, Japan). After an additional three weeks of culture, the cells were stained with 0.5% Alizarin Red solution. To induce adipogenesis, the cells were cultured in an adipogenic medium (StemPro adipogenesis differentiation kit, Life Technologies) and antibiotics. After 14 days, the cultures were stained with 0.3% Oil Red-O solution. For chondrogenesis, 5 × 105 cells were placed in a 15 ml polypropylene tube and pelleted by centrifugation at 500 × g for five minutes. The pellets were cultured for 21 days in the chondrogenic media (StemPro chondrogenesis differentiation kit, Life Technologies) and antibiotics. For histological analysis, the pellets were embedded in paraffin, cut into 6 µm sections, and stained with Safranin-O/Fast Green.

MRI

Three rabbits in the defect and minced meniscus groups were anaesthetized by xylazine (3 mg/kg) and ketamine (10 mg/kg) at four, eight, and 12 weeks, and MRI was performed using a 4.7 T superconducting magnet system (BioSpec47/40USR; Bruker BioSpin, Germany) with a transmit quadrature volume coil (154 mm inner diameter) and 30 mm receiver surface coil. The rabbits were placed on the animal bed in a supine position, and the knees were extended and fixed using a dedicated device; the animals were held inside of the magnet centre. The sagittal MR sequence consisted of Rapid Acquisition with Refocused Echoes (RARE) images (repetition time (TR): 2,000 ms, echo time (TE): 7.4 ms, RARE factor: 6, flip angle (FA): 90° to 180°, matrix: 256 × 256, field of view (FOV): 35 × 35 mm, thickness: 1 mm, number of slices: 10, number of averages: 12, and acquisition time: 12 minutes 48 seconds), T2*-weighted gradient recalled echo (GRE) images (TR: 500 ms, TE: 11 ms, FA: 30°, matrix: 256 × 256, FOV: 35 × 35 mm, thickness: 1 mm,

number of slices: 10, number of averages: 7, and acquisition time: 10 minutes 2 seconds), and Multi-Slice Multi-Echo (MSME) images obtained with the spin-echo technique (TR: 1,800 ms, TE: 9.3 ms to 93.0 ms with interval; 9.3 ms, FA: 90° to 180°, matrix: 256 × 256, FOV: 35 × 35 mm, thickness: 1 mm, number of slices: 10, number of averages: 2, and acquisition time: 15 minutes 22 seconds). The medial meniscus defect was evaluated by two orthopaedic surgeons with over ten years of experience (TN and MI). All measurements of the sagittal section images were performed blindly and independently.

Evaluation of the multipotent cells in the regenerated tissue

To evaluate the contribution of multipotent cells to the regenerative tissue, specimens from postoperative eight weeks were stained with CD44 and CD271, which are recognized markers of MSC. Immunostaining was performed using the anti-CD44 antibody (ab157107; Abcam, UK) and anti-CD271 antibody (14-9400-82, ThermoFisher Scientific, USA). As the secondary antibody for CD44, Alexa Flour 568-conjugated anti-rabbit IgG (Molecular Probes; Invitrogen, USA) was used, and Alexa Flour 488-conjugated anti-rabbit IgG was used for CD271. For nuclear staining, 4′,6-diamidino2-phenylindole (DAPI) solution (Dojindo Laboratories, Japan) was used. As positive controls, rabbit femoral or tibial bone marrow were stained as well.

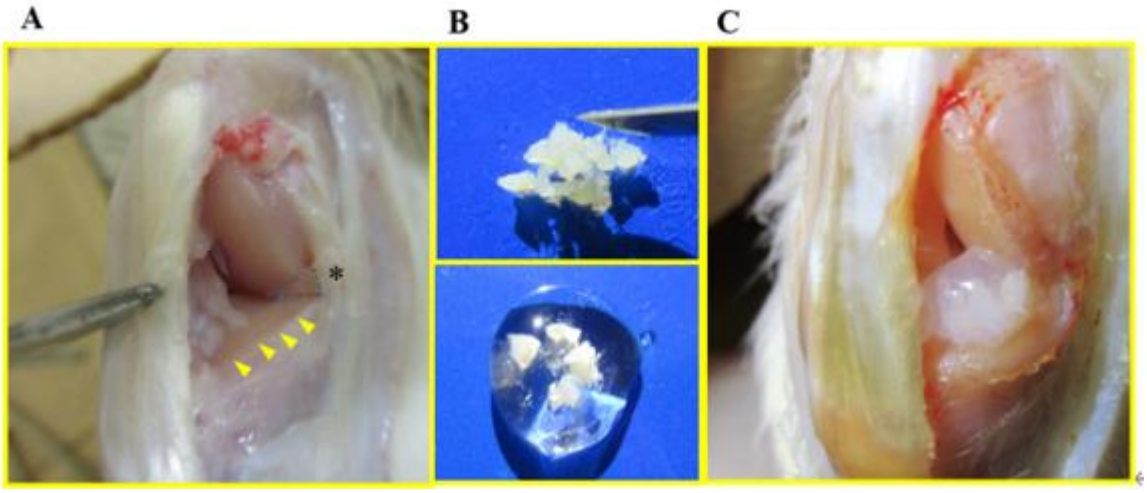

Fig a. a) Anterior half of meniscus was resected. Yellow arrowheads indicate the defect area. Triangle indicates a cross-section of the resected meniscus. * indicates medial collateral ligament. b) Resected meniscus was minced under 1 mm by a scalpel and 25 mg of the minced meniscus was

embedded in 100 µl of atelocollagen gel. c) Solidified gel with minced meniscus was transplanted to the defect site.

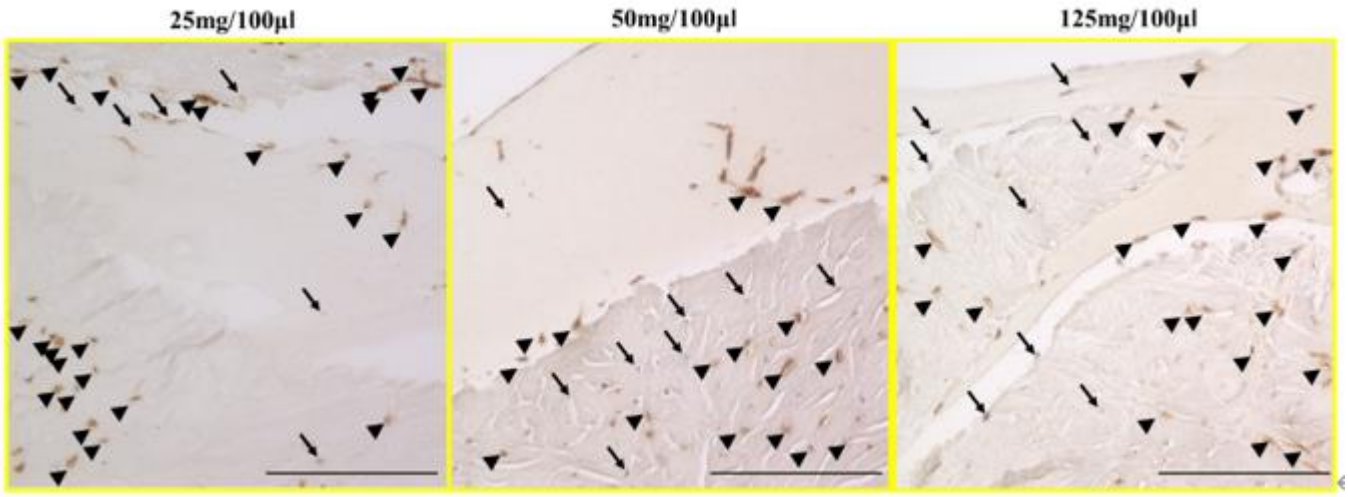

Fig. b. Each group stained by anti-Ki-67 antibody. The negative cells especially exist in the minced meniscus fragment. Most of cells in the gel were positive. Arrows indicate negative cells. Arrowheads indicate positive cells. Bar = 300 µm.

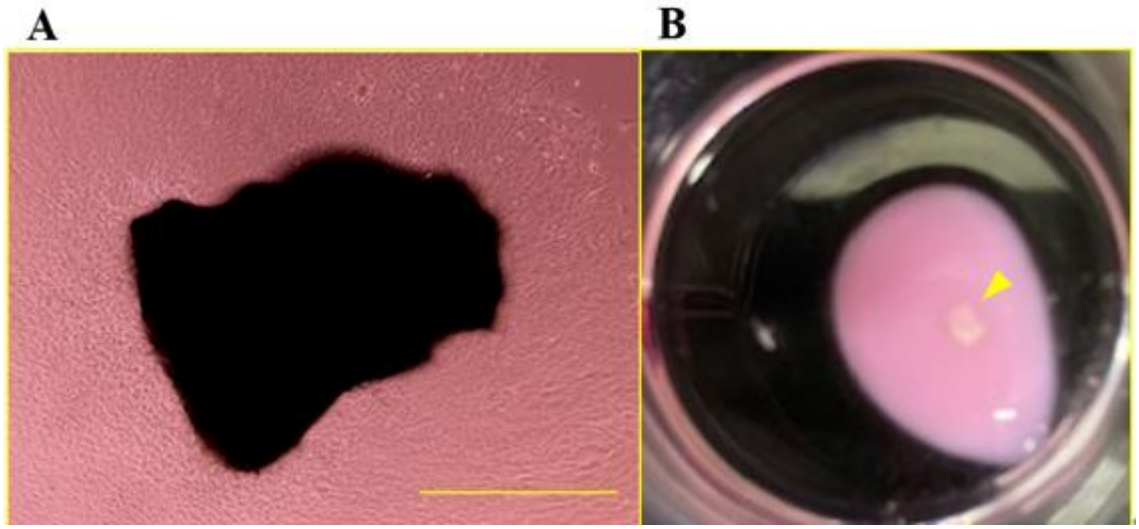

Fig. c. a) Outgrowth of meniscal cells from one piece of minced meniscus in 2D culture. Theoretically, 7.5×10^4 of meniscal cells will outgrowth from 10 mg of meniscus fragment in a 2D culture. Bar = 100 µm. b) Outgrowth of meniscal cells from one fragment of minced meniscus a in 3D culture using atelocollagen gel. Theoretically, 11.3×10^4 of meniscal cells will outgrowth from 10 mg of meniscus fragment in a 3D culture. Yellow arrowhead indicates one fragment of minced meniscus.

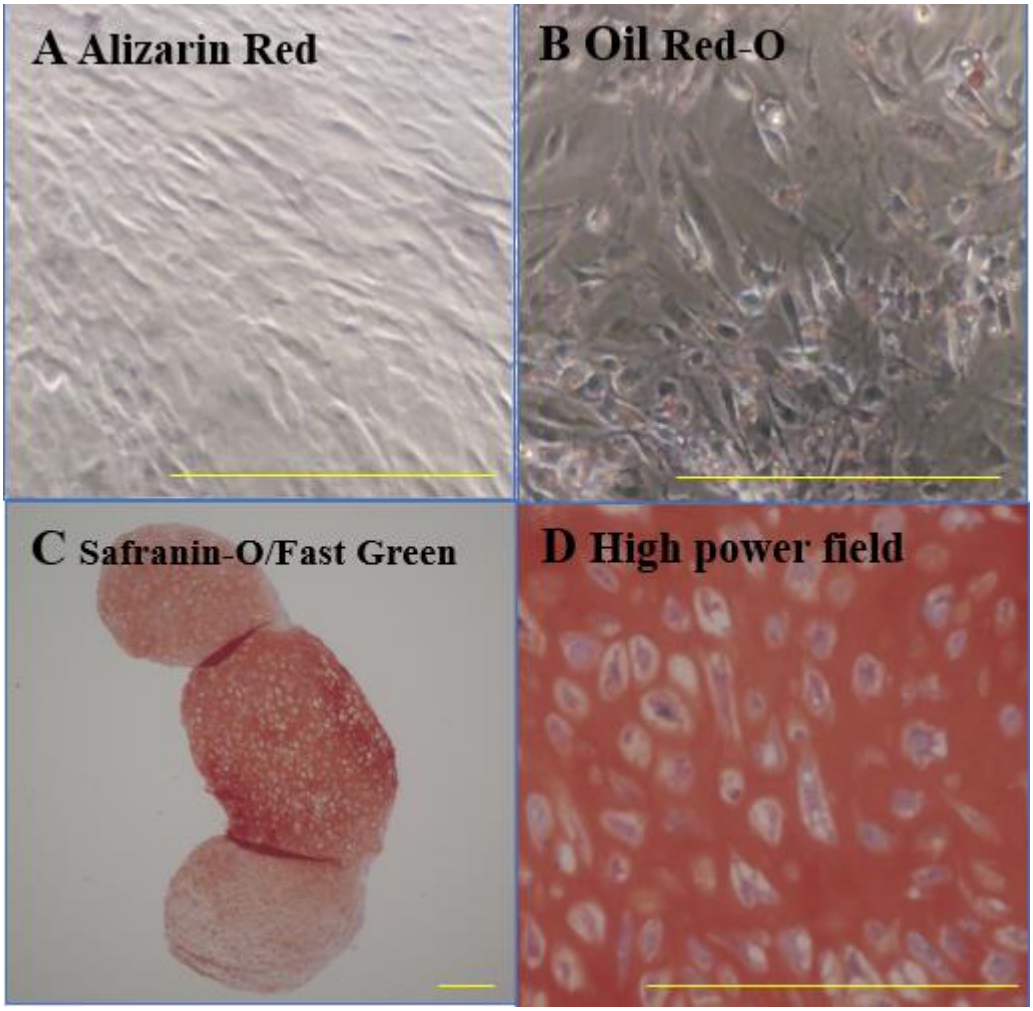

Fig. d. Isolated and cultured meniscus cells were induced for osteogenesis, adipogenesis and chondrogenesis. a) osteogenesis, b) adipogenesis, c) chondrogenesis, lower power field 20×, and d) chondrogenesis, higher power field 200×, bar = 300 μm.

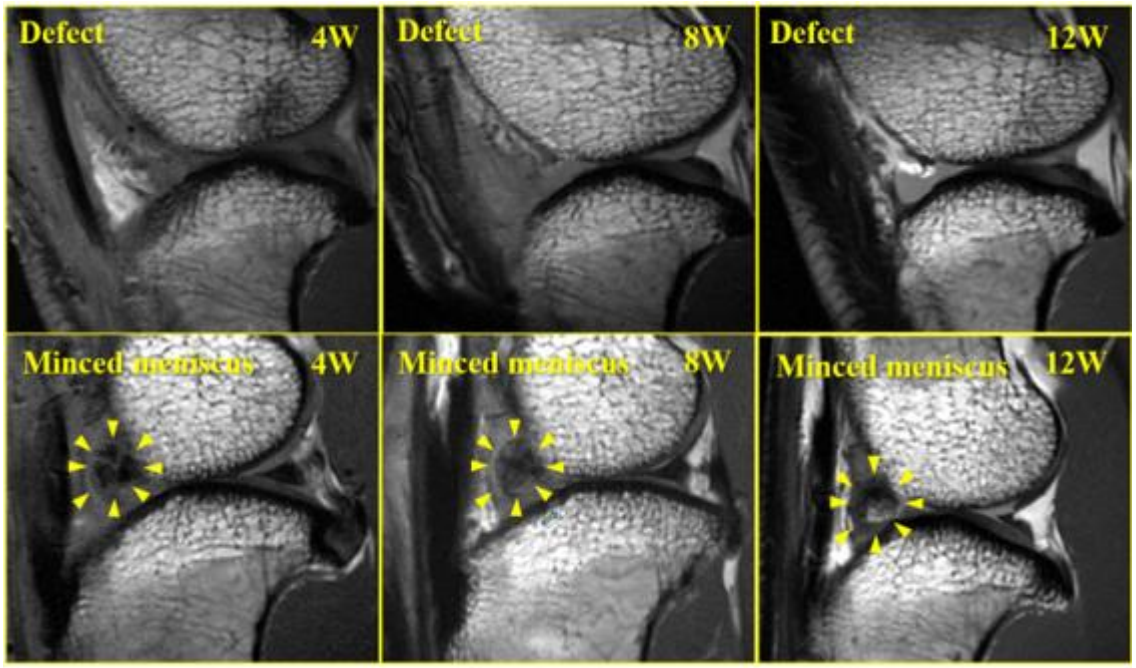

Fig. e. Upper sections (defect groups); the defect sites remained at 12 weeks. Lower sections (minced meniscus groups); implanted tissues exist at the transplanted sites at 12 weeks.

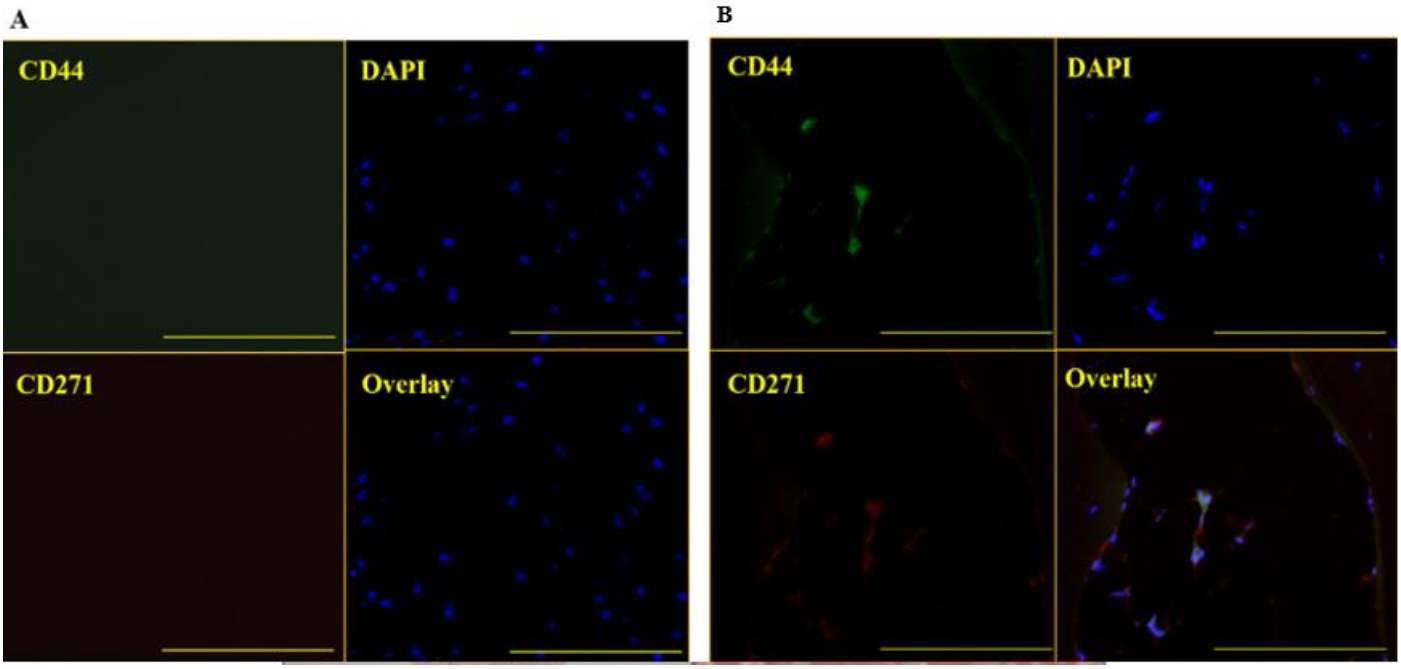

Fig. f. a) Regenerated tissue at eight weeks. b) bone marrow (positive control). CD44 and CD271 positive cells were not observed in the regenerative tissue while there were CD44 and CD271 positive cells in the bone marrow. Bar = 100 μm.

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

Carol Kilkenny[1], William J Browne[2], Innes C Cuthill[3], Michael Emerson[4] and Douglas G Altman[5]

[1]The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, [2]School of Veterinary Science, University of Bristol, Bristol, UK, [3]School of Biological Sciences, University of Bristol, Bristol, UK, [4]National Heart and Lung Institute, Imperial College London, UK, [5]Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Page 1 title
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Page1 Abstract/ paragraph 1
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	a.Page2 Introduction paragraph 1～Page2 paragraph61
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Page2 Introduction
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Page2 paragraph89 ～94
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	Methods Page 2 paragraph 63 ～66
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	Methods animals Page2 paragraph 66～68 ,84～85
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Methods Animals Page2 paragraph 63～66

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	Page2 Methods Animals paragraph89 ～94
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	Page2 Methods Animals paragraph63
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	Page2 Methods Animals paragraph70
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Immnunoche mistry in vitro
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	Page 3 Methods statistical analysis
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Page2 Methods Animals
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	Page2 Methods Animals
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Result Page 3
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	There was no adverse event.
DISCUSSION			
Interpretation/ scientific implications	18	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 any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	RESULT Page14 paragraph 201～202
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Discussion Page6 paragraph 2
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Page 8 Funding

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.