Bone & Joint Research

Supplementary Material

10.1302/2046-3758.131.BJR-2023-0016.R2

Supplementary methods

Reagents and antibodies

Saponin and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma–Aldrich (USA). Antibody against collagen II was from Abcam (USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit was obtained from Biodee Biotechnology (China).

Immunofluorescence assay

Immunofluorescence staining in guinea pig chondrocytes of different ages was performed as previously described.[1] Briefly, cells were fixed with 4% paraformaldehyde for ten minutes, followed by permeabilization with 0.1% saponin for ten minutes. After blocking with normal goat serum for 30 minutes, cells were stained with anti-collagen II antibody for two hours at room temperature followed by FITC-conjugated goat anti-rabbit secondary antibody for one hour at room temperature. Nuclei were counterstained with DAPI. Fluorescence staining was examined by an Olympus IX-71 microscope (Japan).

Densitometry analysis of the immunoblotting data of p-CREB1 regulation of ADAMTS4 expression

Densitometry was performed on the immunoblots shown in Figures 5d and 5e using Image J Software (National Institutes of Health, USA).

Haematoxylin and eosin staining

The knee joint specimens were embedded in paraffin in the coronal plane and cut into 5 μm-thick sections for haematoxylin and eosin staining. The synovitis score was evaluated for synovial lining cell thickness (0 to 3), synovial stroma density (0 to 3), and inflammatory infiltrate degree (0 to 3).[2]

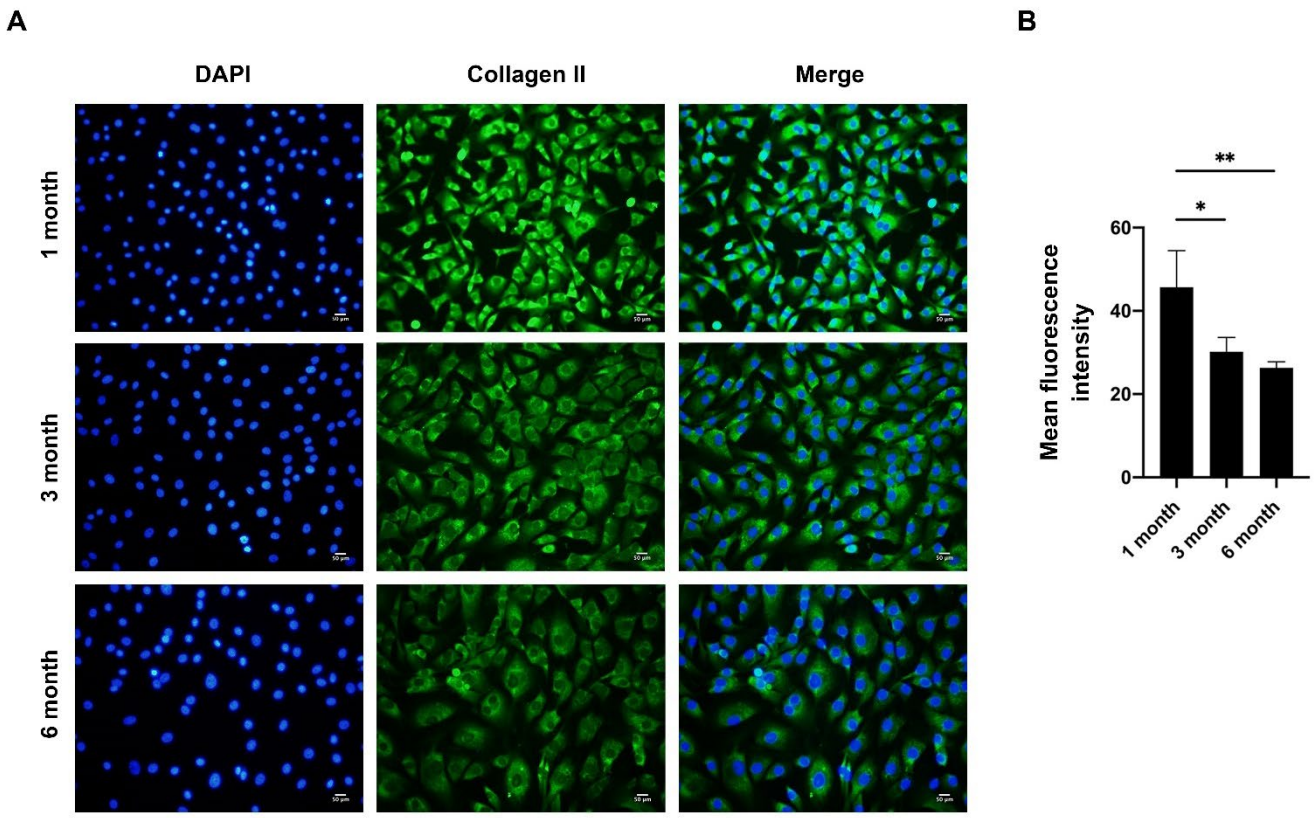

Fig. a. a) Expression of collagen II in primary chondrocytes derived from guinea pigs of different ages was determined by immunofluorescence assay (original magnification = 100×, scale bars = 50μm). b) Quantification of immunofluorescence staining of collagen II. One-way analysis of variance test was used to compare means among groups, and the Fisher's least significant difference multiple comparisons test was performed for the multiple comparisons. *p = 0.014, **p = 0.005. DAPI, 4′,6-diamidino-2-phenylindole.

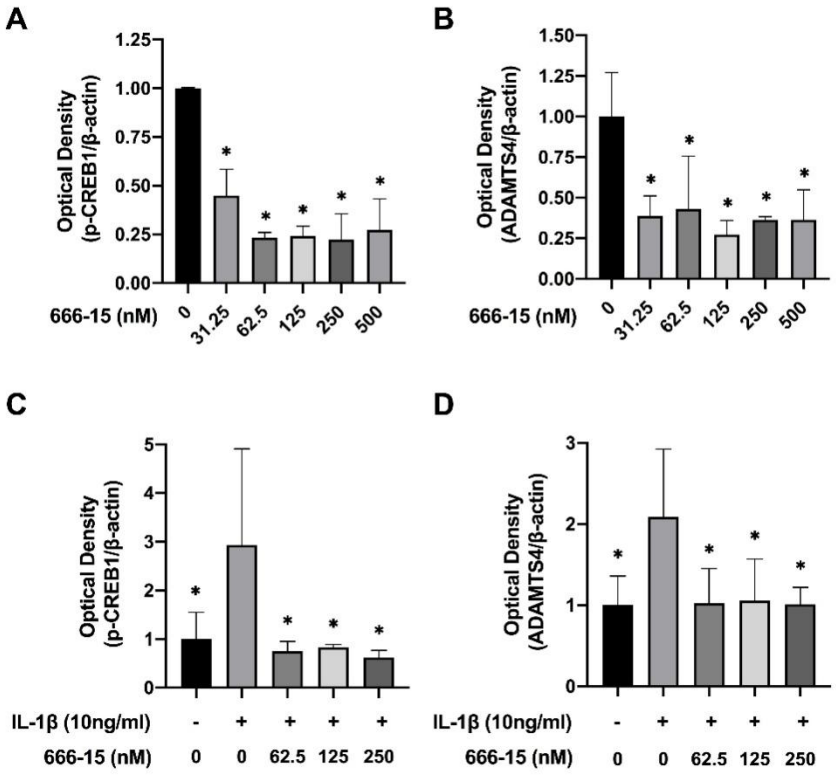

Fig. b. a) to b) 666-15 significantly inhibited a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) expression. c) to d) Interleukin-1β (IL-1β) induced upregulation of ADAMTS4 was significantly suppressed by 666-15. One-way analysis of variance test was used to compare means among groups, and the Fisher's least significant difference multiple comparisons test was performed for the multiple comparisons. The asterisks indicate a significant difference in values compared to the controls ($p < 0.05$). Error bars represent standard deviation of three samples from three independent culture experiments. p-CREB1, cAMP response element binding protein.

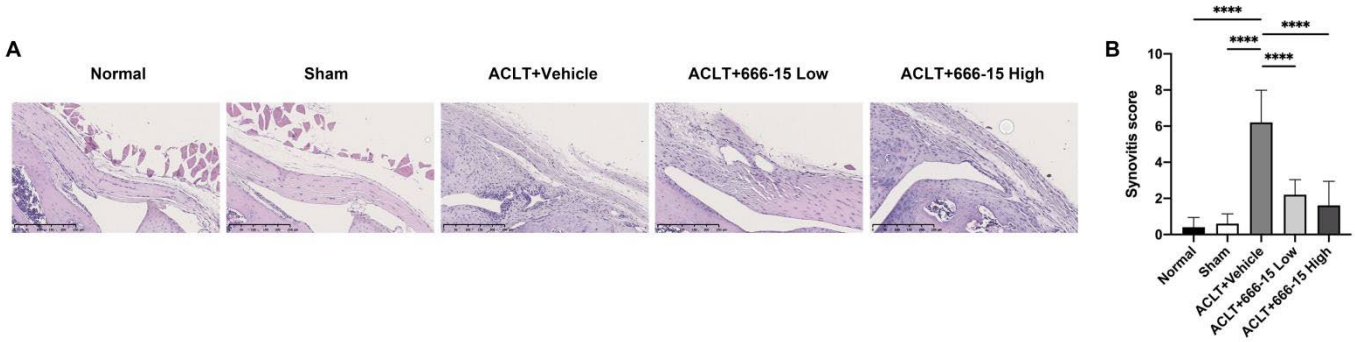

Fig. c. 666-15 alleviated anterior cruciate ligament transection (ACLT)-induced lesions in synovial membrane. a) Representative micrographs (scale bars = 250 μm) of haematoxylin & eosin staining, and b) the synovitis score of the joint tissues of each group (n = 5 in each group). One-way analysis of variance test was used to compare means among groups, and the Fisher's least significant difference multiple comparisons test was performed for the multiple comparisons. ****$p < 0.0001$.

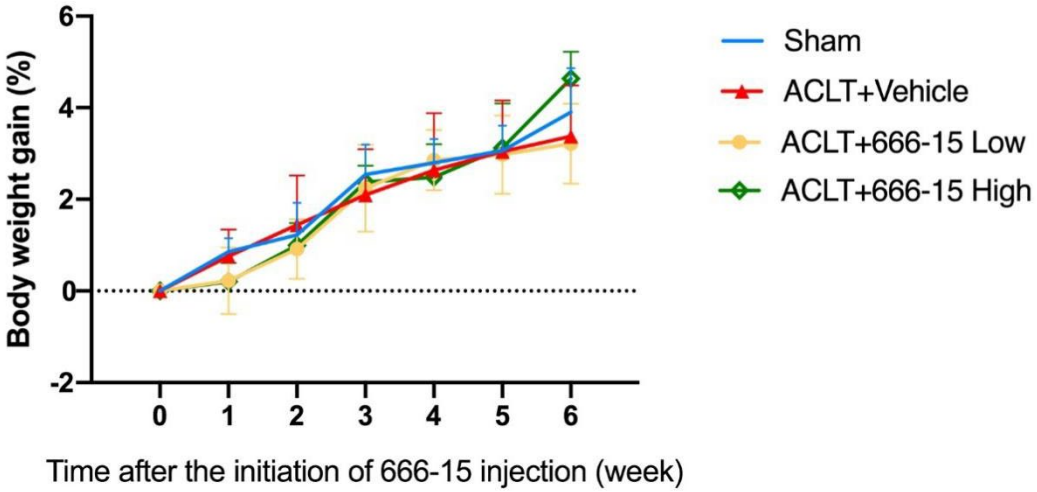

Fig. d. Body weight gain (%) of each experimental group was recorded weekly after the initial injection of 666-15. ACLT, anterior cruciate ligament transection.

References

1. **Wang Y, Li C, Zhang Y, et al.** Aberrant mTOR/autophagy/Nurr1 signaling is critical for TSC-associated tumor development. *Biochem Cell Biol*. 2021;99(5):570-577.

2. **Krenn V, Morawietz L, Burmester GR, et al.** Synovitis score: discrimination between chronic low-grade and high-grade synovitis. *Histopathology*. 2006;49:358e64.

NOTE: Please save this file locally before filling in the table, DO NOT work on the file within your internet browser as changes will not be saved. Adobe Acrobat Reader (available free here) is recommended for completion.

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.	Methods
		b. The experimental unit (e.g. a single animal, litter, or cage of animals).	Methods
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.	Methods
		b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	Methods
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.	Methods
		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.	No exclusions
		c. For each analysis, report the exact value of *n* in each experimental group.	Methods
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.	Methods
		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.	Methods
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).	Methods
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes).	Abstract
		b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	Abstract
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used.	Methods
		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
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.	Methods
		b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	Methods
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including:	Methods
		a. What was done, how it was done and what was used.	Methods
		b. When and how often.	Methods
		c. Where (including detail of any acclimatisation periods).	Methods
		d. Why (provide rationale for procedures).	Methods
Results	10	For each experiment conducted, including independent replications, report:	Methods
		a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range).	Methods
		b. If applicable, the effect size with a confidence interval.	

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.	Abstract
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
Housing and husbandry	15	Provide details of housing and husbandry conditions, including any environmental enrichment.	Methods
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 No observed adverse events Methods
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).	Discussion
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.	An ethical agreement on animal welfare was signed with the animal experiment center.
Data access	20	Provide a statement describing if and where study data are available.	Data sharing
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.	The authors have declared that no competing interest exists. Funding