

Original Article

ESTABLISHMENT OF A COCULTURE SYSTEM WITH OSTEOCHONDRAL AND SYNOVIAL EXPLANTS AS AN *EX VIVO* INFLAMMATORY OSTEOARTHRITIS MODEL

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Abstract

Osteoarthritis (OA) is a whole-joint disorder involving inflammation in cartilage, synovium, and subchondral bone. An *ex vivo* inflammatory OA model incorporating various intra-articular tissues could facilitate the development of new OA therapeutics targeting whole joint tissue. In this study, an inflammatory *ex vivo* coculture system with bovine osteochondral explants (OCEs) and synovial explants (SEs) was induced using 1 ng/mL interleukin one beta and tumor necrosis factor alpha. Furthermore, 5-aminosalicylic acid (5-ASA), a promising OA drug reported previously, was assessed in the coculture system to evaluate the potential of the system to be applied in drug screening for OA treatment. Under inflammatory stimulation, cartilage and synovium tissues in monoculture displayed a strong inflammatory response transcriptionally, biochemically, and histologically. Under inflammatory conditions, coculture elevated the gene expression of *IL-6* in synovium tissue compared with monocultured synovium. The synovium tissue in the coculture inflammatory group showed the highest synovitis score. The IL-8 release in the coculture medium was significantly higher than the additive amount in the OCE and SE monoculture groups under inflammatory microenvironment. For drug assessment, 5-ASA-treated group could promote the gene expression of *CD163/CD86* and inhibit *CD86* expression in synovium compared with inflammatory group. Moreover, the cumulative release of IL-6 into the medium was mitigated upon 5-ASA treatment. This study revealed the interaction between OCEs and SEs in an *ex vivo* coculture system. This preclinical coculture system could be used to evaluate the effects of novel OA drugs on both cartilage and synovium and explore the crosstalk mechanisms between intra-articular tissues.

Keywords: Coculture, inflammation, osteoarthritis model, osteochondral explant, synovium.

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Introduction

Osteoarthritis (OA) is the most common degenerative diarthrodial disorder, affecting over 500 million people globally, especially the elderly (> 65 years of age) (Hunter *et al.*, 2020). Common clinical OA symptoms include recurrent joint pain, swelling, and morning stiffness, which is directly associated with synovial inflammation (Sanchez-Lopez *et al.*, 2022; Sellam and Berenbaum, 2010). To date, no disease-modifying OA drugs have been clinically approved to inhibit OA progression. Patients with end-stage OA usually undertake joint replacement surgery due to loss of joint function and progressive pain.

Only recently, researchers have appreciated that chronic low-grade inflammation plays a pivotal role in the pathogenesis of OA (Robinson *et al.*, 2016). This has led OA to be considered as a whole-joint disease, involving

cartilage degeneration, synovial inflammation (synovitis), subchondral bone remodeling, and joint capsule hypertrophy (Robinson *et al.*, 2016). In OA pathophysiology, cartilage degradation products released into the synovial fluid are phagocytosed by synovial cells, triggering synovium inflammation, hypertrophy and hyperplasia (Sellam and Berenbaum, 2010). In turn, catabolic and pro-inflammatory mediators generated by the activated synovial cells result in cartilage breakdown, forming a vicious feedback loop and promoting OA progression (Sellam and Berenbaum, 2010). Meanwhile, cartilage and synovium produce anti-inflammatory cytokines to counteract the inflammation response (Sellam and Berenbaum, 2010). In addition, synovial macrophages can differentiate into functional osteoclasts, contributing to remodeling of subchondral bone in OA (Adamopoulos *et al.*, 2006; Sellam and Berenbaum,

2010). Therefore, when establishing an OA model to explore the underlying mechanisms and screen novel drugs, it is important to incorporate these intra-articular tissues.

Currently, most of the *in vitro* OA-like models with 2D or 3D cultured chondrocytes do not take the interactions among intra-articular tissues into account. *In vivo* OA models with small animals involve mice and rats, whose anatomical structure and mechanical properties of cartilage are quite different from human joints (McCoy, 2015). On the other hand, *in vivo* OA studies with large animals are expensive and time consuming. Osteochondral explants (OCEs) from bovine or equine joints serve as alternative preclinical tools. A comparative study of cartilage thickness in stifle joints in different animal species concluded that the average articular cartilage thickness of horse is 1.5 to 2 mm, which is the closest to 2.2 to 2.5 mm in humans (Frisbie *et al.*, 2006). Further comparison of biochemical characteristics of equine and human OCEs from the medial and lateral femoral condyles revealed their similarities (Malda *et al.*, 2012). As sources of equine joints are usually limited, bovine joints are suitable alternatives. Thanks to the joint size in large animals, it is also feasible to extract sufficient synovium explants (SEs) to establish a coculture system with OCEs. Moreover, using stifle joints obtained from abattoirs conforms with the 3R principles, i.e. replace, reduce, refine animal experimentation.

An *ex vivo* coculture system with OCEs and SEs could better mimic the *in vivo* joint milieu than monoculture setups. Though such preclinical models have previously been investigated (Beekhuizen *et al.*, 2011; Byron and Trahan, 2017; Fell and Jubb, 1977; Haltmayer *et al.*, 2019; Lee *et al.*, 2009; Patwari *et al.*, 2009; Swärd *et al.*, 2017), no study has applied interleukin-1 β (IL-1 β) in combination with tumor necrosis factor- α (TNF- α) as proinflammatory stimuli, which plays a parallel role in OA development (Chevalier *et al.*, 2013; Robinson *et al.*, 2016). In previous coculture studies, most focus has been put on effects of coculture conditions on cartilage or culture medium, while no study analyzed the transcriptional and histological changes in synovium (Beekhuizen *et al.*, 2011; Byron and Trahan, 2017; Fell and Jubb, 1977; Haltmayer *et al.*, 2019; Lee *et al.*, 2009; Mehta *et al.*, 2019; Swärd *et al.*, 2017). In addition, when comparing cytokine profiles secreted in the culture medium in OCE monoculture and synovium-cocultured groups, the cytokines released by synovium tissue must be considered to draw conclusions on the extent of tissue interaction.

Based on these considerations, this study aims to establish an inflammatory OA model with coculture of osteochondral explant and synovium tissue *ex vivo*, and to investigate the crosstalk between these tissues within such system. We have designed an *ex vivo* inflammatory bovine OCE and SE coculture system with IL-1 β and TNF- α stimulation. By analyzing cartilage, synovium tissue, and the culture medium, the interaction between cartilage and syn-

ovium tissue in the *ex vivo* coculture system under an inflammatory OA condition was investigated. Furthermore, 5-aminosalicylic acid (5-ASA) was assessed in the coculture system, which was previously determined as a potential drug for OA treatment (Li *et al.*, 2023).

Methods

Experimental Aim and Design

The whole study was designed with three experiments. The aim of the first part (Fig. 1a) was to establish inflammatory OCE and SE models individually. Either OCEs or SEs were divided into two groups in the absence (Control) or presence (OA) of 1 ng/mL bovine IL-1 β (King-Fisher Biotech, St Paul, MN, USA) and 1 ng/mL bovine TNF- α (R&D systems, Minneapolis, MN, USA). After one day preculture, samples were cultured in high glucose DMEM (Gibco, Waltham, MA, USA) medium supplemented with 2.5 % HEPES (Gibco), 1 % nonessential amino acids (Gibco), 50 μ g/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 1 % ITS + Premix (Corning, Corning, NY, USA), 100 U/mL penicillin and 100 μ g/mL streptomycin (PAN Biotech, Aidenbach, Germany). For the inflammatory groups, 1 ng/mL IL-1 β and 1 ng/mL TNF- α were added at each medium change on day 0, day 1, day 3, and day 5 (Fig. 1d). Explants were collected on day 0 and day 7 and culture media were collected on day 0, day 1, day 3, day 5, and day 7 for further analysis (Fig. 1d).

The aim of the second part (Fig. 1b) was to establish a more biomimetic coculture model with OCEs and SEs and explore their crosstalk. This experiment was designed with six groups: OCE monoculture without IL-1 β and TNF- α (OCE Control); OCE monoculture with 1 ng/mL IL-1 β and 1 ng/mL TNF- α (OCE OA); SE monoculture without IL-1 β and TNF- α (SE Control); SE monoculture with 1 ng/mL IL-1 β and 1 ng/mL TNF- α (SE OA); coculture of OCE and SE without IL-1 β and TNF- α (Coculture Control); coculture of OCE and SE with 1 ng/mL IL-1 β and 1 ng/mL TNF- α (Coculture OA). The explants from the same animal were randomly divided and transferred into a 12-well plate. For the coculture groups, a 0.4 μ m pore size translucent PET membrane transwell (Corning, Falcon, Cat. No. 353494) was inserted into the wells to avoid direct cell-cell contact between OCE and SE. One OCE was placed in the upper compartment, while two SEs were placed in the lower compartment according to the surface area ratio of synovium to cartilage *in vivo* (Byron and Trahan, 2017; Hewitt and Stringer, 2008). For OCE and SE monoculture groups, 1.5 mL medium was added into each well, while 3 mL medium was supplemented into the coculture group. The frequency of medium change and sample collection methods were the same as the first part.

In the third part (Fig. 1c), a potential OA drug, 5-ASA (Sigma-Aldrich) (Li *et al.*, 2023), was assessed in the inflammatory coculture system. Four groups were designed: coculture of OCE and SE without IL-1 β and TNF- α (Con-

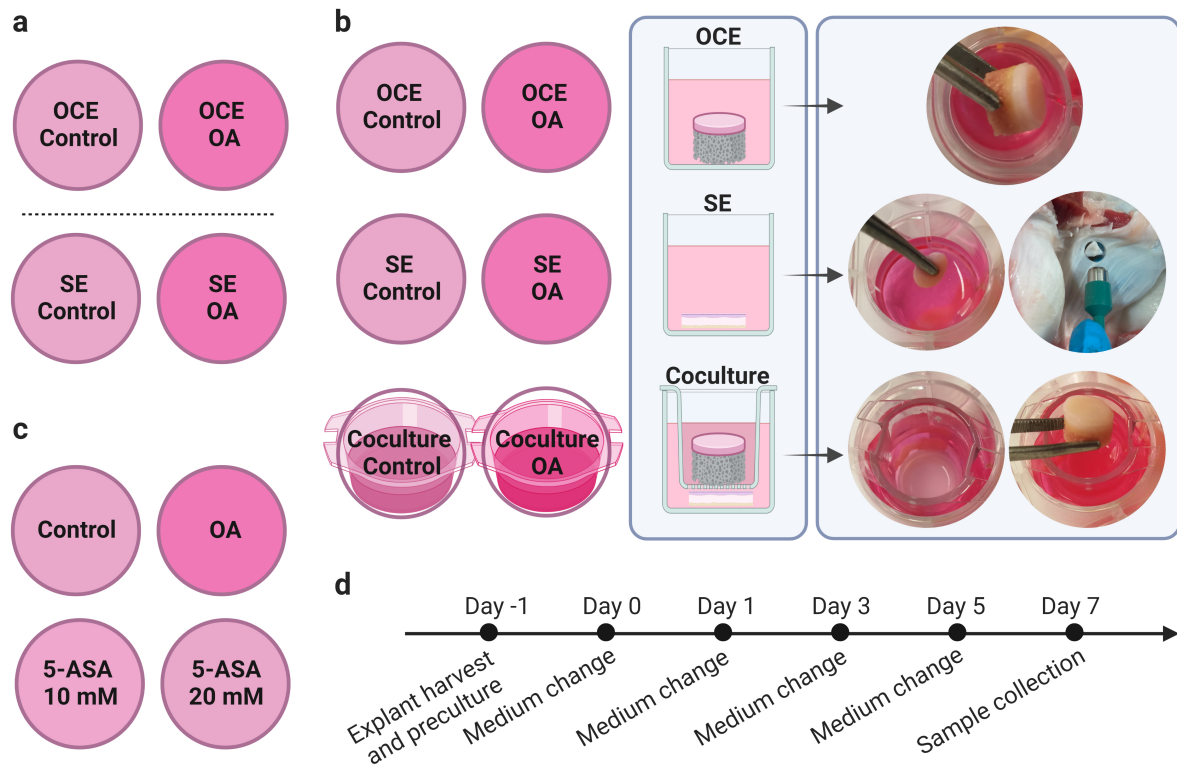


Fig. 1. Experimental groups and design. (a) Groups in experiment part 1. (b) Groups in experiment part 2 and representative images of OCE, SE, and their coculture system. (c) Groups in experiment part 3. (d) Timeline and workflow of the study. Figure made with Biorender. OCE, osteochondral explant; SE, synovial explant; Control, non-inflammatory group; OA, inflammatory group; 5-ASA, 5-ASA-treated inflammatory group.

control); coculture of OCE and SE with 1 ng/mL IL-1 β and 1 ng/mL TNF- α (OA); coculture of OCE and SE with 1 ng/mL IL-1 β , 1 ng/mL TNF- α , and 10 mM 5-ASA (5-ASA 10 mM); coculture of OCE and SE with 1 ng/mL IL-1 β , 1 ng/mL TNF- α , and 20 mM 5-ASA (5-ASA 20 mM). The frequency of medium change and sample collection methods were the same as the first part.

Osteochondral and Synovial Explant Harvest

Bovine (12 to 18.5-month-old) stifle joints were obtained from a local slaughterhouse. Within 60 hours after slaughter, explant samples were aseptically collected from the joints. Synovial tissue with partial fibrous capsule was harvested with an 8 mm diameter biopsy punch (KAI medical, Seki-shi, Gifu Prefecture, Japan) from the lateral parapatellar region of the joint (Fig. 1b). OCEs were collected from the femoral groove as previously described (Vainieri *et al.*, 2018). Briefly, an 8 mm diameter trephine drill (Peertools AG, Ftan, Switzerland) was used to obtain cylindrical osteochondral explants, which were then trimmed to a total height of 6 mm by a circular saw.

Sample Preparation for Histology

Full thickness cartilage tissue was excised from the OCE with a scalpel. The cartilage and SE were embedded in cryocompound (Leica, Wetzlar, Germany) for about 30 minutes at room temperature and then snap frozen in liquid nitrogen. Cryosections with thickness of 6 μ m were cut for further histological analysis.

Cell Viability Evaluation

The viability of chondrocytes and synoviocytes in cartilage and synovium was evaluated with lactate dehydrogenase/ethidium homodimer (LDH/EthD) staining as previously described (Li *et al.*, 2021). Briefly, unfixed cryosections were incubated in ethidium homodimer (Sigma) solution for 45 min at room temperature after removing cryocompound by washing in deionized water. After rinsing in phosphate buffered saline (PBS), slides were incubated in LDH solution at 37 $^{\circ}$ C for 3 h. After washing in 50 $^{\circ}$ C deionized water, sections were fixed with 4 % formalin for 15 min and mounted with an aqueous mounting reagent (Dako, Glostrup, Denmark). Blue-stained or counterstained (blue and red) cells were regarded as live, while only red-stained cells were dead. The number of live/dead cells was automatically counted with ImageJ soft-

ware (ImageJ 1.53t, National Institute of Health, Bethesda, MD, USA). Cell viability was calculated by dividing the number of living cells by the number of total cells.

Safranin O/Fast Green Staining

Cryosections of cartilage tissue were fixed with 70 % methanol for 10 min and then 100 % methanol for 10 min, followed by air drying overnight. As previously described (Li *et al.*, 2021), the staining steps were in following order: Weigert's Hematoxylin (Sigma) for 10 min, tap water for 10 min, 0.02 % Fast Green (Sigma) for 6 min, 1 % acetic acid (Fluka BioChemika, Ronkonkoma, N.Y., Buchs, Switzerland) for 30 s, 0.1 % Safranin O (Sigma) for 12 min, deionized water for 3 times. Slides were then differentiated with 70 % ethanol for 10 s and mounted with Eukitt (Sigma) after ascending dehydration. The mean integrated optical density (IOD) of the cartilage area in each section was quantified with ImageJ (Shi *et al.*, 2019).

Hematoxylin and Eosin Staining

Cryosections of synovium tissue were fixed with 70 % methanol for 10 min. After rinsing in deionized water, slides were incubated in Mayer's Hematoxylin solution (Sigma) for 10 min and blued in 25 °C tap water for 10 min. Then, slides were transferred into 1 % Eosin solution for 10 min and differentiated in tap water for 2 min. Slides were then differentiated with 70 % ethanol for 10 s and mounted with Eukitt (Sigma) after dehydration. In histological grading of synovitis *in vivo*, three basic morphological parameters had usually been included: hyperplasia of synovial intimal lining layer, degree of inflammatory infiltration, and subintimal resident cell activation (Krenn *et al.*, 2002). Here, we only included hyperplasia of synovial lining layer because there was no blood supply to synovium tissue *ex vivo*. Thickened lining layer was graded from 0 (one cell layer thick), 1 (two to three cell layers thick), 2 (four to five cell layers thick) to 3 (equal to or more than six cell layers thick).

Gene Expression Analysis

Fresh cartilage tissue was minced into small pieces and pre-digested in 2 mg/mL pronase (Roche, Basel, Switzerland) in DMEM for 2 h at 37 °C before proceeding. After freezing in liquid nitrogen, cartilage fragments and synovium tissue were pulverized and transferred into TRI Reagent (Molecular Research Center, MRC). The tissue in TRI was then homogenized with a tissue lyser. The supernatant was transferred into fresh tubes after centrifugation. Then, 1-bromo-3-chloropropane (BCP, Sigma-Aldrich) was added into each tube. After vigorously shaking and centrifuging, the upper aqueous phase containing RNA was transferred into fresh tubes for purification. For cartilage tissue rich in polysaccharides, RNA was then purified with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to producer's instructions. For synovium tissue,

isopropanol (Sigma) was added to the aqueous supernatant to precipitate RNA. After centrifugation, the RNA precipitate was washed with 75 % ethanol twice and dissolved in DEPC-treated water after air drying. The RNA concentration was measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was conducted with SuperScript Vilo RT Kit (Thermo Fisher Scientific). Quantitative polymerase chain reaction (qPCR) to analyze gene expression levels was performed with QuantStudio Flex 7.0 instrument and TaqMan Gene Expression Master Mix (Applied Biosystems, Waltham, MA, USA). The primer and probe sequences of target genes were listed in Table 1. RPLP0 ribosomal RNA was used as endogenous control. RNA fold change in different samples was analyzed using the $2^{-\Delta\Delta C_t}$ method.

Enzyme Linked Immunosorbent Assay

The concentrations of bovine IL-6 and IL-8 in the medium were measured by Enzyme Linked Immunosorbent Assay (ELISA, KingFisher Biotech) according to the manufacturer's instructions.

Sulfated Glycosaminoglycan Release

The content of sulfated glycosaminoglycan (GAG) released into the medium was assessed with 1,9-dimethylmethylene blue (DMMB, Sigma-Aldrich) color reagent as previously described (Li *et al.*, 2021). Briefly, 50 μ L samples or chondroitin sulfate standards (Fluka BioChemika) were distributed into designed wells of a 96-well plate in duplicate, followed by 200 μ L DMMB reagent. The content of GAG in samples was calculated with respective absorbance values at 535 nm according to the linear standard curve (content range 0 to 2.5 μ g per well).

Nitric Oxide Release

The content of nitric oxide (NO) in the medium was measured by Griess Reagent System (Promega Corporation, Madison, WI, USA) according to the producer's instructions.

Statistical Analysis

All data were presented as mean + standard deviation (SD). The n value in this study refers to the number of biological replicates. Statistical analysis was performed using GraphPad Prism 8.4 (GraphPad Software, Inc., San Diego, CA, USA). Shapiro–Wilk test was first applied to evaluate the normality of data distribution in each group. Unpaired Student's *t*-test was used to compare the means of data in two groups. For data in more than three groups, one-way analysis of variance (ANOVA) was performed for data with normal distribution, while Kruskal–Wallis test was used for non-normally distributed data. Values of $p < 0.05$ were considered statistically significant.

Table 1. Oligonucleotide primers and probes used for qPCR.

Gene	Primer & Probe/Assay ID	Sequence/Catalogue number
<i>COL2</i>	Forward Primer (5'→3')	AAG AAA CAC ATC TGG TTT GGA GAA A
	Reverse Primer (5'→3')	TGG GAG CCA GGT TGT CAT C
	Probe (5'FAM/3'TAMRA)	CAA CGG TGG CTT CCA CTT CAG CTA TGG
<i>ACAN</i>	Forward Primer (5'→3')	CCA ACG AAA CCT ATG ACG TGT ACT
	Reverse Primer (5'→3')	GCA CTC GTT GGC TGC CTC
	Probe (5'FAM/3'TAMRA)	ATG TTG CAT AGA AGA CCT CGC CCT CCA T
<i>ADAMTS4</i>	Forward Primer (5'→3')	CCC CAT GTG CAA CGT CAA G
	Reverse Primer (5'→3')	AGT CTC CAC AAA TCT GCT CAG TGA
	Probe (5'FAM/3'TAMRA)	AGC CCC CGA AGG GCT AAG CGC
<i>ADAMTS5</i>	Forward Primer (5'→3')	GAT GGTCAC GGT AAC TGT TTG CT
	Reverse Primer (5'→3')	GCC GGG ACA CAC CGA GTA C
	Probe (5'FAM/3'TAMRA)	AGG CCA GAC CTA CGA TGC CAG CC
<i>MMP1</i>	Forward Primer (5'→3')	TTC AGC TTT CTC AGG ACG ACA TT
	Reverse Primer (5'→3')	CGA CTG GCT GAG TGG GAT TT
	Probe (5'FAM/3'TAMRA)	TCC AGG CCA TCT ACG GAC CTT CCC
<i>MMP3</i>	Forward Primer (5'→3')	GGC TGC AAG GGA CAA GGA A
	Reverse Primer (5'→3')	CAA ACT GTT TCG TAT CCT TTG CAA
	Probe (5'FAM/3'TAMRA)	CAC CAT GGA GCT TGT TCA GCA ATA TCT AGA AAA C
<i>MMP13</i>	Forward Primer (5'→3')	CCA TCT ACA CCT ACA CTG GCA AAA G
	Reverse Primer (5'→3')	GTC TGG CGT TTT GGG ATG TT
	Probe (5'FAM/3'TAMRA)	TCT CTC TAT GGT CCA GGA GAT GAA GAC CCC
<i>VEGF</i>	Forward Primer (5'→3')	GGC TGC TGT AAT GAC GAA AGT CT
	Reverse Primer (5'→3')	TGT TAT GCT GTA GGA AGC TCA TCT CT
	Probe (5'FAM/3'TAMRA)	CAC CAT GCA GAT TAT GCG GAT CAA ACC T
<i>RPLP0</i>	Assay ID	Bt03218086_m1
<i>CD86</i>	Assay ID	Bt03231528_m1
<i>CD163</i>	Assay ID	Bt04281409_m1
<i>IL-6</i>	Assay ID	Bt03211905_m1
<i>IL-8</i>	Assay ID	Bt03211906_m1

Note: Primers and probes presented with sequences were self-designed, while others with catalogue numbers were from Applied Biosystems.

Results

Cell Viability in Cartilage and Synovium

To determine the cell viability of *ex vivo* cultured tissues on day 7, we performed LDH/EthD staining on unfixed cryosections of tissues collected on day 0 (after one day preculture) and day 7. The average cell viability of chondrocytes in cartilage on day 0 was 93 %, which was comparable to the viability of cartilage in monoculture or cocultured with synovium with or without cytokine stimulation after 7 days (Fig. 2a,b). Similarly, the cell viability of synovium remained around 90 % in all groups after 7 days, which is comparable with day 0 (Fig. 2c,d). These results demonstrated that one-week *ex vivo* culture did not affect the cell viability of cartilage and synovium tissue.

Establishment of an Inflammatory Osteochondral Explant Model

In experimental part 1, we established inflammatory OCE and SE models separately. The inflammatory group (OA) of OCE was stimulated with 1 ng/mL IL-1 β and 1

ng/mL TNF- α for 7 days (Fig. 3). The anabolic genes *ACAN* ($p = 0.0448$) and *COL2* ($p = 0.0350$) were significantly downregulated in the OA group compared with the untreated group (Fig. 3a). The catabolic genes *MMP13* and *ADAMTS4* presented a 20.16-fold ($p = 0.0429$) and 27.84-fold ($p = 0.0346$) upregulation in the OA group (Fig. 3a). In the OA group, the cumulative release of IL-6 (from 11.23 ng to 149.3 ng, $p = 0.0063$), IL-8 (from 1.165 ng to 19.62 ng, $p = 0.0138$), NO (from 19.66 nmol to 146.8 nmol, $p = 0.0288$), and GAG (from 618.3 μ g to 1354 μ g, $p = 0.0392$) in the medium were significantly elevated compared with the Control group (Fig. 3b-d). Additionally, the OA cartilage displayed an evident proteoglycan loss in the upper and middle zones compared with the untreated group (Fig. 3e). These findings indicate a successful inflammatory OCE model was induced with 1 ng/mL IL-1 β and 1 ng/mL TNF- α after 7 days.

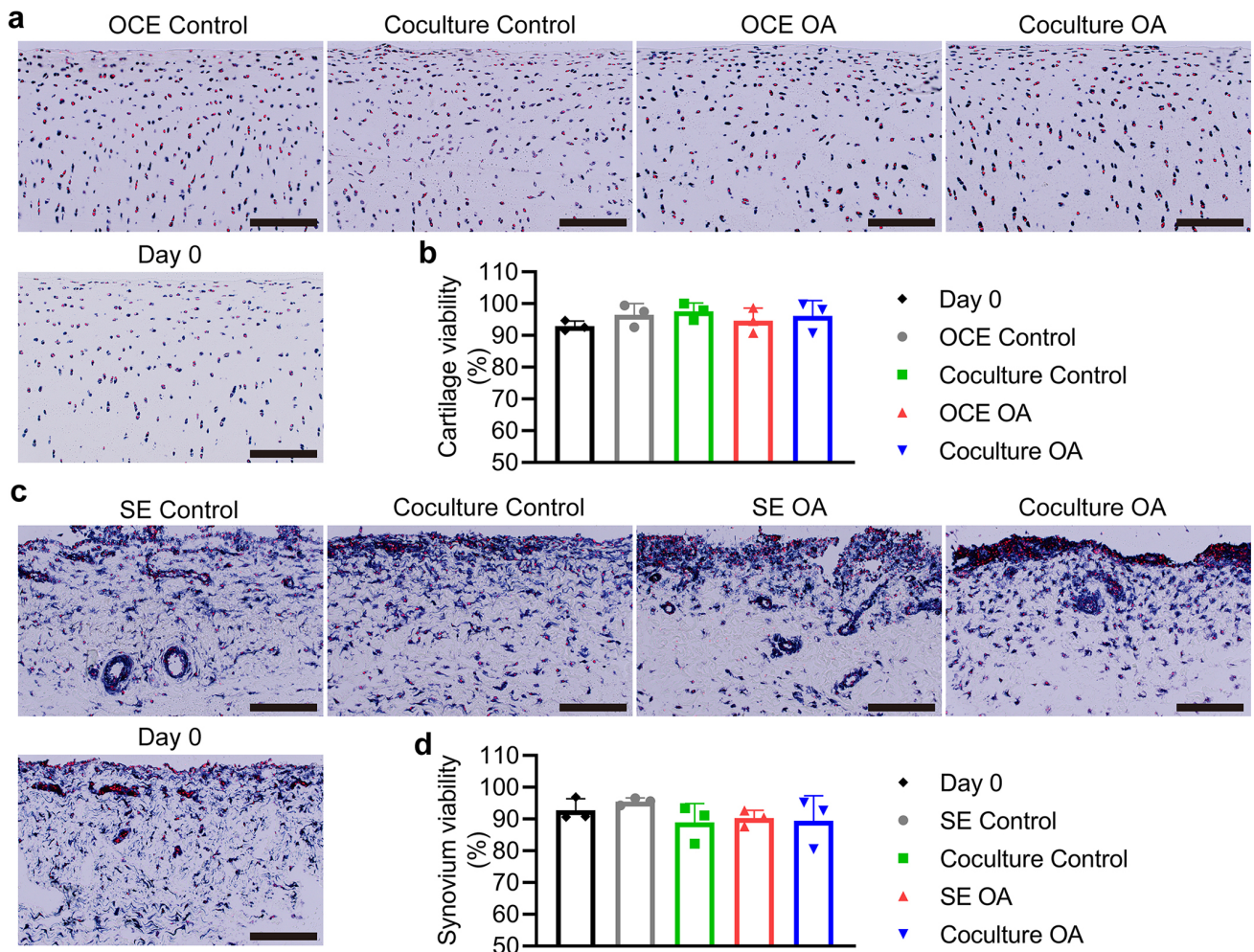


Fig. 2. Cell viability evaluated with LDH/ethidium homodimer staining on snap-frozen sections of cartilage and synovium tissue. (a) Representative images of stained cartilage tissue collected on day 0 and day 7. Scale bars, 200 μ m. (b) Quantification of cartilage cell viability ($n = 3$). (c) Representative images of stained synovium tissue collected on day 0 and day 7. Scale bars, 200 μ m. (d) Quantification of synovium cell viability ($n = 3$). Live cells, blue or blue/red stained cells; dead cells, red-only-stained cells. Data presented as means + SD. Statistical analysis was performed by one-way analysis of variance (ANOVA).

Establishment of an Inflammatory Synovium Model

In experimental part 1, an inflammatory SE model was established simultaneously. Likewise, stimulation with 1 ng/mL IL-1 β and 1 ng/mL TNF- α for 7 days was applied to investigate the inflammatory response in SE. Gene markers of inflammation (IL-6, IL-8), proteinases (MMP1, MMP3, ADAMTS5), and angiogenesis (VEGF) exhibited a 9.40-fold ($p = 0.0070$), 8.64-fold ($p = 0.0014$), 23.33-fold ($p = 0.0478$), 50.48-fold ($p = 0.0094$), 2.83-fold ($p = 0.0185$), and 2.187-fold ($p = 0.0427$) elevation respectively in the OA group compared with the Control group (Fig. 4a). Furthermore, cumulative IL-6 release in the conditioned medium was upregulated from 105.5 ng in the Control group to 435.9 ng ($p = 0.0103$) in the OA group, while IL-8 from 16.72 ng to 67.85 ng ($p = 0.0049$) (Fig. 4b). Hyperplasia of the synovium intimal lining layer is a characteristic of synovitis. In our study, the intimal layer in the

OA group was markedly thickened (2-3 layers) (Fig. 4c) compared with the Control group (1 layer). These results demonstrate that synovitis was induced in the synovium tissue in the *ex vivo* inflammation model.

Influence of Osteochondral and Synovial Explants Coculture on Cartilage

Following establishment of inflammatory OCE and SE models, we performed a second set of experiments to explore the crosstalk between cartilage and synovium with an *ex vivo* coculture system. As described in experimental design, there were six groups included: OCE Control, OCE OA, SE Control, SE OA, Coculture Control, and Coculture OA. Under conditions without cytokine stimulation, mRNA levels of chondrocytes in Coculture Control group presented a marked downregulation of *ACAN* ($p = 0.0086$) and *COL2* ($p = 0.0015$) compared with OCE Control, while being comparable between the two groups in other tested

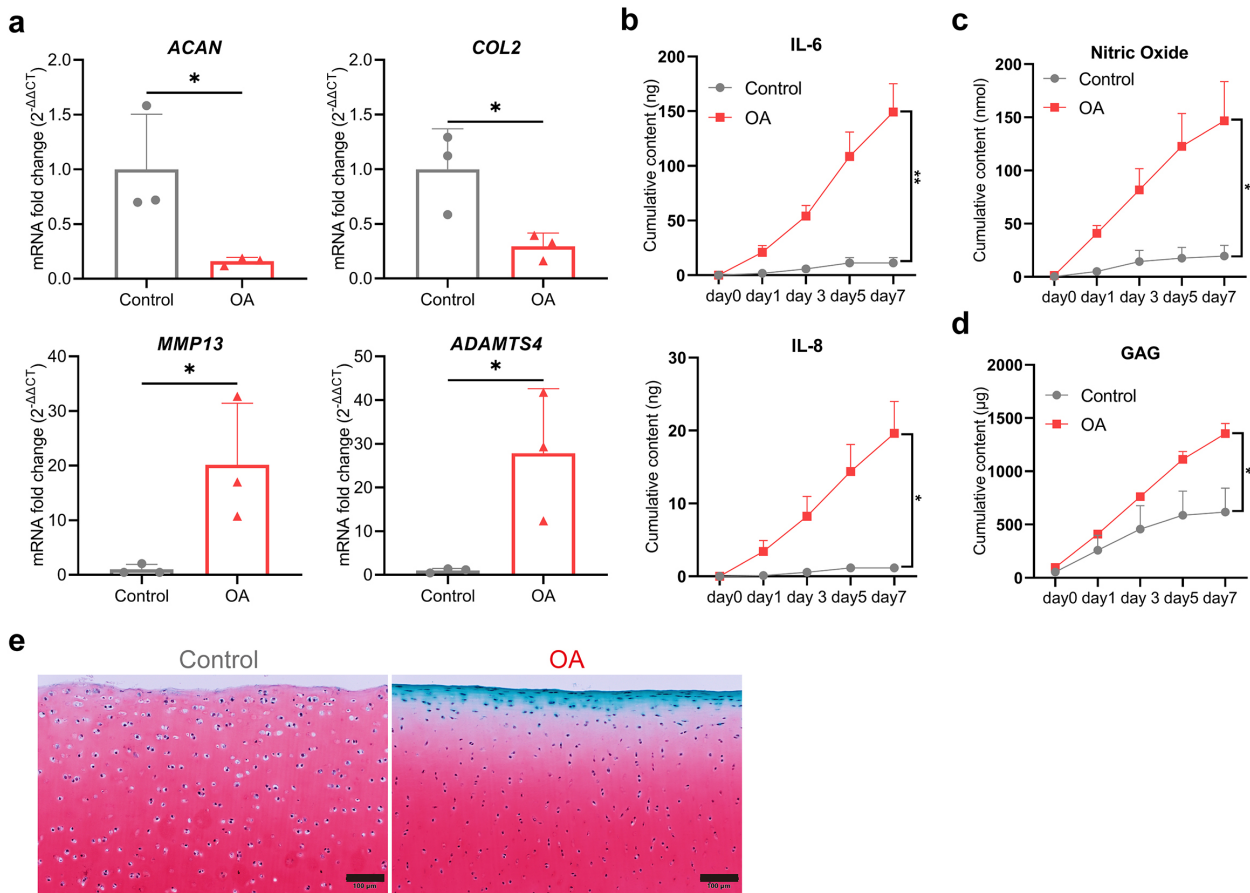


Fig. 3. Establishment of an inflammatory osteochondral explant model induced by 1 ng/mL IL-1 β and 1 ng/mL TNF- α for 7 days. (a) Gene expression levels of *ACAN*, *COL2*, *MMP13* and *ADAMTS4* in chondrocytes untreated (Control) or treated (OA) with cytokines (n = 3). **(b-d)** Cumulative IL-6 **(b)**, IL-8 **(b)**, NO **(c)**, and GAG **(d)** release into the medium in untreated or cytokine-treated groups (n = 3). **(e)** Representative Safranin O/Fast Green staining images of cartilage slides from cytokine-untreated or treated groups. Scale bars, 100 μ m. Data presented as means + SD. Statistical analysis was performed by two-tailed *t*-test. * $p < 0.05$, ** $p < 0.01$.

genes (Fig. 5a). Under inflammatory condition, inflammatory (*IL-6*, *IL-8*) and catabolic (*MMP3*, *ADAMTS4*, and *ADAMTS5*) gene expression levels of chondrocytes presented no difference between Coculture OA group and OCE OA group (Fig. 5a). The mean IOD value of proteoglycan staining in cartilage tissue from Coculture OA group was comparable with OCE OA group (Fig. 5b,c).

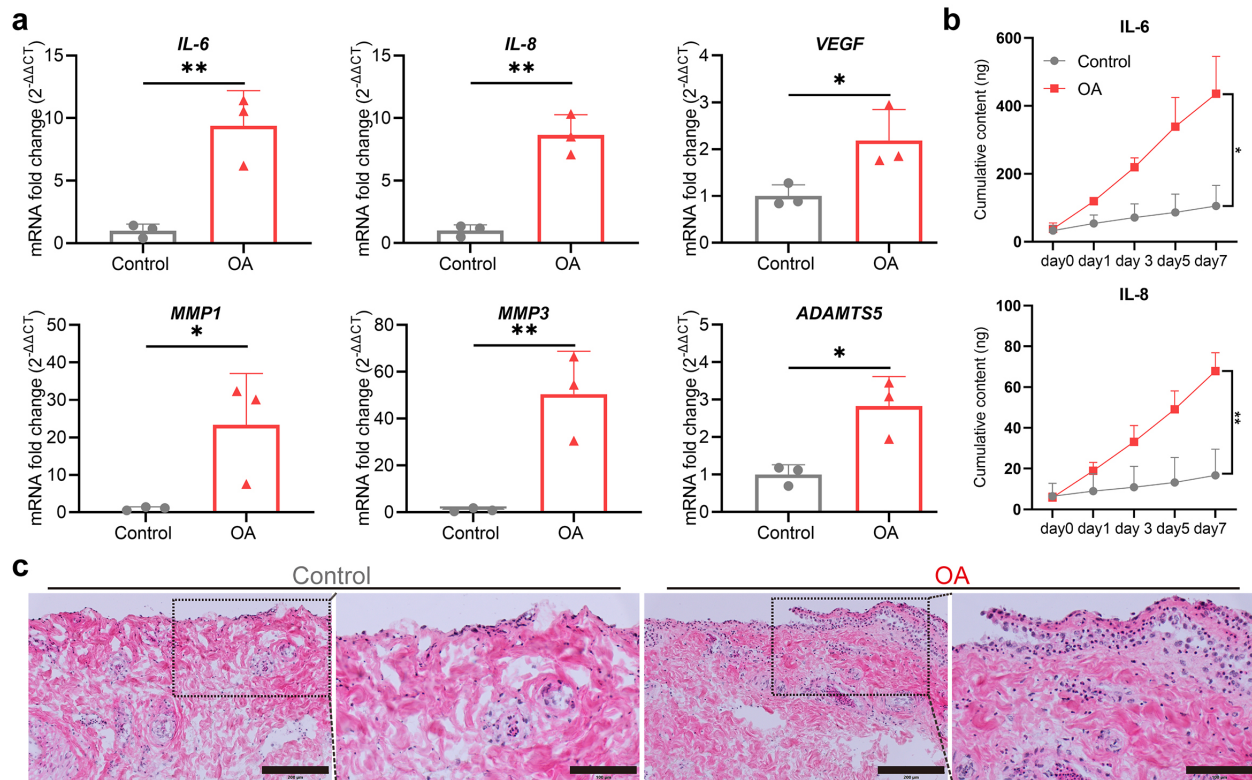
Influence of Osteochondral and Synovial Explants Coculture on Synovium

We further analyzed the effects of SE coculturing with OCE on synovium. Without cytokine treatment, mRNA levels of tested genes in synovium in Coculture Control group were comparable with SE Control (Fig. 6a). Under inflammatory conditions, mRNA level of *IL-6* in Coculture OA were significantly upregulated ($p = 0.0341$) compared with SE OA (Fig. 6a), while the mRNA levels of other tested genes were comparable between the two groups. Histologically, synovium in Coculture OA exhibited a thicker lining layer compared with SE OA, with the highest synovitis score presented among all the four groups (Fig. 6b,c).

Synovium coculturing with OCE could result in a more severe synovitis than monoculture.

Coculture of Osteochondral and Synovial Explants Aggravating the Release of Inflammation Mediators in Medium

Mediators and metabolites in the medium could be generated by OCE as well as SE. Thus, we compared the protein release in the coculture group with the total protein release in OCE and SE monoculture groups (OCE+SE) to evaluate the effects of their crosstalk. Under inflammatory stimulation, the amount of IL-8 in the medium was significantly enhanced ($p = 0.0147$) in Coculture OA group (mean content 120.0 ng) compared with the total amount in OCE+SE OA (mean content 75.54 ng) (Fig. 7a). IL-6, NO and GAG release did not show a difference between the Coculture OA group and OCE+SE OA group (Fig. 7a). Under normal conditions, all the metabolites above were comparable in coculture and monoculture groups (Fig. 7a). Further analysis for the OCE+SE OA group revealed that the percentage of IL-6, IL-8, NO and GAG released by SE were



66.91 %, 89.0 %, 32.8 %, and 5.5 %, respectively (Fig. 7b). The data above showed that there existed crosstalk between synovium and cartilage under *ex vivo* inflammatory conditions, and synovium played a pivotal role in regulating the inflammatory microenvironment in the coculture system.

Effects of 5-ASA on Osteochondral and Synovial Explants Coculture System

To test the validity of the coculture system as a drug-screening tool, we assessed the effects of 5-ASA at the concentration of 10 mM and 20 mM, which demonstrated anti-inflammatory effect on inflammatory chondrocytes pellet model and osteochondral explant model previously (Li *et al.*, 2023). At gene expression level, *IL-6* was significantly upregulated in the OA group compared with the Control group, which showed a downregulation trend upon 20 mM 5-ASA treatment in both cartilage and synovium (Fig. 8a,b). In the conditioned medium, the IL-6 release rose from 74.81 ng in the Control group to 296.6 ng in the OA group ($p < 0.0001$) and dropped to 195.3 ng in the 5-ASA 20 mM group ($p = 0.0029$, 5-ASA 20 mM vs OA) (Fig. 8c). Compared with OA group, *MMP13* gene expression was downregulated upon 20 mM 5-ASA treatment in

the synovium ($p = 0.0065$) (Fig. 8b). Another catabolic gene, *ADAMTS5*, displayed a downregulation trend upon 5-ASA treatment in comparison with the OA group in the synovium without significance (Fig. 8b). Moreover, the gene expression level of *CD86* (M1 macrophage marker) was significantly downregulated ($p = 0.0031$) and the ratio of *CD163/CD86* (M2 macrophage marker/M1 macrophage marker) was upregulated ($p = 0.0345$) in the 5-ASA 20 mM group in comparison with the OA group in the synovium tissue (Fig. 8b). These findings suggested 5-ASA inhibits the inflammation response and catabolic gene expression in the coculture system and repolarize macrophage from M1 to M2 phenotype in the synovium.

Discussion

To better represent the native OA joint microenvironment, this study aimed to establish an *ex vivo* inflammatory coculture system with bovine OCEs and SEs. In rheumatoid arthritis, IL1 β production was driven by TNF, while IL1 β and TNF act in a parallel manner in OA (Chevalier *et al.*, 2013). However, IL-1 β and TNF- α play a parallel and pivotal role in OA (Chevalier *et al.*, 2013; Robinson *et al.*, 2016), they are commonly used as a stimulator for *in vitro*

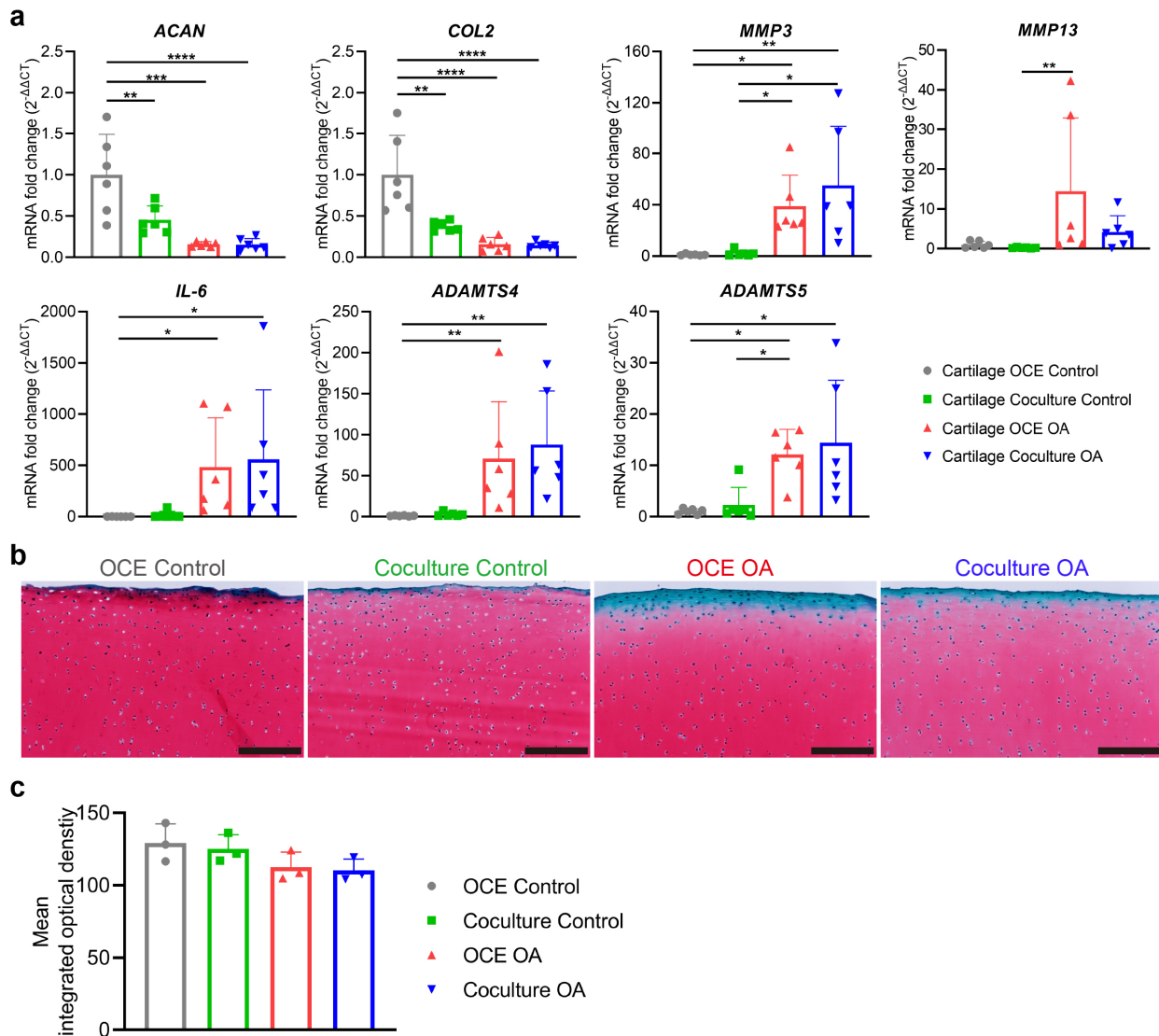


Fig. 5. Gene expression and histological morphology of cartilage in monoculture or synovium-cocultured groups with or without IL-1 β and TNF- α stimulation for 7 days. (a) *ACAN*, *COL2*, *MMP3*, *MMP13*, *IL-6*, *ADAMTS4*, and *ADAMTS5* expression levels of cartilage tissue in monoculture or cocultured groups in the absence or presence of cytokines (n = 6). (b) Representative images of Safranin O/Fast Green staining on cartilage samples collected from above groups. Scale bars, 200 μ m. (c) Mean integrated optical density of Safranin O staining of cartilage in b (n = 3). Data presented as means + SD. Statistical analysis was performed by Kruskal-Wallis test (*ACAN*, *COL2* in a) or one-way analysis of variance (*IL-6*, *MMP3*, *MMP13*, *ADAMTS4*, and *ADAMTS5* in a; histological analysis in c). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

OA-like models. Targeting a single cytokine was encouraging in animal studies, but showed disappointing results in human clinical trials, suggesting blocking one cytokine might not be sufficient to halt OA progression (Chevalier *et al.*, 2013). Inhibiting IL-1 and TNF simultaneously may be required to suppress the activity of collagenases and aggrecanases for OA treatment (Bondeson *et al.*, 2010). Therefore, we applied the same amount of IL-1 β and TNF- α in our coculture system, which could in future be used to evaluate the efficacy of novel disease-modifying OA drugs.

Under non-inflammatory conditions, the expression levels of *ACAN* and *COL2* in chondrocytes were significantly downregulated in the coculture system compared with OCE monoculture, while the expression of inflammatory and catabolic genes was comparable among the two groups, implying synovium tissue could inhibit anabolic gene expression in cartilage. Under inflammatory conditions, the tested genes in cartilage tissue showed no difference between Coculture OA group and OCE OA group, indicating the effects of synovium tissue on cartilage might be masked by the high concentrations of cytokine stimulation.

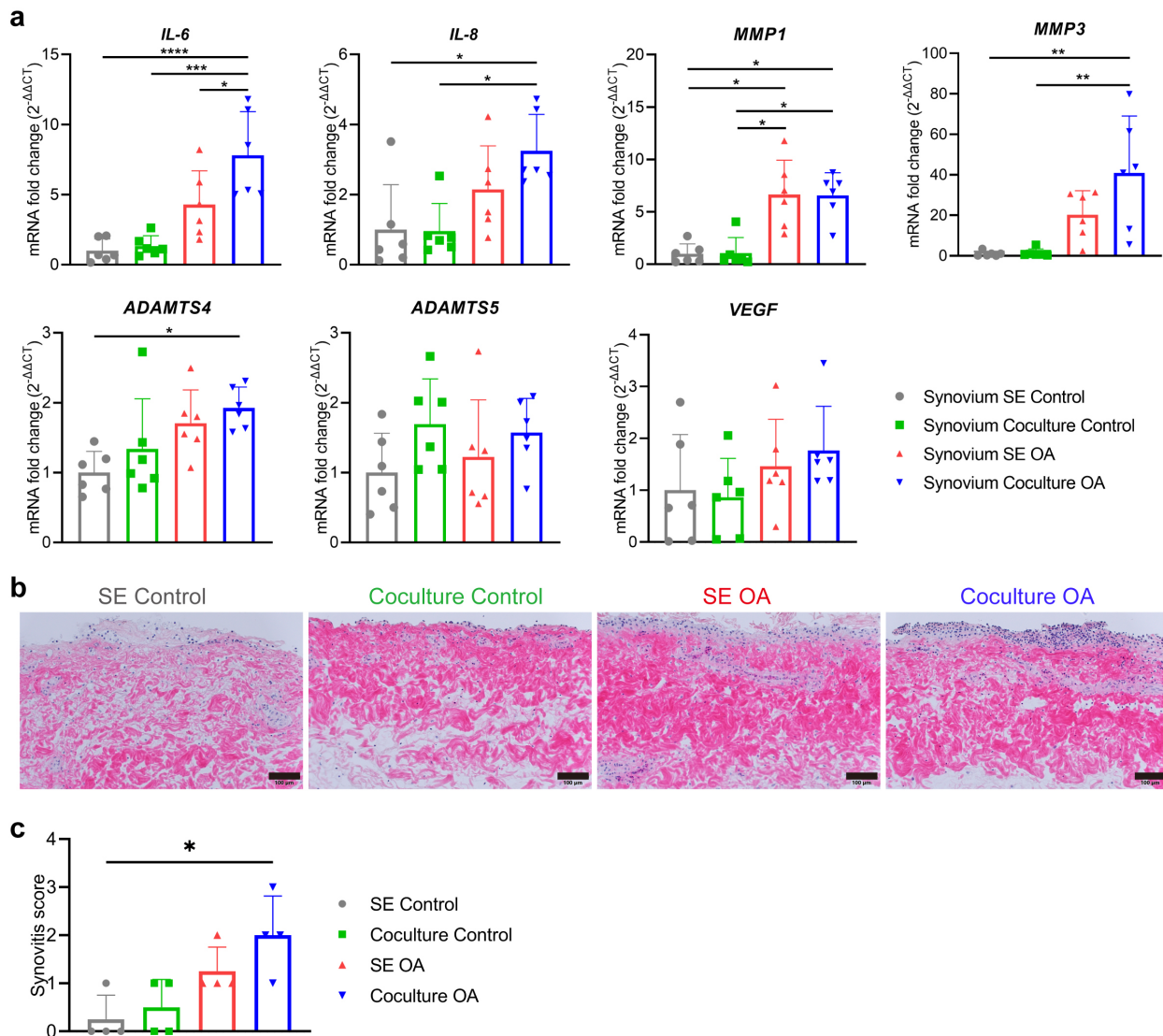


Fig. 6. Gene expression and histological morphology of synovium in monoculture or osteochondral explant-cocultured groups with or without $IL-1\beta$ and $TNF-\alpha$ stimulation for 7 days. (a) Gene expression levels of *IL-6*, *IL-8*, *MMP1*, *MMP3*, *ADAMTS4*, *ADAMTS5* and *VEGF* in synovium from monoculture or coculture groups in the absence or presence of cytokines ($n = 6$). (b) Representative images of Hematoxylin and Eosin staining on synovium tissue of above groups. Scale bars, 100 μm . (c) Synovitis score quantified on Hematoxylin and Eosin staining images ($n = 4$). Data presented as means + SD. Statistical analysis was performed by one-way analysis of variance (*IL-6* and *ADAMTS5* in a) or Kruskal-Wallis test (*IL-8*, *MMP1*, *MMP3*, *ADAMTS4*, and *VEGF* in a; histological analysis in c). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Previously, *MMP13* and *ADAMTS4* expression in cartilage tissue was significantly increased by coculture in a model established with bovine cartilage and joint capsule (including synovial lining layer) under mechanical injury (Lee *et al.*, 2009). In another coculture model using bovine cartilage with synovium and fibrous joint capsule, increased activities of aggrecanases and MMPs in the coculture group were demonstrated by analyzing the proteolytic fragments of aggrecan (Swärd *et al.*, 2017). In these two previous studies, intervention on cartilage was mechanical injury rather than cytokine stimulation as in our work. Mechanical cartilage injury could produce cartilage wear particles and

induce inflammatory cascade in synovium tissue (Estell *et al.*, 2019), which in turn could make catabolic gene changes in cartilage. In histological semi-quantification analysis, no remarkable proteoglycan loss was observed after 7 days when comparing the cartilage staining in coculture and respective monoculture group. However, Beekhuizen *et al.* (2011) observed that synovial tissue extracted from human OA knee joints inhibited proteoglycan production in cocultured cartilage after 21 days (Lee *et al.*, 2009). Hence, a long-term culture period and alternative GAG quantification method, rather than histological staining, may reveal further information on cartilage matrix changes.

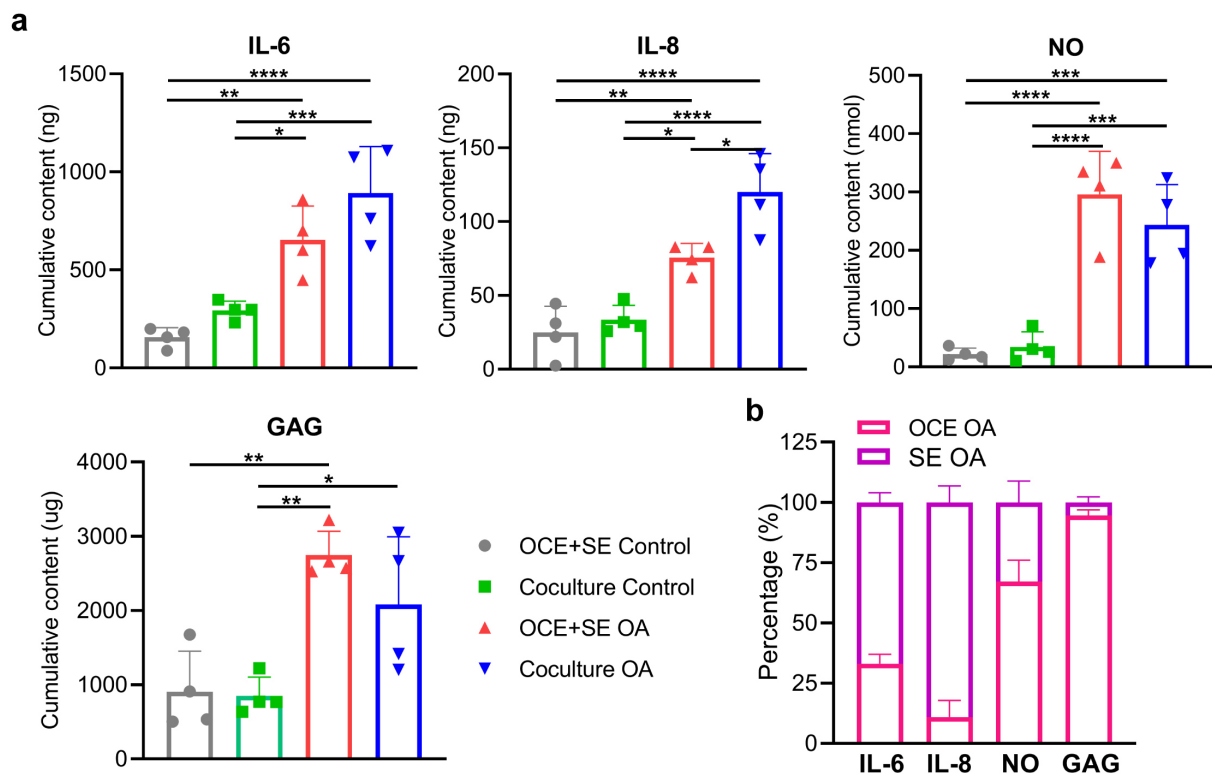


Fig. 7. Medium analysis of osteochondral and synovial explants monoculture and their coculture system with or without cytokine stimulation. (a) IL-6, IL-8, NO, and GAG release into the medium in OCE+SE groups and Coculture group. OCE+SE, total cumulative amount in the medium of OCE and SE monoculture systems from the same donor. Coculture, total cumulative amount in the medium of upper and lower compartments in the coculture system ($n = 4$). (b) The percentage of IL-6, IL-8, NO, and GAG release by monocultured OCE or SE in the OCE+SE group under inflammatory conditions. Data presented as means + SD. Statistical analysis was performed by one-way analysis of variance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In previous coculture studies with OCEs and SEs, little attention has been paid to the synovium (Byron and Trahan, 2017; Greenberg *et al.*, 2006; Lee *et al.*, 2009; Swärd *et al.*, 2017). In the current study, synovium tissue was analyzed to investigate the effects of coculture with OCE. Under non-inflammatory conditions, the tested genes showed no difference between Coculture Control group and SE Control group, implying healthy cartilage has no adverse effects on synovium tissue. Under inflammatory conditions, gene expression of *IL-6* in synovium showed a marked upregulation in the coculture group compared with SE monoculture, suggesting inflamed cartilage in the coculture system could aggravate inflammatory and catabolic responses in the synovium. The thickened lining layers in the Coculture OA group were more abundant than in the SE OA group, suggesting a stronger inflammatory response in synovium in Coculture OA group. These findings revealed that *ex vivo* coculture with inflammation-activated OCEs could promote synovitis in synovium tissue, in line with previous *in vivo* studies (Sellam and Berenbaum, 2010). Clinically, OA is a disease with high heterogeneity that could be divided into at least three phenotypes according to the most active tissue type at a certain stage of OA, like degenerative

cartilage, remodeling subchondral bone, or inflamed synovium (Karsdal *et al.*, 2016). This might be an important reason why a drug targeting cartilage fails in a patient with OA phenotype of inflamed synovium. Thus, when screening novel drugs, their effects on synovium and/or bone tissue should also be tested in a preclinical model. The coculture system in our study could be beneficial for evaluating the effects of a potential drug on cartilage and synovium simultaneously.

When analyzing the content of mediators or metabolites in the medium, the secretion from both cartilage and synovium tissue should be taken into account. As expected, due to release from synovium, the amount of a mediator or metabolite in the coculture group is higher than the OCE monoculture group, which was the case in a previous study (Byron and Trahan, 2017). The comparison between these two groups does not reveal the interaction between cartilage and synovium tissue. To evaluate the crosstalk between tissues in the coculture system, we summed up the total amount of mediators or metabolites in the OCE and SE monoculture groups (namely OCE+SE group) and compared it to the coculture group. As shown in Fig. 7a, a significant elevation of IL-8 release in the medium was ob-

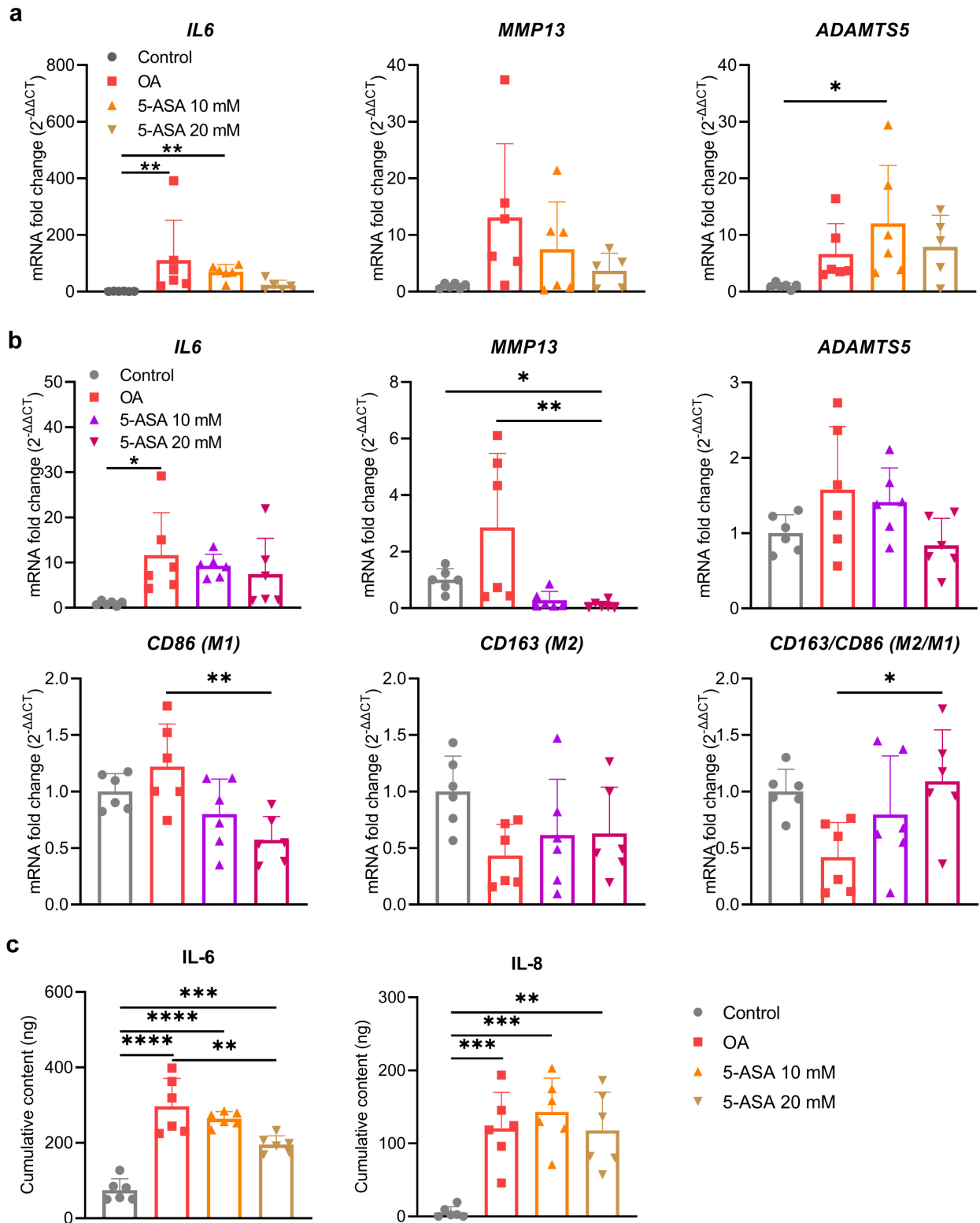


Fig. 8. Effects of 5-aminosalicylic acid on the inflammatory coculture system established with bovine osteochondral and synovial explants. The gene expression changes in the cartilage (a) and synovium tissue (b) upon 5-aminosalicylic acid treatment (n = 5-6). (c) the cumulative content of IL-6 and IL-8 in the conditioned medium. Data presented as means + SD. Statistical analysis was performed by one-way analysis of variance (*MMP13* in a; *IL-6*, *ADAMTS5*, *CD86*, *CD163*, and *CD163/CD86* in b; *IL-6* and *IL-8* in c) or Kruskal-Wallis test (*IL-6*, and *ADAMTS5* in a; *MMP13* in b). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

served in the coculture group in comparison to the OCE+SE group under inflammatory conditions, indicating that coculture of OCE and SE explants have a synergistic effect on inflammatory mediator secretion. In another coculture model with equine cartilage and synoviocytes, Gregg *et al.* (2006) concluded that GAG loss induced by IL-1 β was less in coculture than cartilage monoculture. However, in Beekhuizen's study, coculture reduced final GAG content in human OA cartilage by inhibiting GAG production rather than promoting GAG release, which was comparable in coculture and monoculture groups (Beekhuizen *et al.*, 2011). The effects of coculture on GAG content, production and release needs further investigation due to the contradictory results. In the inflammatory OCE+SE group, the percentage of a mediator or metabolite released by synovium was calculated (Fig. 7b). The results indicated that synovium tissue play a bigger role in the IL-6 and IL-8 cytokines secretion, which are key mediators for the disease progression in knee OA (Sellam and Berenbaum, 2010).

Our previous study showed that 5-ASA had anti-inflammatory and pro-anabolic effects on human inflammatory chondrocytes pellet model and could mitigate OA development in human inflammatory OCE model. To assess the effects of 5-ASA in a model closer to native OA milieu, it was further tested in the current *ex vivo* coculture system established with OCE and SE. Healthy bovine stifle joints are good tissue sources since the access to human synovium tissue is limited. In the bovine inflammatory coculture system, 5-ASA showed an anti-inflammation effect as indicated by inhibition of inflammatory gene expressions and inflammatory mediator release in OCE and SE. Furthermore, the M1 macrophage marker *CD86* in the synovium was inhibited and the ratio of *CD163/CD86* (M2/M1) was upregulated upon 5-ASA, indicating that 5-ASA might mitigate the inflammation in the coculture system via repolarization of macrophages from M1 to M2.

As a whole joint disease, subchondral bone also contributes to development and remodeling in OA (Hu *et al.*, 2020). In our study, the subchondral bone is not analyzed due to the following reasons. First, the *ex vivo* inflammatory medium is to mimic the *in vivo* synovial fluid, which is in contact with cartilage and synovium tissue. The subchondral bone in native joints is in bone marrow cavity and insulated from synovial fluid (Li *et al.*, 2022). Secondly, the remodeling changes of subchondral bone during OA development is mainly caused by excessive mechanical loads, which is not included in the present study (Hu *et al.*, 2020). Thirdly, the included subchondral bone is to reduce cartilage damage during OCE extraction and to keep oxygen and nutrient gradients in the cartilage during *ex vivo* culture. Last but not the least, bone marrow components, including osteocytes, osteoblasts and osteoclasts, may be lost during *ex vivo* culture without the protection of a closed cavity (Li *et al.*, 2022). To further study the role of subchondral bone in an *ex vivo* OA model in the future, we propose a two-

compartment culture system, which separates the articular cartilage and subchondral bone and is fed by different culture media (Li *et al.*, 2022). Moreover, detrimental biomechanical loading can be applied to the OCEs to better simulate the native OA milieu (Li *et al.*, 2022), in which it is more meaningful to analyze the subchondral bone and its crosstalk with articular cartilage.

There are some limitations in our study. Firstly, only mediators of IL-6, IL-8, and NO in the medium were analyzed. Measurement of other mediators, especially protective factors, might help to better understand the role of synovium in the coculture system and the mechanism of synovitis triggered in the coculture system. Secondly, only GAG release in the medium was measured in this study. GAG content retained in the cartilage tissue and newly synthesized GAG maybe measured with biochemical and radioactive assays to quantify GAG retention and production in the future. Thirdly, high cytokine concentration (at ng/mL level) was applied to induce detectable changes in a short term as generally performed. However, the physiological levels of IL-1 β and TNF- α in the synovial fluid are much lower (at pg/mL level) (Chevalier *et al.*, 2013; Rutgers *et al.*, 2010). The pathological changes induced with cytokines at artificially high concentrations may not well represent the OA mechanism *in vivo*. Since OCEs and synovial tissues have demonstrated a high cell viability after 7 days of culture in this study, a longer culture period and lower concentration of inflammatory cytokines stimulation, closer to the *in vivo* progression of this chronic disease, may be applied in the future.

Conclusions

Monocultured bovine OCE and SE showed a strong inflammatory response upon cytokine stimulation. Coculture of OCE and SE induced significant changes in cartilage and synovium compared with their respective monoculture, indicating the interaction between OCE and SE in this *ex vivo* system. The inflammation responses in the coculture system could be reversed by 5-ASA, demonstrating the OCE and SE coculture system might be used as a preclinical model to screen potential disease-modifying OA drugs.

List of Abbreviations

OA, osteoarthritis; OCEs, osteochondral explants; SEs, synovial explants; 5-ASA, 5-aminosalicylic acid; IL-1 β , interleukin-1 β ; TNF- α , tumor necrosis factor- α ; LDH, lactate dehydrogenase; EthD, ethidium homodimer; PBS, phosphate buffered saline; IOD, integrated optical density; MRC, Molecular Research Center; BCP, 1-bromo-3-chloropropane; qPCR, quantitative polymerase chain reaction; ANOVA, analysis of variance; GAG, glycosaminoglycan; NO, nitric oxide; ELISA, Enzyme Linked Immunosorbent Assay; SD, standard deviation.

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Not applicable.

Author Contributions

KL, SG, and ZL contributed to the design of the work; KL and ZL contributed to the acquisition, analysis, and interpretation of data for the work; KL drafted the manuscript; KL, YZ, MA, MS, SG, and ZL revised the manuscript critically; and all authors provided approval for publication of the content.

Ethics Approval and Consent to Participate

The bovine tissue used in this study were obtained from a local slaughterhouse. No ethical approval is required for the use of these tissue.

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Conflict of Interest

All authors declare no conflict of interest. MA, MJS, SG are serving the Editorial Board members of this journal. We declare that MA, MJS, SG had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to CE.

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