Research Article

A Human Osteoarthritis Mimicking Goat Cartilage Explant-Based Disease Model for Drug Screening

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Abstract

Although osteoarthritis (OA) is the most prevalent human joint disease with a large socioeconomic burden, it remains a neglected disease with no clinically approved disease modifying therapies. One of the key reasons for this is that the available disease models poorly recapitulate human OA-like traits, possibly because of the challenge of mimicking the disease in an ECM-rich cartilage tissue. In this study, we report the establishment and validation of a clinically relevant ex vivo OA model using IL1β-treated goat articular cartilage explants. Treatment with IL1β induced OA-like traits in goat cartilage explants and caused a shift in cartilage homeostasis towards enhanced catabolism, resulting in higher matrix degradation, overexpression of degradative and inflammatory mediators, and chondrocyte hypertrophy. We then validated the developed disease model for drug response using the drugs celecoxib, BMP7, and rapamycin, all of which demonstrated concentration-dependent disease amelioration in the model. Finally, we evaluated the translational relevance of the developed ex vivo OA model by comparing it with late-stage OA patient samples and observed a striking resemblance in terms of matrix degradation, expression of degradative enzymes, chondrocyte hypertrophy, and inflammation. Overall, the goat ex vivo OA model elicited a biological response to cytokine treatment that mirrors human OA-like traits and may reduce discordance between preclinical and clinical studies in OA drug development.

1 Introduction

Osteoarthritis (OA) is one of the fastest-growing diseases and affects approximately 7% of the population worldwide (Hunter et al., 2020). Despite its prevalence, no cure for OA is available, and joint replacement by prosthesis is the only option for the treatment of late-stage OA patients (Zhang et al., 2016). Recently, disease modifying osteoarthritis drugs (DMOADs) capable of preventing disease progression and/or restoring normalcy are being actively explored for OA treatment (Lohmander and Roos, 2019).

One of the important bottlenecks for the effective discovery of DMOADs is the lack of a disease model that accurately mirrors the multifaceted human OA pathology (Cope et al., 2019). This often leads to failure of candidate drugs in expensive clinical trials (Le Graverand-Gastineau, 2009; Karsdal et al., 2016). Any disease model used for drug screening should be easy to use, cost-effective, reproducible, and fast. Further, in developing a new model, the 3R principles of replacing, reducing, and refining animal experimentation should be considered (Occhetta et al., 2019).

OA develops as a multifactorial joint disease characterized by excessive degradation of cartilage matrix, inflammation in the synovium, and remodeling of subchondral bone (Roos and Arden, 2016). Risk factors for OA disrupt joint homeostasis by unbalancing the anabolism and catabolism in resident chondrocytes, causing inflammation and eventual cartilage degradation as well as adverse effects in other joint tissues (Mobasher et al., 2017). Pro-inflammatory cytokines such as IL1β regulate these events via NFκB-mediated activation of the downstream inflammatory response (Robinson et al., 2016). Therefore, IL1β has been widely explored, either alone (Corciulo et al., 2017) or in combination with TNFα (Murab et al., 2013), for the establishment of OA-like conditions in in vitro models of OA.

Although cytokine-treated in vitro models of OA are suitable as simple models of disease pathology, they fail to accurately recapitulate human OA conditions due to a lack of extracellular...
matrix (ECM) or having a non-physiological ECM (Cope et al., 2019). While OA is a disease of the whole osteochondral unit, the progression of OA is mainly mediated by active remodeling of cartilage ECM by chondrocytes, leading to cartilage destruction. ECM-rich cartilage tissue mainly contains type II collagen and proteoglycans that maintain the biomechanical properties of articular cartilage necessary for the load-bearing functions of cartilage (Wieland et al., 2005). In OA, the homeostatic balance is tilted in favor of catabolism, causing ECM remodeling that leads to an altered biomechanical milieu, which in turn drives the advancement of the disease. Therefore, OA can be considered a disease of the ECM where aberrant cross-talk between chondrocytes and ECM leads to alteration and destruction of matrix components. Hence, to mirror human OA pathology, disease models should closely mimic human ECM along with recapitulating key disease hallmarks. Based on the above points, ex vivo disease models that can mirror human OA pathology could improve the effectiveness of drug screening.

For the development of an ex vivo OA model, cartilage explants from bovine (Wang et al., 2009), porcine (Siengdee et al., 2015), ovine (Chan et al., 2011), and feline (Gabriel et al., 2010) sources have been explored. However, they poorly mimic human cartilage or OA pathologies. Caprine cartilage, which is highly similar to human cartilage, has surprisingly not been investigated as an OA disease model. The knee cartilage of a skeletally mature goat possesses a striking resemblance to adult human cartilage in terms of anatomy, zonal structure, cellularity, ECM composition, and thickness (Cope et al., 2019; Little et al., 2010). Further, goat cartilage explants are readily available and are relatively inexpensive, which augments their suitability as an ex vivo OA model.

To assess translatability, an OA model for DMOAD screening should be extensively characterized for hallmarks of human OA. However, demonstrating OA-like traits in the developed disease model alone may not be sufficient and a comparison of the models with human OA samples may provide even higher confidence regarding its clinical relevance.

In this study, we report the establishment and validation of an ex vivo OA model using goat articular cartilage explants that mimic human osteoarthritis. The developed disease model mirrored human OA in terms of cartilage ECM loss, overexpression of degradative and inflammatory markers, and increased hypertrophy of chondrocytes.

# Materials and methods

## Induction of OA-like conditions in goat articular cartilage explants

For the establishment of an ex vivo OA model, skeletally mature goat (male, 18-24 months old) stifle joints were collected from a slaughterhouse, and cartilage explants (4 mm diameter) were harvested from femoral condyles under aseptic conditions with a biopsy punch (Kai Medical, Japan). These cartilage pieces were rinsed thoroughly with phosphate-buffered saline (PBS) containing 200 U/mL penicillin-streptomycin (HiMedia Labs, India), 5 µg/mL ciprofloxacin, and 5 µg/mL amphotericin B (HiMedia Labs, India). The explants were then cultured for 48 h in 48-well plates using DMEM (high glucose, Sigma Aldrich, United States) supplemented with 1 mM sodium pyruvate, penicillin-streptomycin (100 U/mL), ciprofloxacin (2.5 µg/mL), and amphotericin B (2.5 µg/mL) for acclimatization. For induction of OA-like conditions, explants were incubated with 10 and 20 ng/mL of IL1β or medium (Sino Biological, United States) as per the protocol adapted from a previous publication (Corciulo et al., 2017). IL1β was replenished twice a week during each medium change, and the medium from each well was harvested at predetermined time points and frozen until further investigation. IL1β-treated explant samples were harvested after 3 weeks of culture and frozen until characterization for OA-like conditions.

**Estimation of sulfated glycosaminoglycan (sGAG) and total collagen release from IL1β-treated goat cartilage explants**

The amount of sGAG and total collagen released from the cartilage explants into the medium was estimated by 1,9 dimethylmethylene blue (Barbosa et al., 2003) and picrosirius red (Marotta and Martino, 1985) assay respectively as per previously reported protocols.

**Histology and immunofluorescence**

For histological and immunofluorescence staining, explants were carefully washed with PBS, fixed with 4% neutral buffered formalin (NBF) for 4 h, embedded in OCT (Sakura Japan), and kept at -80°C until further study. Samples were then sectioned using a cryotome (Leica 1860UV, Germany) at a thickness of 10 µm. Histological staining for sGAG (safranin O-fast green (SOFG) and Alcian blue) and total collagen (picrosirius red) was performed using standard histology protocols. Immunofluorescence staining for chondroitin sulfate (ChS), collagen type II (Col II), and aggrecan (Aggn) (DSHB, USA) was performed following a previously published protocol (Bhattacharjee and Katti, 2018) and imaged using a confocal microscope (LSM780NLO, Carl Zeiss GmbH). For immunofluorescence staining of Collagen type X (Coll X) (Sigma Aldrich, USA), decorin (DSHB, USA), MMP13, ADAMTS4, NFkB, p38 MAPK, iNOS, cyclooxygenase 2 (Cox-2), and osteopontin (OPN) (Sino Biologicals, USA), the manufacturers’ respective protocols were followed (see Tab. S2 for antibody details).

**Quantification of immunofluorescence staining by image analysis**

Quantification of the intensity of immunostaining was performed using ImageJ software (Schneider et al., 2012) in at least

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1 doi:10.14573/altex.2107071s
Gene expression analysis of ex vivo OA model

The expression of matrix degradative and hypertrophic marker genes was investigated after 7 days of IL1β treatment of goat articular cartilage explants. For this, total RNA was extracted using TRI reagent following the manufacturer’s protocol. Briefly, the cartilage explants were harvested after 7 days of IL1β treatment with or without drug treatment and were snap-frozen in liquid nitrogen. The frozen explants were then minced into small pieces using a surgical blade, maintaining ice-cold temperature. The samples were homogenized using a microfuge (samples were re-frozen in liquid nitrogen before this process to facilitate the grinding process). The homogenized tissue samples were then treated with 700 μL TRI reagent (Sigma Aldrich) and stored at -80°C until further use. For the extraction of RNA, the samples were thawed, and 140 μL chloroform was added and centrifuged at 13,000 rpm for 15 min at 4°C. The top aqueous layer was collected in a fresh tube, and an equal volume of isopropanol was added to the aqueous phase to precipitate the RNA, followed by centrifugation at 13,000 rpm for 15 min to obtain the RNA pellet. The RNA pellet was washed twice with 75% chilled ethanol followed by drying at room temperature and resuspension in RNase-free water. Total RNA present in the samples was quantified using a Nanodrop spectrophotometer (Thermo Scientific).

Preparation of cDNA was performed with 500 ng total RNA using a high-capacity cDNA reverse transcription kit (Puregene, catalog no.-PGK162B) following the manufacturer’s protocol. Quantitative PCR (QuantStudio™ 5, Applied Biosystems) was performed using SYBR Green Master Mix, and the fold change present in the samples was quantified using a Nanodrop spectrophotometer (Thermo Scientific).

Culture of human osteoarthritic cartilage explants

Human knee cartilage samples were collected from OA patients undergoing knee arthroplasty as per the approved protocol (EC no:20/E.C. Acad./01.05.2019) by the institutional ethics committee of GSVM (Ganesh Shankar Vidyarthi Memorial) Medical College, Kanpur, India with prior informed consent from the patients (see Tab. S1 for patient characteristics). Femoral condylar cartilage explants of 4 mm diameter were collected and cultured in high-glucose DMEM (Sigma Aldrich, United States) supplemented with 1 mM sodium pyruvate, penicillin-streptomycin (100 U/mL), ciprofloxacin (2.5 μg/mL), and amphotericin B (2.5 μg/mL) for 2 days for acclimatization. Human OA explants were then cultured for 14 more days followed by harvesting of explants, fixation in 4% NBF, and embedding in OCT followed by characterization for OA-specific markers.

Statistical analysis

All data is presented as mean ± standard deviation; at least 3 biological replicates for each experiment were performed, and representative data from the 3 repeats is provided. All statistical analysis was performed using GraphPad Prism 7 with Student’s t-test or one-way analysis of variance (ANOVA) with Tukey’s post-hoc analysis based on the number of study groups. P < 0.05 was considered statistically significant. For human OA explant studies, data from each donor were provided unless otherwise mentioned.
3 Results

3.1 IL1β treatment induces matrix loss in goat cartilage explants

Goat cartilage explants were treated with 10 or 20 ng/mL IL1β for 3 weeks to induce OA-like conditions as depicted in Figure 1A. To confirm the induction of OA-like traits, the goat cartilage explants were characterized for different markers of human OA cartilage such as ECM degradation, overexpression of matrix degradative enzymes, chondrocyte hypertrophy, and inflammatory mediators. While differences between control and IL1β-treated explants are described in detail below, differences between the two IL1β concentrations did not reach statistical significance in any of the assays.

We observed that IL1β treatment reduces the sulfated glycosaminoglycan (sGAG) content in cartilage explants when compared to control explants as observed via SOFG staining (Fig. 1B). Quantification of staining intensity using image analysis revealed a statistically significant reduction in sGAG content for IL1β-treated explants (Fig. 1B) versus control explants. To corroborate these results, we estimated the amount of sGAG released into the medium following IL1β treatment. As expected, IL1β treatment of explants resulted in enhanced sGAG levels in the medium compared to medium from control cartilage explants (Fig. 1C). Similarly, IL1β treatment of explants also caused greater release of total collagen into the medium compared to control explants (Fig. 1C). However, picrosirius red staining of cartilage explants for total collagen demonstrated comparably intensive staining in control as well as IL1β-treated cartilage explants (Fig. 1B).

To further confirm the IL1β induced matrix loss from goat articular cartilage explants, we performed immunostaining for specific cartilage matrix markers. Staining of chondroitin sulfate (ChS), the major sGAG present in the articular cartilage extracellular matrix, was reduced in IL1β-treated explants when compared to control (Fig. 2A). Quantification of fluorescence intensity revealed significantly less retention of ChS in IL1β-treated explants as compared to control (Fig. 2B). Similarly, IL1β treat-
Immunofluorescence staining of cartilage explants revealed enhanced expression of MMP13 and ADAMTS4 in IL1β-treated cartilage explants compared to control explants (Fig. 3A). Further, percent MMP13 and ADAMTS4 positive cells was significantly higher in IL1β-treated groups compared to control (Fig. 3B). These results were corroborated by analyzing the expression of Mmp13 and Adamts4 mRNA in goat cartilage explants using qPCR. Expression of both Mmp13 and Adamts4 was significantly enhanced in IL1β-treated cartilage explants when compared to control (Fig. S8A,B).

Further, we performed fluorescence-based quantification of MMP13 activity in the medium of cartilage explants after IL1β treatment and observed a significant enhancement in specific MMP13 activity (Fig. 3C) compared to untreated control. Therefore, it appears that IL1β treatment significantly enhances the expression and activity of matrix degrading proteases in goat cartilage explants.

3.3 IL1β treatment upregulates chondrocyte hypertrophic markers in goat cartilage explants

Human OA is strongly associated with hypertrophic changes in resident chondrocytes. Therefore, we evaluated the effect of IL1β treatment on chondrocyte hypertrophy in goat cartilage explants. Immunostaining of collagen type X (Coll X) and Osteo-
opontin (OPN), two key hypertrophic markers associated with human OA (Van der Kraan and Van den Berg, 2012), demonstrated higher marker expression in IL1β-treated cartilage explants compared to control (Fig. S3A). Image analysis-based quantification of fluorescence intensity corroborated this observation, with IL1β-treated cartilage explants showing a significantly higher intensity of Coll X (Student’s t-test, P = 0.0349) and OPN (Fig. S3B). Further, quantification of mRNA expression also showed significantly higher expression of Coll X in the cartilage explants treated with IL1β compared to control (Fig. S8C). Runx2 expression was also increased in response to IL1β treatment, but this did not reach statistical significance (Fig. S8D). Overall, treatment with IL1β led to the upregulation of the chondrocyte hypertrophic markers Coll X and OPN in goat articular cartilage explants.

### 3.4 OA-associated signaling pathway markers are expressed in IL1β-treated goat cartilage explants

The NFκB signaling pathway is a key catabolic regulator in human OA cartilage. NFκB stimulates the expression of genes responsible for the degradation of cartilage, inflammation of the synovium, and enhanced resorption of subchondral bone (Chang et al., 2019). Therefore, we evaluated the status of NFκB expression in goat cartilage explants after treatment with IL1β. As expected, a high expression of NFκB was observed in the IL1β-treated cartilage explants with negligible staining in control explants (Fig. 4A). Quantification of percent NFκB positive chondrocytes revealed a significantly higher fraction of NFκB expressing cells in the IL1β-treated groups compared to control (Fig. 4B).

The p38 signaling pathway is connected to enhanced apoptosis and inflammatory responses in OA. Immunofluorescence staining revealed enhanced expression of p38 MAPK in the IL1β-treated cartilage explants as compared to control explants (Fig. 4A), with a higher percentage of p38 positive chondrocytes observed in cartilage explants treated with IL1β (Fig. 4B).

Further, enhanced NFκB expression in cartilage often leads to overexpression of iNOS, which in turn causes the initiation of inflammatory cascades. As IL1β-treated cartilage explants demonstrated higher expression of NFκB, we evaluated the expression of iNOS. As expected, IL1β-treated cartilage explants demonstrated enhanced iNOS expression (Fig. 4A) compared to control. Quantification of percent iNOS positive chondrocytes revealed a significantly higher fraction of iNOS expressing cells in the IL1β-treated groups compared to control (Fig. 4B).

Taken together, we showed that treatment of goat cartilage explants with IL1β led to higher expression of OA-associated signaling pathways such as NFκB, p38, and iNOS.

### 3.5 Treatment with IL1β alone induces OA-like traits comparable to a combination of IL1β and TNFα

In our initial experiments, we found no difference between treatment with 10 or 20 ng/mL IL-1β in any of the assessed param-
Pharmacological validation of the established disease model was performed by assessing the efficacy of the anti-osteoarthritis drugs/drug candidates celecoxib, BMP-7 and rapamycin. Celecoxib is a standard anti-inflammatory OA drug whose anti-catabolic effects have previously been established (Zweers et al., 2011). The goat ex vivo OA model was treated with IL1β and different concentrations of celecoxib (0.1 µM and 1 µM), and cartilage matrix degradation was evaluated. Celecoxib enhanced the retention of sGAG in cartilage explants treated with IL1β in a concentration-dependent manner, as observed by SOFG and Alcian blue staining (Fig. 6A). This finding was substantiated by measuring the loss of cartilage matrix components from ex vivo OA explants into the medium after treatment with celecoxib. Cartilage sGAG and total collagen loss from ex vivo OA explants was significantly reduced, indicating efficacy of celecoxib in ameliorating OA-like conditions (Fig. 6B). Further, pharmacological validation of the established disease model was performed by assessing the efficacy of the anti-osteoarthritis drugs/drug candidates celecoxib, BMP-7 and rapamycin. Celecoxib is a standard anti-inflammatory OA drug whose anti-catabolic effects have previously been established (Zweers et al., 2011). The goat ex vivo OA model was treated with IL1β and different concentrations of celecoxib (0.1 µM and 1 µM), and cartilage matrix degradation was evaluated. Celecoxib enhanced the retention of sGAG in cartilage explants treated with IL1β in a concentration-dependent manner, as observed by SOFG and Alcian blue staining (Fig. 6A). This finding was substantiated by measuring the loss of cartilage matrix components from ex vivo OA explants into the medium after treatment with celecoxib. Cartilage sGAG and total collagen loss from ex vivo OA explants was significantly reduced, indicating efficacy of celecoxib in ameliorating OA-like conditions (Fig. 6B). Further,
treatment with both concentrations of celecoxib significantly reduced mRNA expression of Mmp13 (Fig. S9A) compared to IL1β-treated cartilage explants, whereas, treatment with the higher concentration of 1 µM celecoxib significantly reduced Adamts4 expression (Fig. S9B). Moreover, treatment with celecoxib reduced the expression of Cox-2 based on immunofluorescence, confirming its established mechanism of action in the goat ex vivo OA model (Fig. 6C).

Similarly, we evaluated the responsiveness of the ex vivo OA model to BMP-7, a known anabolic factor in cartilage, and rapamycin, an autophagy initiator. Both these agents are potential disease modifying OA drugs and are in clinical trials for OA treatment. Treatment with BMP-7 and rapamycin demonstrated concentration-dependent retention of cartilage sGAG as visualized by SOFG and Alcian blue staining in the goat ex vivo OA model (Fig. 7A). This finding was further substantiated by biochemical estimation of cartilage matrix components in medium from explants after BMP-7 and rapamycin treatment (Fig. 7D). Further, treatment with the drugs led to a reduction in the expression of key OA degradative enzymes MMP13 and ADAMTS4 in the ex vivo OA model (Fig. 7B).

Fig. 5: Treatment with IL1β alone induces OA-like conditions comparable to a cocktail of IL1β and TNFα (A) SOFG and Alcian blue staining of cartilage explant sections for sGAG after 15 days of treatment with IL1β, TNFα, or their combination (scale bar – 50 µm); (B) biochemical estimation of cumulative sGAG release; (C) immunofluorescence micrographs of cartilage sections stained for ADAMTS4 (red, top), MMP13 (red, bottom), and nuclei (blue) (scale bar – 100 µm). *** P < 0.001 with respect to control; ns, not significant. N = 4 (one-way ANOVA with Tukey’s correction for multiple comparisons).

Quantification of percent MMP13 and ADAMTS4 positive cells in cartilage explants confirmed significant reduction upon drug treatment (Fig. 7C). These results were supported by gene expression analysis, which showed a trend towards concentration-dependent reduction of the expression of both the degradative markers (Fig. S9).

To understand the effect of drug treatment on the viability of chondrocytes in the cartilage explants, live/dead staining was performed. We observed no marked difference in the viabi-
ty of chondrocytes between different treatment groups at both time points studied, except for 1µM celecoxib at 14 days, which showed a relatively higher number of dead chondrocytes (Fig. S7\textsuperscript{1}). These results demonstrated that cartilage matrix components were retained by treatment with BMP-7 and rapamycin, whereas the expression of matrix degradative enzymes was significantly reduced, indicating efficacy of the drugs in the amelioration of OA-like conditions.

### 3.7 Comparison of goat ex vivo OA model with human OA cartilage

To determine the clinical relevance of the developed goat ex vivo OA model, we compared it with late-stage human patient OA cartilage samples in terms of expression of matrix degrading, inflammatory, hypertrophic, and cartilage matrix-specific markers.

The expression of MMP13 and ADAMTS4 in the goat ex vivo OA model and in OA cartilage from samples from three different patients was comparable (Fig. 8A). This observation was corroborated by the quantification of percent chondrocytes positive for MMP13 and ADAMTS4, which demonstrated no significant difference between IL1β-treated goat cartilage explants and human patient OA explants (Fig. 8B). Similarly, the expression of the inflammatory mediator NFκB and its downstream effector iNOS in IL1β-treated goat cartilage explants and clinical OA samples was comparable (Fig. S4\textsuperscript{1}).

The expression of OPN, a marker for chondrocyte hypertrophy, was also comparable between IL1β-treated goat cartilage explants and human patient samples (Fig. 8A). This result was confirmed by the quantification of percent chondrocytes that were positive for OPN expression (Fig. 8B). Moreover, aggre-
The present study reports an attempt towards reducing the discord between preclinical success and clinical failure of candidate drugs by developing a clinically relevant ex vivo disease model. Most of the available OA disease models fail to recapitulate the multifactorial pathogenesis and heterogeneity of human OA (Little and Hunter, 2013; Cope et al., 2019). Therefore, in this study, we attempted to model human OA-like traits using pro-inflammatory cytokine treatment of goat cartilage explants that closely resemble the human equivalent in terms of anatomy and collagen type 2 (Coll II) expression in the IL1β-treated goat cartilage explants was comparable to clinical OA samples (Fig. S5). While no significant difference in Coll II and aggrecan staining was observed between IL1β-treated goat cartilage explants and donor 2 and donor 3, there was a difference observed for donor 1.

Overall, the ex vivo OA model developed in this study recapitulates human OA in terms of major OA hallmarks such as matrix degradation and chondrocyte hypertrophy and is responsive to drugs that are part of the standard treatment protocol for OA.

### 4 Discussion

The present study reports an attempt towards reducing the discord between preclinical success and clinical failure of candidate drugs by developing a clinically relevant ex vivo disease model. Most of the available OA disease models fail to recapitulate the multifactorial pathogenesis and heterogeneity of human OA (Little and Hunter, 2013; Cope et al., 2019). Therefore, in this study, we attempted to model human OA-like traits using pro-inflammatory cytokine treatment of goat cartilage explants that closely resemble the human equivalent in terms of anatomy...
The focus of the present study was to develop a disease model that recapitulates the major hallmarks of human OA. The use of a recombinant proinflammatory cytokine is a simple yet effective strategy to initiate OA-like traits (Corciulo et al., 2017; Murab et al., 2013; Shi et al., 2019). IL1β is strongly related to the initiation and progression of human OA and is aberrantly expressed in OA cartilage as well as synovium (Corciulo et al., 2017; Kapoor et al., 2011). 10 ng/mL of IL1β treatment was adequate to induce osteoarthritis-like changes in goat articular cartilage explants. IL1β-treated goat cartilage explants demonstrated (Fig. 1C) a strong matrix degradative response in terms of significant loss of sGAG and total collagen, which concurs with previous studies in another explant model (McNulty et al., 2013) as well as in human OA (Wieland et al., 2005; Shi et al., 2019; Glyn-Jones et al., 2015). In OA, enhanced MMP13 activity mediates degradation of the cartilage matrix (Kapoor et al., 2011). Similarly, we demonstrated enhanced MMP13 activity (Fig. 3C) in the medium of IL1β-treated goat cartilage explants, suggesting the activation of an analogous OA-like degradative response in goat explants. This enhanced MMP activity is in line with our current understanding that IL1β can increase with MMP activity.

To the best of our knowledge, this is the first report in which goat cartilage explants were used for the establishment of an ex vivo OA model. Despite being relatively well explored as an in vivo model for OA research, it is intriguing to note the absence of caprine explants used as ex vivo OA disease models (Little et al., 2010). Therefore, considering the striking similarity between goat and human knee joints as well as the extensive use of goat as an in vivo OA model, we reasoned that caprine cartilage explants could be a suitable basis for the development of a clinically relevant ex vivo OA disease model. An ex vivo system provides an opportunity to implement the 3R principle, i.e., replace, reduce and refine animal use in scientific research, as joints can be procured from slaughterhouses, and the model can potentially reduce and replace in vivo experiments.

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(Little et al., 2010), size (Little et al., 2010), cellularity (Cope et al., 2019), zonal structure (McCoy, 2015), and thickness (Little et al., 2010). The developed ex vivo disease model showed a close similarity to human OA as characterized by enhanced matrix degradation, overexpression of degradative proteases, over-expression of OA-associated inflammatory signaling pathway markers, and chondrocyte hypertrophy.

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(Mengshol et al., 2001). Further, overexpression of MMP13 and ADAMTS4 protein (Fig. 3A,B) and mRNA (Fig. S8) were detected in IL1β-treated cartilage explants, demonstrating overexpression of matrix degrading proteases, a characteristic of OA.

This increase in matrix degrading proteases was associated with reduced retention of specific cartilage matrix markers like chondroitin sulfate and aggrecan (Fig. 2A,B; Fig. S1). In OA, degradation of cartilage matrix generally commences with the degradation of proteoglycans, followed by loss of collagen (Li et al., 2015), which may explain the extensive sGAG loss and mild collagen degradation (Fig. 1, 2) observed in our study. This holds importance in disease pathology as the degraded proteoglycan fraction, when released into the synovial space, further enhances the catabolic response in the joint. We also demonstrated that treatment with IL1β results in the degradation of decorin (Fig. S1), a small proteoglycan responsible for aggrecan network integrity and biomechanical function of cartilage ECM (Han et al., 2019). Moreover, in post-traumatic OA, decorin mediates degeneration and fibrillation of the cartilage matrix (Li et al., 2020). Therefore, we speculate that IL1β-induced decorin loss might initiate matrix degeneration in goat cartilage explants analogous to human OA. Further, as decorin is known to mediate biomechanical function to maintain cartilage matrix integrity, its reduction can lead to abnormal mechanical behavior, which in turn may mimic aberrant biomechanical responses observed in OA.

Since we have demonstrated that extensive sGAG degradation and mild collagen degradation occur in the ex vivo OA model, we speculated that resident chondrocytes display a characteristic heightened anabolic response to counter the matrix loss. It has been demonstrated previously that treatment with IL1β results in the modulation of cartilage ECM composition (Corciulo et al., 2017; Shi et al., 2019). We observed enhanced expression of the chondrocyte hypertrophic markers Coll X and OPN (Fig. S3), which are also upregulated in human OA (Aigner et al., 2006) as well as in experimental animal models of OA (Van der Kraan and Van den Berg, 2012), in IL1β-treated goat cartilage explants.

As disruption of joint homeostasis mediates initiation and progression of OA (Lories, 2008), various catabolic and inflammatory factors are upregulated in OA (Mobasher et al., 2017; Robinson et al., 2016). Amongst these, the NFκB signaling pathway is stimulated by proinflammatory cytokines, which in turn lead to the activation of multiple factors responsible for cartilage destruction and synovial inflammation (Saito and Tanaka, 2017). Overexpression of NFκB was observed in goat cartilage explants after treatment with IL1β (Fig. 4), which in turn may have led to overexpression of MMP13 and ADAMTS4 degrading cartilage matrix components. Additionally, this might also lead to the expression of several NFκB-mediated catabolic cytokines such as TNFα, IL1β, and IL6 that enhance the production of matrix degrading proteases, reduce proteoglycan and collagen synthesis, and augment NFκB activation through a positive feedback loop (Kapoor et al., 2011). Moreover, NFκB can further enhance the degradation of cartilage via overexpression of its downstream effectors such as iNOS (Fig. 4) and Cox-2 (Fig. 6C) as observed in our study as well as in a previous study (Ulivi et al., 2008).

Further, we also observed overexpression of p38 MAPK, which is known to regulate the degradation of collagen, apoptosis in chondrocytes, and inflammation in human OA in IL1β-treated goat cartilage explants (Fig. 4) (Feng et al., 2017). Conversely, inhibition of the p38 signaling pathway resulted in reduced apoptosis and proinflammatory cytokine production in chondrocytes. Moreover, upregulation of p38 is also reported in mechanical loading-based cartilage explant OA models, which leads to overexpression of matrix degrading proteases and reduction in collagen type 2 and aggrecan gene expression (Ding et al., 2010). Therefore, this study suggests a similar biological response of cartilage explants to cytokine treatment.

In all, we observed that treatment of goat articular cartilage explants with IL1β led to enhanced matrix degradation with a concurrent increase in the activity and expression of matrix degrading proteases, overexpression of OA-associated signaling pathways, and enhanced chondrocyte hypertrophy. Therefore, the IL1β-treated goat articular cartilage explant recapitulates major human OA-like traits and can be a potential disease model for screening for disease modifying OA drugs.

Our data exhibited robust evidence in support of the development of OA-like traits in IL1β-treated goat articular cartilage explants. However, treatment with a cocktail of pro-inflammatory factors is often envisioned to have a stronger effect than treatment with a single cytokine (Gabriel et al., 2010) for the induction of OA. Interestingly, we found that IL1β alone was sufficient to induce OA-like traits in goat articular cartilage explants (Fig. 5) and was not augmented by TNFα. Individual treatment of TNFα showed a negligible effect (Fig. 5) on goat cartilage explants, although previous studies have shown that TNFα can cause induction of OA-like conditions in cartilage explants and chondrocytes of other species (Johnson et al., 2016). This observation might be explained by a species-specific difference in chondrocyte density and proteoglycan content in the articular cartilage of different organisms (Kamisan et al., 2013).

To confirm the drug response of the developed ex vivo OA model, we demonstrated the efficacy of the established OA drug celecoxib (Ochetta et al., 2019) as well as the two potential DMOADs BMP-7 (Hunter et al., 2010) and rapamycin (Ochetta et al., 2019) using the disease model. The reduction of matrix degradation by celecoxib was probably mediated by decreasing the inflammatory milieu as observed by the downregulation of Cox-2 expression (Fig. 6C) in goat cartilage explants. BMP-7 is a known anabolic factor in cartilage that is responsible for the synthesis of cartilage ECM components (Chubinskaya et al., 2007). Our data suggest that BMP-7 can potentially regulate the expression of matrix degrading enzymes such as MMP13 and ADAMTS4 (Fig. 7). However, the exact mechanism of this regulation needs further elucidation. Further, rapamycin regulates autophagy and consequently controls matrix composition (Caramés et al., 2012). Our results demonstrate that rapamycin can control matrix retention and reduces the expression of key matrix proteases (Fig. 7). This data demonstrates that the ex vivo disease model can be used for the screening of disease modifying OA drugs targeting various OA pathological factors such as joint ho-
meostasis, inflammation, autophagy, senescence, and hypertrophy. Taken together, the ex vivo OA model demonstrates appropriate responsiveness to OA drugs (standard as well as drugs in clinical trials), confirming the suitability of the model for screening of novel disease modifying OA drugs.

Poor translatability of preclinical drug candidates to the clinical setting might be one of the major reasons behind the absence of effective disease modifying therapeutic approaches for the treatment of OA. The lack of appropriate disease models that recapitulate human OA conditions is one of the key factors that affect the translatability of drug candidates. A useful disease model preferably displays all the major pathological processes observed in actual disease. To better appreciate whether the developed disease model mimicked OA, we compared it with samples from late-stage OA patients and observed a strong similarity in terms of markers of matrix degradation, inflammation, and hypertrophy. Therefore, the goat ex vivo OA model may be well suited for understanding disease pathology with high accuracy as well as for successful drug screening. Since the developed model shows striking similarity with clinical samples, it might mitigate discordance between preclinical success and clinical failure, thereby improving the translatability of drug candidates. The high degree of resemblance between the disease model and OA patient samples (Fig. 8) might partially stem from the similarity between human cartilage and goat cartilage in terms of anatomy (Little et al., 2010), size (Little et al., 2010), cellularity (Cope et al., 2019), architecture (McCoy, 2015), and thickness of cartilage (Little et al., 2010).

This ex vivo OA model implements the 3R principle of replacement, reduction, and refinement of animal use for scientific research. Moreover, for culturing of goat cartilage explants we have not used any animal-derived products, including serum, unlike most of the available in vitro or ex vivo disease models. Besides, this model overcomes the major limitations of both explant-based and cytokine-based in vitro and ex vivo OA models such as cell death at explant edge (Cope et al., 2019), reduced reproducibility in case of explant-based models (McCoy, 2015), and poor disease mimicking due to change in phenotype in the case of cytokine-based cellular models (Johnson et al., 2016).

All cartilage-based in vitro disease models lack intertissue cross-talk, and involvement of other osteochondral tissues such as subchondral bone, and synovium cannot be studied using such disease models. Although the developed ex vivo OA model demonstrated OA-like traits, showed appropriate drug response, and was comparable with clinical OA samples in terms of major OA pathological markers, assertion of disease recapitulation by OA models should be made carefully. Moreover, consensus biomarkers for different stages of human OA are still not available. In addition, our understanding of OA-associated signaling pathways and their role in disease pathogenesis needs further exploration. Given this, bridging the gap between in vitro disease models and actual disease scenarios not only needs careful design and development of the model but also an extensive comparison of the developed model with clinical samples to mirror the disease pathology and to counter disease heterogeneity.

Taken together, we have demonstrated the development of a stable, cost-effective, and reproducible OA disease model using goat cartilage explants which elicits a biological response to cytokine treatment that mirrors human OA-like traits. This model recapitulates OA pathological changes such as degradation of cartilage ECM components, enhanced expression of degradative and inflammatory mediators, and enhanced chondrocyte hypertrophy. The model is responsive to drug treatment and predicts the effect of a standard OA medication in agreement with previously published reports. More importantly, this model is highly comparable to late-stage OA patient samples in terms of major OA-like traits. Therefore, the developed model promises to reduce the need for animal models and facilitate the screening of disease modifying OA drugs while expediting drug discovery and bridging the discord between preclinical success and clinical failures of drug candidates.

References


**Conflict of interest**

There are no conflicts of interest to report.

**Data availability statement**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Author contributions**

AB: conceptualization, formal analysis, investigation, methodology, resources, validation, visualization, writing (original draft, review, and editing); DSK: conceptualization, funding acquisition, methodology, project administration, supervision, validation, writing (review and editing).

**Acknowledgments**

The authors acknowledge the infrastructural and financial support provided by IIT Kanpur for the conduct of this research. AB acknowledges the financial assistance provided by MoE, Government of India, and DSK acknowledges the Gireesh Jankinath Chair Professorship. AB also acknowledges Dr Aditya Arora for helpful discussions during the inception of the project. The authors acknowledge Dr Praganesh Kumar and Dr Sanjay Kumar of GSVM Medical College Kanpur for their help in the procurement of human OA samples. The authors also acknowledge The Mehta Family Center for Engineering in Medicine, IIT Kanpur for generous support.