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

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Abstract

Osteoarthritis (OA) is one of the fastest-growing diseases, which affects approximately 7% of the population worldwide (Hunter et al., 2020). Despite its huge prevalence, currently, no known cure for OA is available, with joint replacement by prosthesis being the lone possible 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, is being actively explored for OA treatment (Lohmander and Roos, 2019).

However, one of the important bottlenecks for the effective screening of DMOADs is the non-availability of a disease model that mirrors human OA pathology which often leads to failure of candidate drugs in clinical trials (Le Graverand-Gastineau, 2009; Karsdal et al., 2016). Therefore, for the development of effective DMOADs, the OA model should closely recapitulate human OA pathology. Moreover, OA is a chronic, insidious and heterogeneous disease with clinical trials often being prohibitively expensive making the requirement of a suitable model almost mandatory and irreplaceable. Unfortunately, as of now, no single disease model has been able to aptly recapitulate the conditions in human OA because of the multifaceted pathophysiology of the disease (Cope et al., 2019). Further, any disease model used for drug screening should also be easy to develop while being cost-effective, reproducible and less time-consuming. Moreover, one should also consider the applications of 3R principles of replacing, reducing and refinement of animal suffering while developing a disease model (Occhetta et al., 2019). Therefore, while it is desirable to develop a suitable disease model for OA, it remains a challenge.

In response to a variety of risk factors, 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). These factors disrupt joint homeostasis by unbalancing the anabolism and catabolism in resident chondrocytes causing the commencement of inflammation and eventual cartilage degradation as well as adverse effects in other joint tissues (Mobasheri et al., 2017). Pro-

1 Introduction

Osteoarthritis (OA) is one of the most prevalent human joint disease with a massive global disease burden. Despite having a large socioeconomic burden, OA 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. Therefore, 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 three different drugs viz. BMP7, rapamycin and celecoxib, all of which demonstrated concentration-dependent disease amelioration in the explant 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 which mirrors human OA-like traits that may reduce discordance between preclinical and clinical studies in OA drug development.
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 vitro.

Although cytokine-treated in vitro models of OA are suitable for preliminary understanding of disease pathology, they fail to accurately recapitulate human OA conditions due to either lack of extracellular matrix (ECM) or having dissimilar 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. The ECM rich cartilage tissue mainly contains type II collagen and proteoglycans that maintain 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. This imbalance causes ECM remodeling that leads to an altered biomechanical milieu which in turn drives the advancement of the disease. Therefore, OA can be considered as a disease of ECM where aberrant cross-talk between chondrocyte and ECM leads to destruction and alterations of matrix components. Hence, to mirror human OA pathology, disease models should closely mimic human ECM along with recapitulating key disease hallmarks. Keeping the above points in mind, ex vivo disease models that can mirror human OA pathology might be an appropriate choice for 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 as well as OA pathologies. Whereas caprine cartilage which offers high similarity with 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 less expensive, which augments their suitability as an ex vivo OA model.

To enhance translatability, the OA model for DMOAD screening should be extensively characterized for hallmarks of human OA. However, demonstrating the OA-like traits in the developed disease model alone may not be enough. Therefore, we speculated that a comparison of the developed disease models with human OA samples might provide 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.

2 Materials and methods

2.1 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 then rinsed thoroughly with 1X 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 containing 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 (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 media change and the spent medium from each well was harvested at all the predetermined time points and kept frozen until further investigation. Furthermore, IL1β treated explant samples were harvested after 3 weeks of culture and stored under frozen conditions followed by characterization for OA-like conditions.

2.2 Estimation of sGAG and total collagen release from IL1β treated goat cartilage explants

The amount of sulfated GAG and total collagen released from the cartilage explants (both untreated and IL1β treated) into media at all the predetermined time points were estimated through DMMB (Barbosa et al., 2003) and picrosirius red (Marotta and Martino, 1985) assay respectively as per previously reported protocols.

2.3 Histology and Immunofluorescence

For histological and immunofluorescence staining, all the explants harvested at predetermined timepoints were carefully washed with PBS, fixed with 4% neutral buffered formalin (NBF) for 4h, embedded in OCT (Sakura Japan), and kept at -80°C till further study. These embedded samples were then sectioned using a cryotome (Leica 1860UV, Germany) at a thickness of 10 µm. Histological staining for sGAG (safranin O/fast green and alcian blue) and total collagen (picrosirius red) was performed using standard histology protocols. Further, immunofluorescence staining for Chondroitin sulfate (ChS), Collagen type II (Col II), and Aggrecan (Agg) (DSHB, USA) was performed following a previously published report (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, iNOS, Cox 2 and osteopontin (OPN) (Sino Biologicals, USA), the manufacturer’s protocol was followed (see the Table S2 for antibody details).
2.4 Quantification of immunofluorescence staining by image analysis
Quantification of the intensity of various immunostaining was performed using the analysis of immunofluorescence by ImageJ software (Schneider et al., 2012). Quantification was presented as a minimum of 12 images from 4 different samples for each group. Further, quantification of percentage cells positive for any staining was performed using a Cell counter (ImageJ plugin) relative to the total number of cells available.

2.5 Quantification of MMP activity by fluorescent assay
MMP activity in the IL1β treated cartilage explants was determined by quantifying MMPs released into media using a modification of a previously published fluorescence-based assay (Netzel-Arnett et al., 1991). For this, spent media was incubated with 2.5 mM 4 aminophenylmercury acetate at 37°C for 5 h to activate MMPs present in the media samples. These samples were then incubated at 37°C (dark) for 2 h with a fluorogenic substrate specific for either MMP13 or MMP2/9 in TCNB (50 mM Tris, pH 7.5, with 10 mM calcium chloride, 150 mM sodium chloride, and 0.05% BRIJ 35) buffer. The resultant fluorescence was then recorded at 328 nm excitation and 393 nm emission for MMP13 specific substrate and 280 nm excitation and 360 nm emission for MMP 2/9 specific substrates using a multimode reader (Synergy H4 hybrid reader, Biotek, United States).

2.6 Live dead staining for chondrocytes in IL1β treated goat cartilage explants
The cell viability of explants after treatment was evaluated using Fluorescein Diacetate (FDA) and Propidium Iodide (PI) stains following a previously reported protocol (Bhattacharjee et al., 2016). Briefly, cartilage explants were collected at a predetermined time point, followed by washing with PBS and staining with FDA and PI in a glass-bottom Petri plate and then imaged using a confocal microscope equipped with a live-cell imaging facility.

2.7 Resazurin assay for chondrocyte viability
The cell viability in human OA cartilage was evaluated via resazurin assay, as reported previously (Thudium et al., 2019). Briefly, at predetermined time points, media was removed from the wells containing cartilage explants, followed by washing with PBS. Resazurin assay reagent containing 0.02 mg/mL concentration of resazurin in DMEM was added to each well followed by 5 h incubation. 200 μL of resazurin containing media was transferred to a 96 well black plate, and the fluorescence was recorded using a Synergy H4 multi-mode reader at an excitation wavelength of 540 nm and an emission wavelength of 600 nm (BioTek USA). Resazurin containing media without explants were used as blank controls.

2.8 Pharmacological validation of ex vivo OA model
For pharmacological validation of the ex vivo OA model, the cartilage explants were initially treated with IL1β for the induction of OA-like conditions. On the 4th day of OA induction, the cartilage explants were treated with different concentrations of selected drugs (0.1 µM and 1 µM celecoxib, 50 ng/mL and 100 ng/mL BMP7, and 0.1 µM and 1 µM rapamycin) along with IL1β treatment. The explants were maintained and characterized for OA markers as described above (section 4.1 to 4.7).

2.9 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 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 then homogenized using a micro pestle (samples were re-frozen in liquid nitrogen before this process to facilitate the grinding process). The homogenized tissue samples were then treated with TRI reagent (Sigma Aldrich) (700 μL) and stored at -80°C until further use. For the extraction of RNA, the samples were thawed, and 140 μL of chloroform was added and centrifuged at 13,000 rpm for 15min at 4°C. The top aqueous layer was collected in a fresh tube and an equal volume of isopropanol was added to precipitate the RNA. The RNA pellet was then 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 of the target gene was calculated using the ΔΔCt method. The primer sequences used for the qPCR are listed in Table S3. Further, for the analysis of gene expression in goat ex vivo OA model after treatment with different drugs, the explant samples were harvested after 7 days of drug treatment and processed as described above.

2.10 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 the Tab. S1 for patient characteristics). Femoral condylar

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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) containing media for 2 days for acclimatization to in vitro culture conditions. 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.

2.11 Statistical analysis
All data has been presented as a mean ± standard deviation; however, at least 3 biological replicates for each experiment were performed, and representative data from the 3 repeats has been provided. All statistical analysis has been performed using GraphPad Prism 7 with Student’s t-test or one-way analysis of variance (ANOVA) by 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 different concentrations of 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.

We observed that IL1β treatment reduces the sulfated glycosaminoglycan (sGAG) content in cartilage explants when compared to control explants as observed via safranin O staining (Fig. 1B). This observation was further confirmed by quantification of staining intensity using image analysis, which revealed a statistically significant reduction (one-way ANOVA, F (2, 33) = 21.79, P<0.0001) in sGAG content for IL1β treated explants (Fig. 1B). However, no significant difference in sGAG content was observed between the cartilage explants treated with different concentrations (10 ng/ml and 20 ng/ml) of IL1β. To further corroborate these results, we estimated the amount of sGAG released in the media due to IL1β treatment. As expected, IL1β treatment resulted in an enhanced sGAG loss from cartilage explants when compared to control cartilage explants (Fig. 1C). Similarly, treatment with IL1β also demonstrated increased loss of total collagen from cartilage explants when compared to control explants (Fig. 1C). Although both sulfated GAG (one-way ANOVA, F (2, 6) = 135.2, P<0.0001) and total collagen (one-way ANOVA, F (2, 6) = 18.00, P=0.0025) loss were significantly higher in IL1β treated explants when compared to control, loss of cartilage ECM components was comparable in explants exposed to 10 ng/ml and 20 ng/ml IL1β (Fig. 1C). Interestingly, picrosirius red staining for total collagen demonstrated comparable 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. Expression of chondroitin sulfate, the major sGAG present in the articular cartilage extracellular matrix was reduced in IL1β treated explants when compared to control (Fig. 2A). This observation was further corroborated by quantification of fluorescent intensity using image analysis which revealed significantly (one-way ANOVA, F (2, 21) = 24.5, P<0.0001) lesser retention of chondroitin sulfate in IL1β treated groups as compared to control (Fig. 2B). Similarly, IL1β treatment resulted in reduced expression of other important sGAGs present in articular cartilage such as aggrecan and decorin when compared to control (Fig. S1A). However, no significant loss of collagen type II was detected in IL1β treated cartilage explants (Fig. 2A, and B). Overall, IL1β treatment induces degradation of cartilage matrix components in goat cartilage explants.

Further, we also evaluated the effect of IL1β treatment on viability and metabolic activity of resident chondrocytes of goat articular cartilage explants. Staining of live and dead chondrocytes by FDA/PI revealed a minimal reduction of cell viability in the IL1β treated cartilage explants at day 7 and day 14; however, no concentration-dependent effect on cell viability was observed (Fig. S2A). Nevertheless, chondrocyte metabolic activity determined by resazurin assay demonstrated little or no reduction in cell metabolic activity in the IL1β treated groups when compared to control (Fig. S2B).

3.2 Effect of IL1β treatment on expression and activity of matrix degrading proteases
To evaluate the effect of IL1β treatment on the expression of matrix degrading proteases in goat articular cartilage explants, we performed immunostaining for MMP13 and ADAMTS4, two major proteases causing the cartilage degradation in human OA. Immunofluorescence staining of cartilage explants revealed enhanced expression of MMP13 and ADAMTS4 in the IL1β treated explants as compared to control explants (Fig. 3A). Further, percent MMP13 (one-way ANOVA, F (2, 21) = 34.35, P<0.0001) and ADAMTS4 (one-way ANOVA, F (2, 21) = 52.57, P<0.0001) expressing cell number were significantly higher in IL1β treated groups as compared to control (Fig. 3B). However, no significant difference in percent MMP13 and ADAMTS4 positive chondrocytes was observed between the cartilage explants treated with different concentrations (10 ng/ml and 20 ng/ml) of IL1β. These results were further corroborated by analyzing the expression of Mmp13 and Adamts4 in goat cartilage explants using qPCR. Expression of both Mmp13 (one-way ANOVA, F (2, 9) = 9.17, P= 0.0067) and Adamts4 (one-way ANOVA, F (2, 9) = 7.594, P=
0.0117) was significantly enhanced in the IL1β treated cartilage explants when compared to control (Fig. S8 A & B). However, the expression of these markers was comparable in the cartilage explants treated with different concentrations (10 ng/ml and 20 ng/ml) of IL1β.

Further, we performed fluorescence-based quantification of MMP13 activity in the spent media of cartilage explants after IL1β treatment and observed a significant (one-way ANOVA, F (2, 6) = 26.44, P=0.0011) enhancement in the specific MMP13 activity (Fig. 3C) when compared to untreated control. However, specific MMP13 activity in 10 ng/ml and 20 ng/ml IL1β treated cartilage explants were comparable (Fig. 3C). Therefore, it can be inferred that treatment with IL1β significantly enhances the expression and activity of matrix degrading proteases in goat cartilage explants.

3.3 Effect of IL1β treatment on chondrocyte hypertrophy in goat cartilage explants

Human osteoarthritis 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 Osteopontin (OPN), two key hypertrophic markers associated with human OA (Van der Kraan and Van den Berg, 2012), demonstrated significantly higher expression in IL1β treated cartilage explants when compared to control (Fig. S3A). Image analysis-based quantification of fluorescence intensity further 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 (Student’s t-test, P=0.0073) (Fig. S3B). Further, quantification of mRNA expression also showed significantly higher expression of Coll X (one-way ANOVA, F (2, 9) = 9.482, P=0.0061) in the cartilage explants treated with IL1β when compared to untreated control (Fig. S8C). However, Runx2 expression showed an IL1β concentration dependent increase although the increase was not statistically significant (Fig. S8D).
**Fig. 2: Immunostaining for cartilage matrix markers in IL1β treated goat cartilage explants**

(A) Immunofluorescence micrographs of goat cartilage explant sections stained for Chondroitin Sulfate (ChS) (green), Collagen type II (Coll II) (green), and nuclei (blue) (scale bar - 100 μm); (B) fluorescence intensity quantification using image-based analysis [* indicates statistically significant difference (P<0.05) with respect to control cartilage explants, and ‘ns’ indicates no significant difference between the groups, n=4 (One-way ANOVA with Tukey’s correction for multiple comparisons)].

**Fig. 3: IL1β treatment enhances expression and activity of OA specific matrix degradative markers in goat cartilage explants**

A) Immunofluorescence micrographs of cartilage sections stained for MMP13 (red), ADAMTS4 (red), and nuclei (blue) (scale bar - 100 μm); (B) quantification of percent MMP13 and ADAMTS4 positive cells using image-based analysis; (C) quantification of the specific activity of MMP13 in spent media of cartilage explants after IL1β treatment using fluorescence substrate-based assay[* indicates statistically significant difference (P<0.05) with respect to control cartilage explants, and ‘ns’ indicates no significant difference between the groups, n=4 (One-way ANOVA with Tukey’s correction for multiple comparisons)].
Characterization of OA-associated signaling in IL1β treated goat cartilage explants

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 the control explants (Fig. 4A). Quantification of percent NFκB positive chondrocytes revealed a significantly (one-way ANOVA, F (2, 21) = 39.51, P<0.0001) higher fraction of NFκB expressing cells in the IL1β treated groups as compared to control (Fig. 4B). Moreover, no significant difference in the percent of NFκB expressing chondrocytes was observed between two IL1β treated cartilage explant groups (Fig. 4B).

The p38 signaling pathway is connected to enhanced apoptosis and inflammatory responses in OA. Immunofluorescence staining revealed enhanced expression of p38 in the IL1β treated cartilage explants as compared to control explants (Fig. 4A), with higher percent p38 positive chondrocytes being 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 to understand the influence of NFκB on inflammatory cascades in goat cartilage explants. As expected, IL1β treated cartilage explants demonstrated enhanced...
iNOS expression (Fig. 4A) when compared to control. Further, quantification of percent iNOS positive chondrocytes revealed a significantly (one-way ANOVA, F (2, 21) = 180.9, P<0.0001) higher fraction of iNOS expressing cells in the IL1β treated groups when compared to control (Fig. 4B). Moreover, as observed previously no significant difference in the percent p38 and iNOS expressing chondrocytes was observed between different IL1β treated groups (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

Previous studies demonstrated that a cocktail of pro-inflammatory cytokines such as IL1β and TNFα is better suited for the induction of OA-like conditions in cartilage explants than treatment with an individual cytokine (Gabriel et al., 2010). Therefore, we evaluated the effect of IL1β and TNFα both individually as well as in combination on goat cartilage explants. Surprisingly, we observed that TNFα (10 ng/ml) treatment had a negligible impact on goat articular cartilage explants in terms of cartilage matrix degradation, as determined by histological staining of sGAG retained in the explants as well as biochemical estimation of sGAG released in the media (Fig. 5A and B). However, treatment with IL1β (10 ng/ml) alone demonstrated induction of OA-like traits as observed in the previous experiments, which was also observed in the IL1β and TNFα combination treatment group (Fig. 5A and B). As expected, treatment with either IL1β or TNFα or their combination had a negligible effect on the viability of chondrocytes and all the groups showed comparable cell viability (Fig. S6). Since the effect of IL1β treatment is comparable to the combination (IL1β + TNFα), in terms of induction of OA-like phenotype, we infer that treatment with IL1β alone was adequate for the induction of human OA-like traits in goat articular cartilage explants.

Fig. 5: Treatment with IL1β alone induces OA-like conditions comparable to a cocktail of IL1β and TNFα
(A) Safranin O and alcian blue staining of cartilage explant sections for sGAG after 15 days of treatment with IL1β, TNFα, and their combination (scale bar = 50 μm); (B) biochemical estimation of cumulative sGAG release, ‘+’ and ‘-’ indicate presence and absence of the corresponding bioactive molecule in that particular group; (C) immunofluorescence micrographs of cartilage sections stained for MMP13 (red), ADAMTS4 (red), and nuclei (blue) (scale bar = 100 μm) [ *** indicates statistically significant difference (P<0.001) with respect to control cartilage explants and ‘ns’ indicates no significant difference between the groups, n=4 (One-way ANOVA with Tukey’s correction for multiple comparisons)].
3.6 Pharmacological validation of goat ex vivo OA model

Pharmacological validation of the established disease model was performed by assessing the efficacy of a variety of anti-osteoarthritis drugs such as 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). IL1β treated cartilage explant i.e., the goat ex vivo OA model was treated with different concentrations of celecoxib (0.1 µM and 1 µM), and its effect on cartilage matrix degradation was evaluated. We observed that celecoxib enhanced the retention of sGAG in cartilage explants treated with IL1β in a concentration-dependent manner, as observed by safranin O and alcian blue staining (Fig. 6A). This finding was further substantiated by evaluating the loss of cartilage matrix components from ex vivo OA explants after treatment with celecoxib. The results demonstrated that sGAG and total collagen loss from ex vivo OA explants were significantly reduced indicating the efficacy of celecoxib in the amelioration of OA-like conditions (Fig. 6B). Further, treatment with both the concentrations of celecoxib significantly reduced (one-way ANOVA, F (7, 24) = 25.62, P<0.0001) the mRNA expression of Mmp13 (Fig. S9A) when compared to IL1β treated cartilage explants, whereas, treatment with 1 µM celecoxib significantly reduced Adamts4 expression (Fig. S9B). Moreover, treatment with celecoxib reduced the expression of cyclooxygenase 2, confirming the established mechanism of action in the goat ex vivo OA model (Fig. 6C).
Fig. 7: Validation of goat ex vivo OA model using disease modifying OA drugs (BMP7 and Rapamycin)
(A) Safranin O and alcian blue staining of cartilage explant sections for sGAG after 15 days of treatment with different concentrations of BMP7 and Rapamycin (scale bar - 50 µm); B1-BMP7 50 ng/ml, B2-BMP7 100 ng/ml, R1-rapamycin 0.1 µM & R2- 1 µM; (B) immunofluorescence micrographs of cartilage sections stained for MMP13 (red), ADAMTS4 (red), and nuclei (blue) (scale bar - 100 µm); (C) quantification of percent ADAMTS4 and MMP13 positive cells suing image-based analysis; and (D) biochemical estimation of cumulative sGAG and total collagen release, [*], # and ○ indicate a statistically significant difference (P<0.05) with respect to IL1β, R1 and B1 treated cartilage explants respectively, n=4 (One-way ANOVA with Tukey’s correction for multiple comparisons).
Similarly, we evaluated the drug responsiveness of the developed ex vivo OA model using 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 enhancement in the retention of cartilage sGAG as visualized by safranin O and alcian blue staining in goat ex vivo OA model (Fig. 7A). This finding was further substantiated by biochemical estimation of cartilage matrix components in spent media from ex vivo OA explants after BMP-7 and rapamycin treatment (Fig. 7D). Further, treatment with the selected drugs led to a reduction in the expression of key OA degradative enzymes MMP13 and ADAMTS4 in the ex vivo OA model (Fig. 7B). Quantification of percent MMP13 and ADAMTS4 positive cells in cartilage explants also exhibited significant reduction upon treatment with the selected drugs (Fig. 7C). These results were further corroborated using gene expression analysis which showed a general trend of concentration dependent reduction in the expression of both the degradative markers (Fig. S9).

Moreover, to understand the effect of treatment on the viability of chondrocytes in the cartilage explants, live dead staining was performed. We observed no marked difference in the viability of chondrocytes between different treatment groups at both the time points studied, except 1μM Celecoxib at 14 days, which showed a relatively higher number of dead chondrocytes (Fig. S7). These results demonstrated that retention of cartilage matrix components was enhanced due to treatment with BMP-7 and rapamycin, whereas the expression of matrix degradative enzymes was significantly reduced indicating the efficacy of the drugs in the amelioration of OA-like conditions.

Fig. 8: Comparison of goat ex vivo OA model with human OA cartilage

(A) Immunofluorescence micrographs of cartilage sections stained for MMP13, ADAMTS4 and OPN (red), and nuclei (blue), (scale bar =100 μm); (B) quantification of percent MMP13, ADAMTS4, and OPN positive cells using image-based analysis [* indicates statistically significant difference (P<0.05) with respect to control cartilage explants, and ‘ns’ indicates no significant difference between the groups, n=3, (One-way ANOVA with Tukey’s correction for multiple comparisons)].
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 OA cartilage samples in terms of expression of matrix degrading, inflammatory and hypertrophic cartilage matrix-specific markers.

The expression of MMP13 and ADAMTS4 in the goat ex vivo OA model was comparable with their corresponding expression in OA cartilage from three different patient samples (Fig. 8A). This observation was further 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 OA explants harvested from three different OA patients (Fig. 8B). Similarly, the expression of inflammatory mediator NFκB and its downstream effector iNOS in IL1β treated goat cartilage explants and clinical OA samples was comparable (Fig. S4).

We further demonstrated that IL1β treatment induces hypertrophy in chondrocytes, as observed through OPN overexpression, which was comparable to that observed in 3 different clinical OA samples (Fig. 8A). This result was further validated by the quantification of percent chondrocytes positive for OPN expression (Fig. 8B) which demonstrated comparable overexpression of OPN in goat as well as clinical OA samples. Moreover, aggrecan 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, however, there was a mild 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 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 mirror human OA-like traits using pro-inflammatory cytokine treatment in goat cartilage explants that closely resemble its human equivalent in terms of anatomy (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 high similarity with human OA as characterized by enhanced matrix degradation, overexpression of degradative proteases, overexpression of OA-associated inflammatory signaling pathway markers, and chondrocyte hypertrophy.

To the best of our information, this is the first report where goat cartilage explants were used for the establishment of an ex vivo OA model. Despite being relatively well explored as an appropriate in vivo model for OA research, it is intriguing to note the absence of cartilage explants as an in vitro OA disease model (Little et al., 2010). Therefore, considering its striking similarity with human knee joints as well as its extensive use as an in vivo OA model, we reasoned that cartilage explants might be a suitable tissue source for the development of a clinically relevant ex vivo OA disease model. Further, an ex vivo system provides an opportunity to implement the idea of “replacement, reduction and refinement” of the extent of animal use in scientific research thereby making the ex vivo system a desirable disease model.

The focus of the present study was to develop a disease model that recapitulates major hallmarks of human OA. The use of proinflammatory cytokine is a simple yet effective strategy for the initiation of OA-like traits under in vitro conditions (Corciulo et al., 2017; Murab et al., 2013; Shi et al., 2019). IL1β is one such cytokine that 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). IL1β treated goat cartilage explants demonstrated (Fig. 1C) a sharp matrix degradative response in terms of significant loss of sGAG and total collagen which corroborates well with previous studies in another explant model (McNulty et al., 2013) as well as in 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 have demonstrated enhanced MMP13 activity (Fig. 3C) in spent media of IL1β treated goat cartilage explants suggesting the activation of analogous OA-like degradative response in goat explants as well. Moreover, this enhanced MMP activity is in agreement with our current understanding, wherein an increase in MMP activity due to IL1β treatment has been reported (Mengshol et al., 2001). Further, overexpression of MMP13 and ADAMTS4 protein (Fig. 3A & B) and mRNA (Fig. S8) were detected in cartilage explants, indicating overexpression of matrix degrading proteases, a characteristic of OA.

This increase in matrix degrading proteases led to reduced retention of specific cartilage matrix markers like chondroitin sulfate, and aggrecan (Fig. 2A, B & 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 extensive sGAG loss and mild collagen degradation (Fig. 1&2) observed in our study. This holds importance in disease pathology as the degraded proteoglycan fractions when released in 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 have led to the initiation of 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 speculate that resident chondrocytes would display characteristic heightened anabolic response to counter the matrix loss. Previously, it has been demonstrated that treatment with IL1β results in the modulation of cartilage ECM composition (Corciulo et al., 2017; Shi et al., 2019).

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). A similar response was observed in goat cartilage explants after treatment with IL1β resulting in overexpression of NFkB (Fig. 4), which in turn led to overexpression of MMP13, and ADAMTS4 degrading cartilage matrix components. Additionally, this might also lead to the activation 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 NFkB activation through a positive feedback loop (Kapoor et al., 2011). Moreover, NFκB molecules 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 in IL1β treated goat cartilage explants (Fig. 4), which is known to regulate the degradation of collagen, apoptosis in chondrocytes, and inflammation in human OA (Feng et al., 2017). Conversely, inhibition of the p38 MAPK signaling pathway resulted in reduced apoptosis and proinflammatory cytokine production in chondrocytes. Moreover, upregulation of p38 MAPK 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 by cartilage explants against cytokine treatment.

We observed enhanced expression of chondrocyte hypertrophic markers such as Coll X and OPN (Fig. S3) in IL1β treated goat cartilage explants 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).

Taken together, 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, it can be inferred that IL1β treated goat articular cartilage explant recapitulates major human OA-like traits and can be a potential disease model for screening of disease modifying OA drugs. Further, 10 ng/ml of IL1β treatment was adequate to induce osteoarthritis-like changes in goat articular cartilage explants.

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 better 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 the response was comparable to that observed when a combination of IL1β and TNFα was used. Further, individual treatment of TNFα showed a negligible effect (Fig. 5) on goat cartilage explants, although previous studies have shown TNFα can cause induction of OA-like conditions in cartilage explants and as well as chondrocytes of other species (Johnson et al., 2016). This observation might be explained by the species-specific variation 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 an established OA drug celecoxib (Occhetta et al., 2019) as well as two potential DMOADs (BMP-7 (Hunter et al., 2010) and rapamycin (Occhetta et al., 2019)) using the disease model. The reduction of matrix degradation by celecoxib was probably mediated by decreasing inflammatory milieu as observed by the downregulation of Cox2 expression (Fig. 6C) in goat cartilage explants. On the other hand, BMP-7 is a known anabolic factor in cartilage, which 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 demonstrated that rapamycin can not only control matrix retention but can also reduce 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 homeostasis, inflammation, autophagy, senescence, and hypertrophy. Taken together, the ex vivo OA model demonstrated appropriate responsiveness to OA drugs (standard as well as drugs under 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 settings might be one of the possible 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. The purpose of a disease model is to display preferably 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 better suited for understanding disease pathology with improved accuracy as well as for drug screening with greater success. 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 have partially stemmed 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).

Further, this ex vivo OA model upholds the principle of 3R’s and aims at replacement, reduction, and refinement of animal use for scientific research. Moreover, for in vitro culturing of goat cartilage explants we have not used any animal-derived products including serum unlike most of the available in vitro disease models. Besides, this model overcomes the major limitations of both explant-based and cytokine-based in vitro 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).

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, it is worth mentioning here that 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. However, all the cartilage based in vitro disease models lack the inter tissue cross-talk, and involvement of other osteochondral tissues such as subchondral bone, and synovium cannot be studied using such disease models.

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. More specifically, this model recapitulates OA pathological changes such as degradation of cartilage ECM components, enhanced expression of degradative and inflammatory mediators, and enhanced chondrocyte hypertrophy. Moreover, the model was responsive to drug treatment and predicted the effect of a standard OA medication in agreement with previously published reports. More importantly, this model was highly comparable to late-stage OA patient samples in terms of major OA-like traits. Therefore, the developed model due to its disease mimicking ability will 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.

Author contributions
AB: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing - review & editing; DSK: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing - review & editing.

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