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# Metabolic Responses of Meniscal Explants to Injury and Inflammation Ex Vivo

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**ABSTRACT:** This study was designed to characterize metabolic responses of meniscal tissue explants to injury and inflammation. We hypothesized that impact injury and interleukin (IL-1β) stimulation of meniscal explants would result in significant increases in matrix metalloproteinase (MMP) activity and relevant cytokine production compared to controls. Mature canine meniscal explants ($n = 9$/group) were randomly assigned to: (i) IL-1β (0.1 ng/ml) treated (IL); (ii) 25% strain (25); (iii) 75% strain (75); (iv) 25% + IL-1β (25IL); (v) 75% + IL-1β (75IL); or (vi) 0% + no IL-1β control (NC). Explants were impacted at 100 mm/s to 0%, 25%, or 75% strain and then cultured for 12 days with or without 0.1 ng/ml rcIL-1β. Media were refreshed every 3 days and analyzed for MMP activity, ADAMTS-4 activity, MMP-1, MMP-2, MMP-3, GAG, NO, $PGE_2$, IL-6, IL-8, MCP-1, and KC concentrations. Treatment with IL-1β alone significantly increased NO, PGE2, general MMP activity, IL-6, IL-8, KC, and MCP-1 media concentrations compared to negative controls. Impact at 75% significantly increased PGE2, IL-6, IL-8, and KC media concentrations compared to negative controls. The combination of IL-1β and 75% strain significantly increased production of PGE2 compared to IL-1β or 75% strain alone. Impact injury to meniscal explants ex vivo is associated with increased production of pro-inflammatory mediators and degradative enzyme activity, which are exacerbated by stimulation with IL-1β. © 2018 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 36:2657–2663, 2018.

**Keywords:** meniscus ex vivo; explant; injury IL-1β

Meniscal injury is a significant clinical burden on patients and the health system, and approximately one million meniscal surgeries are performed annually in the US alone.[1] The menisci are key tissues in the knee that play important roles in the health and function of the knee joint organ.[2–4] Meniscal injury is associated with increased risk for osteoarthritis (OA) in the affected joint.[5–12] The increased risk for OA development associated with meniscal injury is often attributed to biomechanical disruption of the tissue and resultant inability to dissipate load, leading to articular cartilage tissue disruption and whole joint disease.[8,12] However, inflammatory and degradative responses after meniscal injury may also play key roles in this disease process as suggested by significant increases in pro-inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α), in synovial fluid of knees with meniscal disease.[13,14] Therefore, in depth characterization of meniscal responses to injury, and how that response is potentially exacerbated by the presence of the inflammatory cytokines often observed in the synovial fluid after meniscal injury and in the osteoarthritic knee,[13,14] is needed to improve our understanding of meniscal injury pathobiology, and it will potentially identify new targets for prevention and treatment of meniscal degeneration and OA following meniscal injury.

Because of the complexity of the knee environment, and the multiple factors that can influence the metabolism of the meniscus after joint injury (i.e., joint movement, synovial fluid, tissue vascularization, and injury to other joint tissues), it can be difficult to identify meniscus specific responses to injury and inflammation in clinical patients and in vivo animal models. Therefore, ex vivo models using tissue explants are often used to identify the metabolic responses of meniscal tissue to a specific stimuli.[15–19] Removing the tissues from the complex in vivo environment allows researchers to study the tissue's responses to specific stimuli alone or in combination. While the data from these models are not directly translatable to the complex in vivo responses to joint injury and the subsequent development of meniscal degeneration and OA, it can be used to guide the development of more robust in vivo animal models and human clinical research studies aimed at understanding these complex disease processes.

This study was designed to characterize the production of inflammatory mediators, cytokines, chemokines, and degradative enzymes by meniscal tissue in

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response to impact injury and pro-inflammatory stimulation, alone or in combination during ex vivo culture. Our study has three main hypotheses. First, that production of degradative enzymes, inflammatory mediators, cytokines and chemokines would be significantly increased in response to cytokine stimulation and/or impact injury to meniscal explants compared to the negative control. Second, that production of degradative enzymes, inflammatory mediators, cytokines, and chemokines would be significantly increased in the cytokine stimulated group compared to the impact injury groups. Finally, the production of degradative enzymes, inflammatory mediators, cytokines, and chemokines would be significantly increased in the combined cytokine stimulation/impact group compared to the impact only and cytokine stimulation only groups. The objective of this work is to characterize metabolic responses of the meniscus to injury and inflammation in a way that optimizes prevention and treatment approaches to this prevalent and costly orthopaedic problem.

## MATERIALS AND METHODS

### Meniscal Explant Harvest

All procedures were performed with Animal Care and Use Committee approval. Medial and lateral menisci were aseptically collected from the knees of skeletally mature dogs ($n = 9$) euthanatized for reasons unrelated to this study. Full-thickness explants were created from the center of the meniscus to avoid the synovium attached to the outer edge of the meniscus using a 4 mm diameter biopsy punch (Sklar Instruments, Rochester, PA). Explants of this size contain tissue from the vascularized (red zone) to the non-vascularized (white zone) portions of the canine meniscus. A total of six explants were created from each animal (three from the medial meniscus and three from the lateral meniscus), and one explant per animal was randomly assigned to one of six groups: (i) IL-1β (0.1 ng/ml) treated (IL); (ii) 25% strain (25); (iii) 75% strain (75); (iv) 25% + IL-1β (25IL); (v) 75% + IL-1β (75IL); or (vi) 0% + no IL-1β control (NC). A total of 9 explants were cultured in each group ($n = 54$).

### Impact Loading

A servo-hydraulic testing machine (model 8821S, Instron, Norwood, MA) was used to apply a single impact load to the tissue. The meniscus explant was placed in a stainless steel well (4 mm diameter) and a 3.9 mm diameter flat punch attached to the ram was used to measure the thickness of the explant. The thickness measurement was used to calculate the parameters to apply a 25% or 75% strain confined impact compressive load at 100 mm/s as previously described.[20] These strain levels were selected based on previous studies outlining normal strain levels experienced by the meniscus in vivo,[21] and previous injury studies on meniscal explants.[22,23]

### Tissue Culture

All explants were cultured in 2 ml of Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA), supplemented with 0.1% ITS, 2 mM L-glutamine, 50 ng/ml ascorbic acid, 1 mM sodium pyruvate, 100 U/ml Penicillin/100 μg/ml Streptomycin/2.5 μg/ml Amphotericin B (P/S/A), and 1X Non-essential Amino Acids (Gibco, Grand Island, NY). The media used for the IL, 25IL, and 75IL groups were cultured with 0.1 ng/ml recombinant canine IL-1β (R&D systems Inc., Minneapolis, MN) while the NC, 25, and 75 groups were cultured without IL-1β. This concentration of IL-1β was selected for this study based on reported synovial fluid concentrations observed in patients with meniscal injury and OA.[14] Explants were incubated at 37°C, 90% humidity, and 5% $CO_2$ for 12 days. Culture media were collected and replenished every 3 days. Media samples were stored at −20°C for subsequent biomarker analysis.

### Media Analysis

The concentrations of nitric oxide (NO, Promega, Madison, WI); prostaglandin $E_2$ (PGE2, Cayman Chemical, Ann Arbor, MI); Interleukin (IL)-6, IL-8, keratinocyte-derived chemokine (KC), and monocyte chemotatic protein-1 (MCP-1, Millipore, Billerica, MA); matrix metalloproteinase (MMP)-1, MMP-2, and MMP-3 (R&D Systems, Minneapolis, MN); Total MMP activity and ADAMTS-4 activity (AnaSpec, Fremont, CA) were measured using commercially available assays according to the manufacturer's protocol. The concentration of GAG released to the media was quantified using the dimethylmethylene blue (DMMB) assay as previously described.[24]

### Statistical Analysis

Statistical analyses were performed using IBM SPSS® Version 24, IBM Corp., Armonk, NY. Data were assessed for normality using Kolmogorov-Smirnov test for normality. Because data were not distributed normally, a Kruskal–Wallis 1-way ANOVA with post hoc Mann–Whitney analysis was performed to determine significant differences between groups at each time point. Bonferroni adjustments were made for multiple comparisons.

## RESULTS

### Inflammatory Mediators

Media NO concentrations were significantly ($p \leq 0.014$) higher in the 25IL and 75IL groups compared to the NC, 25, and 75 groups at all time points except for 75IL compared to 25 on day 3 and 75 on day 6. The NO concentration in the 25IL group was significantly ($p \leq 0.001$) higher than the 75IL group on day 3. NO concentration in the IL group was significantly ($p \leq 0.024$) higher than the NC, 25, 75, and 75IL groups for Days 6, 9, and 12. There were no significant ($p \geq 0.085$) differences in NO concentrations among the NC, 25, and 75 groups at any time point (Fig. 1).

Media PGE2 concentration was significantly ($p \leq 0.019$) higher in the 75IL group compared to the NC, IL, and 25 groups at all time points; the 75 group on days 3, 9, and 12; and the 25IL group on day 9. PGE2 in the 25IL group was significantly ($p \leq 0.006$) higher than the NC group at all time points, but not consistently significantly different than any other group. The 75 group had significantly ($p \leq 0.019$) higher production of PGE2 compared to the NC and 25 groups from day 6 to day 12, and compared to the IL group for days 6 and 9.



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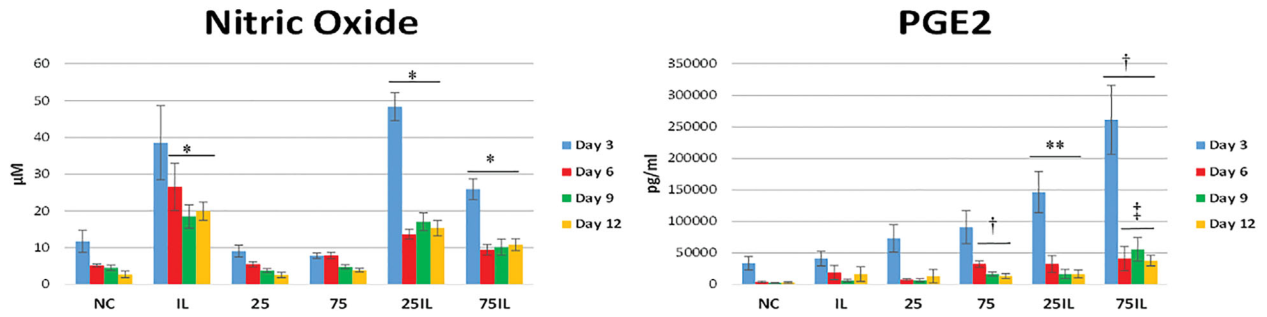


**Figure 1.** Media concentration of PGE2 and NO in the negative control (NC), cytokine treated (IL), Impacted (25 and 75), and the combined cytokine treated and impacted groups (25IL and 75IL) on days 3, 6, 9, and 12. (*) indicates significantly higher than NC, 25, and 75 groups on that day, (**) indicates significant ($p \leq 0.05$) increase compared to NC group on that day, (†) indicates significant ($p \leq 0.05$) increase compared to NC, IL, and\or 25 groups on that day, (‡) indicates significant ($p \leq 0.05$) increase compared to 75 group on that day.

## Degradative Enzyme Production and Activity

The level of MMP activity was significantly ($p = 0.006$) increased in the IL group compared to the NC, 25, 75, and 75IL groups at all time points. The NC group had significantly higher ($p \leq 0.006$) levels of MMP activity compared to the 75 group through day 6. The 25IL group had significantly higher ($p \leq 0.014$) levels of MMP activity compared to the NC, 25, 75, and 75IL groups at all time points. MMP activity in the 75IL group was only significantly higher ($p = 0.019$) than the 75 group throughout culture. There was not a significant difference in ADAMTS-4 activity or MMP-1, MMP-2, or MMP-3 concentration among groups at any time point (Fig. 2).

## Cytokine/Chemokine Production

The production of IL-6 was significantly increased ($p \leq 0.011$) in the 25IL group compared to the NC and 25 groups at all time points. The 75IL group had significantly higher ($p \leq 0.003$) levels of IL-6 compared to the NC group from day 6 to 12, the 75 group at days 9 and 12, and the 25 group at all time points. The media concentration of IL-6 in the IL group was significantly higher ($p \leq 0.019$) than the NC and 25 groups from day 6 to 12 (Fig. 3). The production of IL-8 was significantly ($p \leq 0.003$) higher in the 25IL and 75IL groups compared to the NC and 25 groups at all time points tested, and the 75 group on day 9 and 12. The production of IL-8

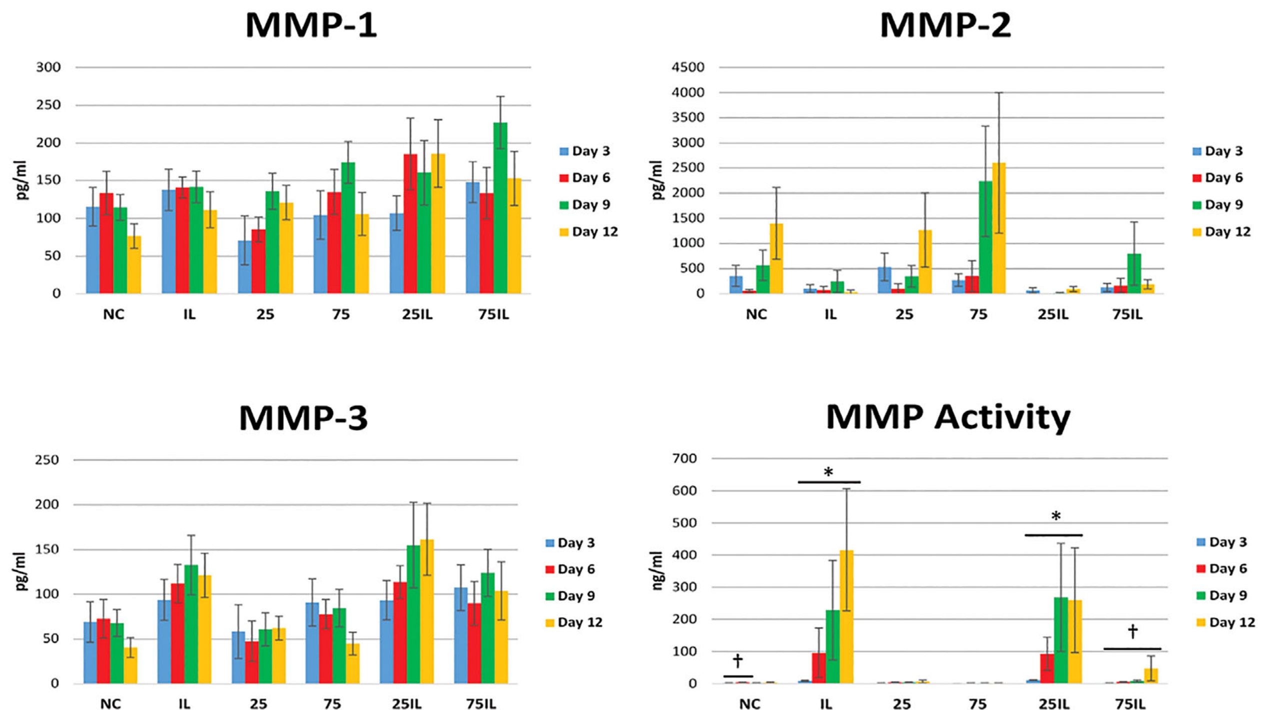


**Figure 2.** Media concentration of MMP-1, MMP-2, MMP-3, and total MMP activity in the negative control (NC), cytokine treated (IL), Impacted (25 and 75), and the combined cytokine treated and impacted groups (25IL and 75IL) on days 3, 6, 9, and 12. (*) indicates significant increase compared to NC, 25, 75, and 75IL groups on that day, (†) indicates significant increase compared to 75 group on that day.

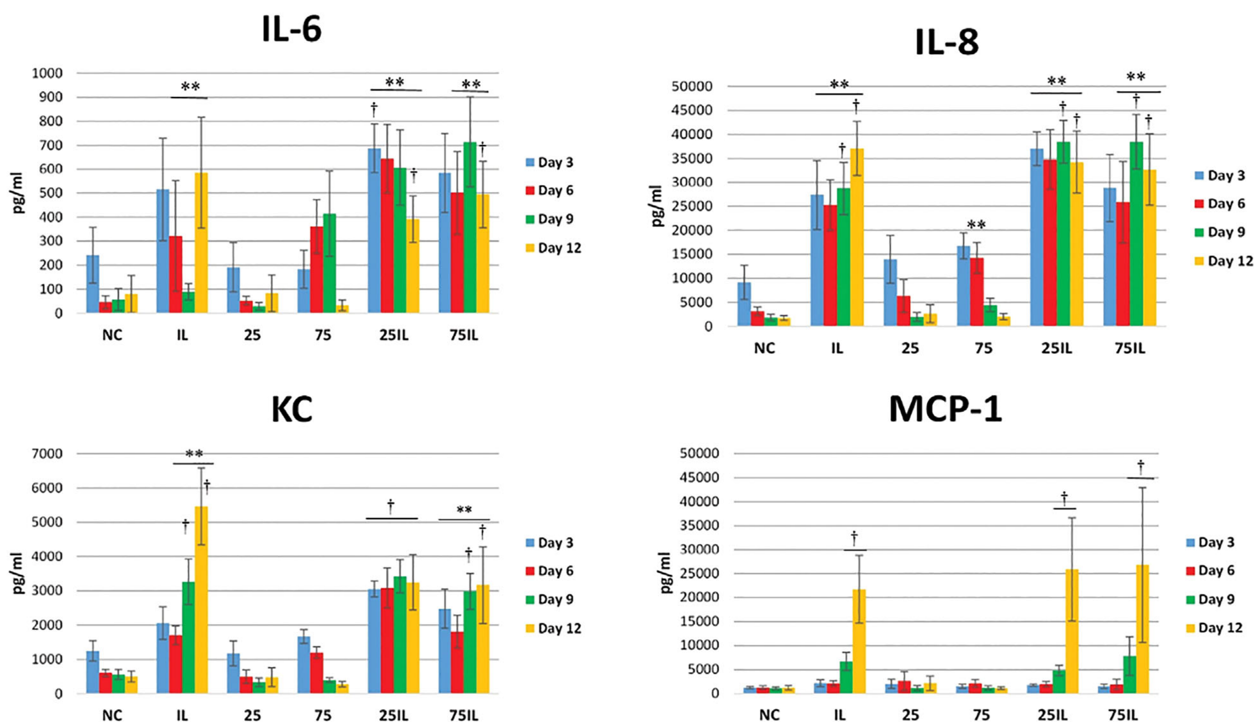


**Figure 3.** Media concentration of IL-6, IL-8, KC, and MCP-1 in the negative control (NC), cytokine treated (IL), Impacted (25 and 75), and the combined cytokine treated and impacted groups (25IL and 75IL) on days 3, 6, 9, and 12. (*) indicates significant ($p \leq 0.05$) increase compared to NC group on that day (**) indicates significant increase over NC and 25 group on that day, (†) significantly different than NC, 25, and 75 groups at that time point.

was significantly higher ($p \leq 0.004$) in the IL group compared to the NC and 25 on days 6 through 12, and the 75 group on days 9 and 12. The production of IL-8 in the 75 group was significantly higher ($p \leq 0.024$) than the NC and 25 groups on day 6. The media concentration of KC was significantly higher ($p \leq 0.004$) in the IL group compared to the NC and 25 groups from days 6 to 12, and the 75 group on days 9 and 12. The media concentration of KC in the 25IL group was significantly higher ($p \leq 0.004$) than the NC, 25, and 75 groups at all time points tested. The media concentration of KC in the 75IL group was significantly higher ($p \leq 0.004$) than the NC and 25 groups from day 6 to 12, and the 75 group from day 9 and 12. The concentration of MCP-1 in the media was significantly higher ($p \leq 0.006$) in the IL, 25IL, and 75IL groups compared to the NC, 25, and 75 groups on day 9 and 12 of culture (Fig.3).

## DISCUSSION

The data from this study indicate that meniscal tissue responds to pro-inflammatory cytokine stimulation and impact injury by increasing production, and activity of degradative enzymes, cytokines, and inflammatory mediators associated with meniscal degradation and OA pathophysiology.[25,26] Many of the responses are specific to inflammation or injury and some responses are exacerbated by the combination of injury and inflammation. The characterization of these responses by meniscal tissue explants during ex vivo culture indicates potential cellular pathways activated after meniscal injury, and indicates targets for future study of the complex metabolic interplay that can lead to meniscal degeneration and OA commonly seen after meniscal injury.

NO and PGE2 have important roles as inflammatory mediators in meniscal degeneration and OA development.[25,27–30] Specifically, NO and PGE2 have been linked to inhibition of collagen and proteoglycan synthesis by chondrocytes.[14,31–33] In agreement with previous studies,[20,27,28,33,34] the data from the present study indicate that the meniscus produces increased levels of NO, but not PGE2, in response to pro-inflammatory cytokine stimulation, and the meniscus increases production of PGE2, but not NO, in response to impact injury. Importantly, the production of PGE2 after injury was exacerbated by the presence of inflammatory cytokines. These data indicate that there is a difference in the meniscal response to a single compressive injury and repeated compressive load observed in previous studies.[3,16,17,33] In these studies, dynamic compressive load applied to the tissue increased the production of NO and PGE2 by the tissue, and decreased the response of the meniscal tissue to cytokine stimulation.[16,17,33] In this study, a single impact compressive load only stimulated the production of PGE2, and the combination of load and cytokine stimulation increased the production of PGE2 by the tissue. Therefore, while dynamic compressive



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load associated with normal joint movement may decrease the response of meniscal tissue to cytokine stimulation, a single injurious compressive load appears to enhance tissue response to cytokine stimulation.

In agreement with previous studies,[18,35–37] proinflammatory stimulation was associated with increased production of IL-6, IL-8, MCP-1, and KC by meniscal tissue in this study. These molecules play important roles in inflammatory and tissue repair processes by recruiting monocytes and neutrophils to the injury site, and may be indicative of attempted repair after insult.[38–41] Production of IL-6, IL-8, and KC by meniscal tissue was also significantly increased after high-strain impact injury. Further, the combination of impact injury and cytokine stimulation resulted in the most consistent significant increases in IL-6, IL-8, and KC production. These findings are in agreement with previous clinical studies that found significantly higher synovial fluid concentrations of IL-6 and IL-8 in patients with meniscal injury compared to uninjured controls,[42] and IL-6 production is increased in injured meniscal tissue obtained from clinical patients.[43] Taken together, the clinical and basic science data suggest that, after injury to the meniscus, there is an initial surge in production of IL-6, IL-8, and KC, which is exacerbated by injury-associated inflammation and/or pre-existing OA.

MMP and aggrecanases are also important therapeutic targets in meniscal degeneration and OA.[44–47] While there is some disagreement among studies with respect to type and timing of degradative enzyme production associated with meniscal injury, meniscal degeneration, and OA, significant alterations in degradative enzyme expression, production, and activity by meniscus in response to inflammation and/or injury are consistent findings.[18,19,22,23,48–53] In our study, a significant increase in the production of MMP-1, MMP-2, or MMP-3 was not observed following stimulation or impact injury. One possible explanation for this finding is that the level of cytokine stimulation was lower than previous studies (0.1 ng/ml), and a higher level of inflammation is required to stimulate a significant increase in the production of these degradative enzymes in vitro.

While the production of the degradative enzymes analyzed in this study were not significantly increased, there were consistent increases in MMP activity associated with IL-1β stimulation, but not load, from meniscal explants. Interestingly, the application of a compressive load at 75% strain to meniscal explants counteracted the increased MMP activity associated with cytokine treatment. ADAMTS-4 activity was not significantly affected by cytokine treatment or impact injury. Previous studies reported that meniscal tissue significantly decreased ADAMTS-4 gene expression when exposed to a single compressive load between 25% and 75% strain or dynamic compressive loading at 10% strain, indicating that load may inhibit the production of ADAMTS-4 by meniscal tissue.[19,22,23] The mechanisms by which loading can ameliorate degradative enzyme activity by meniscal tissue have not yet been determined and deserve further study. The mechanisms may involve direct effects on MMP production, alterations in conversion of MMPs from pro- to active forms, and/or through regulation of MMP activity by tissue inhibitors of MMPs (TIMPS). Our ongoing laboratory research is aimed at determining how acute and chronic exposure to cytokine stimulation, load, and injury combine to initiate, exacerbate, and prolong meniscal degeneration and OA.

The limitations of our study are primarily associated with the use of normal canine meniscal explants in an ex vivo model. The tissues were removed from the joint environment and therefore the responses observed in vitro may not directly reflect the tissues response in vivo. The single compressive impact injury does not directly relate to the complex biomechanical forces applied to the meniscus during injury in vivo, and therefore the responses in response to load observed in this study may not be reflective of what occurs in vivo. Further, the initial concentration of IL-1β (0.1 ng/ml) used for stimulation in this study was based on the concentration observed in synovial fluid after meniscal injury and OA patients clinically, however it was not determined how stable the activity of IL-1β was during the 3 days of stimulation, and stimulation with a single cytokine does not reflect the complex inflammatory response that occurs in the joint after injury and during OA development. Additionally, the tissues were not evaluated histologically to determine how the impact injury affected the structure of the meniscal explant and how the injury correlated to what is observed clinically. Based on this experimental design, we cannot draw definitive conclusions with respect to meniscal pathology in the knees of patients.

## CONCLUSIONS

With these limitations in mind, the results of the present study indicate that a single impact injury applied to a meniscal explant is associated with increases in production of inflammatory biomarkers (PGE2, IL-6, IL-8, KC), but not degradative enzymes (MMP-1, MMP-2, MMP-3). Further, stimulation of the meniscal explants with clinically relevant levels of IL-1β, which is consistently present after joint injury and in the presence of OA, resulted in increased production of inflammatory biomarkers (NO, IL-6, IL-8, MCP-1) and activity of degradative enzymes (total MMP activity). The combination of impact injury (75% strain) and cytokine stimulation applied to meniscal explants ex vivo resulted in a significant increase in the production of inflammatory biomarkers (PGE2, NO, IL-6, IL-8, MCP-1), but significantly reduced the level of total MMP activity produced by the tissue in response to cytokine stimulation. Further studies are required to determine if these findings also pertain to the in vivo setting of meniscus injury.



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## AUTHORS' CONTRIBUTIONS

AS and JC participated in the conception and design of this study. AC, EL, FP, and AS preformed the experiments and acquired data as well as participated in analysis and interpretation of the data. AC drafted the manuscript. Revisions and final approval of manuscript were made by AC, AS, EL, and JC.

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