Results: TLR-1, TLR-2 and TLR-6 were detected along the progression of OA but their expression did not differ significantly with the control group. Remarkable, a significant increased (p < 0.05; p < 0.0001) of TLR4 mRNA expression was observed since day 8 (2.5 fold) of OA induction. By immunohistofluorescence, TLR-4 was detected in the superficial and middle zones of cartilage from day 3 to 20 with highest expression at day 8. In another hand, a notably level of IL-1β expression (19 fold) was observed at day 8 of OA induction (p < 0.0001), while TNF-α and IL-6 did not show a significant difference within the experimental groups. Among the MMPs, the MMP-3 showed the highest expression at the 8 (2.8 fold; p <0.05) and 10 (3 fold; p <0.0001) days of OA. Vitamin D treatment showed a clear reduction of TLR4, IL-1β and MMP-3 during the progression (3, 10 and 20 days) of OA.

Conclusions: TLR4 is expressed in articular cartilage and it is up regulated during the induction of OA. Signaling through TLR-4 might be the pro-inflammatory mechanism in osteoarthritis that results in the expression of IL-1β and MMP-3. Interfering this signaling pathway by the use of vitamin D reduce the progression of OA in the rat model.

430 INHIBITION OF PRO-INFLAMMATORY MEDIATORS IN ACTIVATED HUMAN ARTICULAR CARTILAGE BY THE COMBINATION OF AVOCADO/SOYBEAN UNSAPONIFIABLES, GLUCOSAMINE, AND CHONDROITIN SULFATE AND BY ASPIRIN
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Purpose: We evaluated the anti-inflammatory effect of the combination of avocado/soybean un-saponifiables (ASU), glucosamine (GLU), and chondroitin sulfate (CS) by measuring production of pro-inflammatory mediators by human articular cartilage. These mediators include prostaglandin E2 (PGE2), interleukin-6 (IL-6), interleukin-8 (IL-8), and macrophage chemotactic protein-1 (MCP-1). They are produced excessively in osteoarthritis (OA) and play critical roles in the pathogenesis of the disease. These pro-inflammatory mediators participate in the destruction of articular cartilage by inducing enzymes that degrade extracellular matrix. PGE2 activates cartilage-degrading enzymes while IL-6 mediates bone resorption. These molecules also activate inflammatory pain pathways. However, at physiologic levels, these molecules serve multiple functions in cell signaling, proliferation, tissue repair, and regeneration. Studies have demonstrated that ASU, GLU, and CS inhibit production of inflammatory mediators. They have been shown to reduce inflammation and relieve pain in OA patients with no adverse effects. These compounds have also been reported as chondroprotective and exert structure-modifying effects on osteoarthritic knees. As positive control in our in vitro study, we used aspirin at a clinically relevant concentration. Aspirin is a widely used non-steroidal anti-inflammatory drug (NSAID) that has recently been cited as causing gastro-intestinal side effects.

Methods: Human articular cartilage (Articular Engineering Inc.) was cut into 200 mg/well for explant experiments. Explants were pre-incubated with: (a) control media alone, (b) the combination of ASU (NMX1000®, 8.3 µg/mL) + GLU (FC849®, 11 µg/mL) + CS (TRH122®, 20 µg/mL); or (c) aspirin (100 µg/mL) for 24 hrs to seven days. Explants and chondrocytes were then activated with IL-1β (5 or 10 ng/mL) for 24-48 hrs for measurement of PGE2, IL-6, IL-8, and MCP-1 production by ELISA. Concentrations of mediators were expressed per mg cartilage wet weight in explant culture. Data was analyzed by one-way ANOVA, Tukey post-hoc, with P<0.05 level of significance.

Results: Cartilage explants from normal and OA donors cultured with control media alone produced undetectable to low levels of PGE2, IL-6, IL-8, and MCP-1. Activation with IL-1β (5 and 10 ng/mL) resulted in similar, significantly increased production of pro-inflammatory mediators. These increases were as much as 80-fold for PGE2, 500-fold for IL-6, 300-fold for IL-8, and 700-fold for MCP-1 (all P<0.001). The combination of ASU+GLU+CS reduced PGE2 production down to 10% of IL-1β-activated levels. The effect of the ASU+GLU+CS combination on the cytokine-induced production of other mediators was variable. Inhibition was reduced to 10% of activated levels for IL-6, 40% for IL-8, and 15% for MCP-1. In contrast, aspirin dramatically reduced production of PGE2 down to 1% of activated levels. Aspirin also decreased IL-6 production down 10% of activated levels, 38% for IL-8, and 34% for MCP-1.

Conclusion: The present study shows that the combination of ASU+GLU+CS effectively inhibits cytokine-induced production of PGE2, IL-6, IL-8, and MCP-1 by cartilage explants. In comparison, aspirin nearly obliterated the production of PGE2 and reduced the production of IL-6, IL-8, and MCP-1. This suggests that the combination of ASU+GLU+CS may have similar mechanisms of action as some NSAIDs such as aspirin. However, the observation that ASU+GLU+CS attenuated rather than dramatically suppressed production of PGE2 may help preserve their physiologic functions. The present study supports the notion that the ASU+GLU+CS combination may offer an effective anti-inflammatory and chondroprotective approach for the management of OA. The ASU+GLU+CS combination may help reduce chronic dosing of NSAIDs, thus minimizing deleterious side effects.

431 INHIBITION OF CYTOKINE-INDUCED INFLAMMATORY GENE EXPRESSION IN CHONDROCYTES FROM HORSES AFFLICTED WITH HEREDITARY EQUINE REGIONAL DERMAL ASTHENIA (HERDA) BY THE COMBINATION OF AVOCADO/SOYBEAN UNSAPONIFIABLES, GLUCOSAMINE, AND CHONDROITIN SULFATE
Purpose: Hereditary Equine Regional Dermal Asthenia (HERDA) is an autosomal recessive connective tissue disorder resulting in impaired collagen folding, hypertexsensible skin, and abnormal wound repair. We observed osteoarthritic lesions in tarsal and carpal joints of yearling HERDA horses. HERDA horses show similarities to the human hereditary connective tissue syndrome Ehlers-Danlos (EDS). Many EDS patients suffer from joint pain and osteoarthritis (OA) as adults. The phenotypic similarities between EDS and HERDA suggest that horses suffering from HERDA may also develop OA. In OA, excess production of inflammatory mediators activates enzymes that degrade cartilage as well as inhibit wound healing. These mediators include prostaglandin E2 (PGE2) which produces is regulated by the cyclooxygenase-2 (COX-2) enzyme, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8). The purpose of this study was to characterize articular cartilage from HERDA afflicted horses. We determined the baseline expression of several pro-inflammatory genes and whether their expression could be induced by the cytokine IL-1β similar to what is observed in OA joints. We also determined whether induction of these genes could be inhibited by the combination of avocado/soybean un-saponifiables (ASU), glucosamine (GLU), and chondroitin sulfate (CS) in vitro. These compounds inhibit inflammation and are used for the management of OA in both humans and animals. As a positive control, we used phenylbutazone (PBZ), a non-steroidal anti-inflammatory drug (NSAID) currently used for the management of both HERDA and equine OA.

Methods: Articular cartilage was harvested from tarsal joints of three yearling HERDA horses. Chondrocytes were seeded onto 6-well plates and pre-incubated at 37°C, 5% CO2 with control media alone, PBZ at a clinically relevant concentration (4 µg/mL), or the combination of ASU (NMX1000®, 8.3 µg/mL) + GLU (FC849®, 11 µg/mL) + CS (TRH122®, 20 µg/mL) for 24 hrs. Chondrocytes were activated with IL-1β (10 ng/mL) for 24 hrs. COX-2, IL-6, TNF-α, and IL-8 gene expression was quantified by real-time PCR. Pair-wise multiple comparisons were carried out using one-way ANOVA, Student-Newman-Keuls post-hoc analysis where differences of P<0.05 were considered statistically significant.

Results: Chondrocytes incubated with control media alone expressed low levels of COX-2, IL-6, TNF-α, and IL-8. Cytokine activation induced a hundred-fold increase (P<0.001) in the expression of these pro-inflammatory genes. Pre-treatment with ASU+GLU+CS significantly (P<0.05) decreased COX-2, IL-6, TNF-α, and IL-8 expression by approximately 30%. In comparison, cytokine-induced increases in COX-2, IL-6, TNF-α, and IL-8 pro-inflammatory gene expression were inhibited by PBZ by as much as 60% (P<0.05).

Conclusions: Chondrocytes obtained from horses with HERDA responded to cytokine stimulation with the up-regulation of several key pro-inflammatory markers that have been associated with the pathogenesis
of OA. In this study, ASU+GLU+CS attenuated gene expression of these inflammatory molecules. Our finding suggests that the ASU+GLU+CS combination would be potentially beneficial for reducing chronic joint inflammation in both humans and animals, such as HERDA horses. In addition, utilization of the ASU+GLU+CS combination may help reduce the risk associated with chronic NSAID administration.

**Mechanobiology**

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**MECHANOBIOLOGICAL EFFECTS IN A PLOWING MODEL OF DIARTHRODIAL JOINTS**

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**Purpose:** Human diarthrodial joints (e.g. the temporomandibular joint, TMJ) undergo complex rolling/plowing loading patterns scarcely investigated in live tissue. Yet, joint biological responses are explained by mechanobiological effects also due to plowing, i.e. pressing a round indenter – such as a condyle – against the cartilage surface and displacing it tangentially. Objective of this study was to investigate the response of cartilage tissue models to plowing. In particular, we analyzed the effect of plowing on cell viability, gene expression and glycosaminoglycans (GAGs) release. For this purpose, we used loading data collected in vivo by means of dynamic stereoradiography on the TMJ, i.e. a software reconstruction of real anatomies animated by their real kinematics, that combined with numerical modeling can provide in vivo data on strains, forces, stresses, and work density.

**Methods:** Cartilage plowing was performed by means of a rolling/plowing explant test system previously developed and validated. It consists of an arm moving horizontally by means of linear stages holding a custom-milled indenter pushed vertically by a linear actuator against the cartilage specimen. This is kept in a tank filled with DMEM solution. Adequate sensors measure force and displacement in relevant directions. Live cartilage strips (60 x 17 x 2 mm) were obtained from bovine nasal septum of one year old calves were subjected to plowing for 2 hours at 37°C in DMEM solution by using a cylindrical aluminum indenter (diameter 25 mm) moving tangentially at 10 mm/sec and simultaneously applying normal forces of 25, 50 or 100 N respectively. Control explants (diameter 25 mm) moving tangentially at 10 mm/sec and simultaneously applying normal forces of 25, 50 or 100 N respectively were kept unloaded in the same medium during the whole experiments. Analyses were performed 0, 2, 4 and 24 hours post loading. Cell viability was assessed by means of calcein acetoxymethylster & ethidium homodimer assays. Gene expression was assessed after mRNA extraction by quantitative real-time polymerase chain reactions (qRT-PCR). GAGs measurements were performed by means of 1–9-dimethyl-methylene blue (DMMB) assays. To determine whether GAGs release was mechanically or enzymatically induced, supplementary tests with MMPs activity inhibition before plowing were performed. Statistical differences were analyzed using two-way ANOVAs and Student’s t tests at alpha=0.05.

**Results:** Overall cell viability exceeded 95% for all applied forces, although superficial zones of dead chondrocytes were observed, increasing with the applied normal force. qRT-PCR showed that plowing induces MMP-3 upregulation dependent on the applied force (peaks of 2.3 ×, 4.7 × and 6.3 × for 25, 50 and 100 N respectively). Transcription of other genes involved in cartilage turnover such as TIMP-1, aggrecan, collagen type I and type II and fibronectin were shown not to be significantly affected by plowing. Furthermore, DMMB assays revealed that cartilage strips plowed at 100 N exhibited significantly enhanced GAGs release compared to the control explants at all time points observed.

**Conclusions:** This study shows that (1) bovine nasal septum can be a convenient model to study chondrocyte mechanobiological response to plowing, (2) plowing of cartilage appears to induce significant dose-dependent mechanobiological effects, and (3) the loading parameters studied would promote the production of catabolic enzymes tending to degrade the extracellular matrix.

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**LOW-INTENSITY PULSED ULTRASOUND DOWNREGULATES THE MESSENGER RNA EXPRESSION OF THE MATRIX METALLOPROTEINASES ON AN ARTICULAR CARTILAGE EXPLANTS MODEL**


**Purpose:** Low-intensity pulsed ultrasound (LIPUS) has been utilized for the enhancement of fracture healing because of its bone reconstructive ability. In addition, LIPUS has been reported to be able to increase an anabolic reaction on chondrocytes. However, the effect of LIPUS about a catabolic reaction has not been elucidated, especially on an articular cartilage explants model. The purpose of this study was to investigate the immediate effect of LIPUS to the homeostasis of articular cartilage explants by mRNA analysis. We hypothesized that LIPUS acts as a moderate (physiological) mechanical stress, and downregulates catabolic reactions.

**Methods:** Osteochondral plugs (6 mm in diameter) were isolated from the distal femurs of pigs, and cultured for 1 day in the CO2 incubator. We divided these samples into 3 groups, control group applied sham LIPUS, the 27mW/cm2 group applied LIPUS at 27mW/cm2 intensity (spatial-average-temporal-average, SATA) and the 67mW/cm2 group applied LIPUS at 67mW/cm2 intensity (SATA) (n = 5 plugs/group). Samples of the plugs were treated for 60min each intensity. Total RNA was extracted from these samples, and then the concentration and purity were assayed by spectrophotometer. To investigate the effect of LIPUS about both catabolic and anabolic reaction on articular cartilage explants, we analyzed the mRNA expression of MMP-13 (matrix metalloproteinase-13), MMP-1 (matrix metalloproteinase-1), Col2a1 (type 2a1 collagen), ACAN (aggrecan) and CITED2 (CBP/p300-interacting transactivator with ED-rich tail 2) by Real-time PCR. The final expression value was calculated by dividing the expression level of these target genes by the expression level of beta-actin, and each value at control group was set as 1. All data were expressed as mean ± standard error. Statistical analysis was carried out using Kruskal-Wallis test and Bonferroni test for post hoc analysis. A p value <0.05 was considered to be significant.

**Results:** The expression of MMP-13 was significantly downregulated in both the 27mW/cm2 group and the 67mW/cm2 group in comparison with control group (0.68 ±0.08, 0.38 ±0.08, respectively). The significant downregulation of MMP-1 was found only in the 67mW/cm2 group (0.44 ±0.02). But the expression of Col2a1 was also downregulated significantly in comparison with control group (0.61±0.13, 0.54±0.10, respectively). There was no significant change in mRNA expression of ACAN. The expression of CITED2 was significantly upregulated in the 67mW/cm2 group (1.83±0.48).

**Conclusions:** Our results suggest that LIPUS has an anti-inflammatory effect. This effect may be induced partly through CITED2 upregulation, which plays a negative regulatory role in MMP transcription to compete with MMP transactivator Ets-1 for limiting amounts of co-factor p300, especially at the 67mW/cm2 intensity. However, since the mRNA expression of Col2a1 was downregulated by LIPUS treatment in our experimental condition, further study should be needed for clinical using.

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**CONNEXIN 43 IS DOWNREGULATED IN SCLEROTIC HUMAN SUBCHONDRAL OSTEOBLASTS: A POSSIBLE EXPLANATION FOR DECREASE OF MECHANOSENSITIVITY IN THESE CELLS**

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**Purpose:** In osteoarthritis, subchondral bone remodeling is increased leading to bone sclerosis. In a previous work, we have reported that osteoblasts coming from the sclerotic subchondral bone express a particular phenotype characterized by an increased production of IGF-1, IL-6, IL-8, PGE2, RANKL, MMP-3 and a decreased of OPG. We have also reported that all these genes are mechanosensitive and that osteoblast coming from the sclerotic zone were less sensitive to compression than osteoblasts of the non-sclerotic zone. In this study, we have compared the effects of compression on the expression of membrane receptors by osteoblasts coming from sclerotic and not sclerotic area in response to compression.