chondrocyte secreted proteins using the stable isotope labelling by amino acids in cell culture (SILAC) technique.
Methods: Cartilage obtained from patients undergoing joint replacement, or from patients with no history of joint disease was provided by the Tissue Bank and the Autopsy Service at CHU A Coruña. The study was approved by the local Ethics Committee. Chondrocytes released from cartilage by enzymatic digestion were recovered and plated at low density in basic SILAC medium (Silantes) supplemented with antibiotics and 10% FBS dialyzed. In the case of light media, standard L-lysine (146 mg/L) and L-arginine (28 mg/L) were used, while in the heavy media isotope-labelled L-lysine ($^{13}C_6$), and isotope-labelled L-arginine ($^{13}C_6$,$^{15}N_4$) were used. When complete incorporation of the heavy isotope was achieved in the cells (2-3 weeks), normal (N) chondrocytes were treated with CS 200μg/mL and then stimulated with IL-1β 5ng/mL, while osteoarthritic (OA) chondrocytes were treated with CS 200μg/mL alone. 48 hours later, conditioned media were collected and their proteins were concentrated and quantified. Heavy and light samples were mixed 1:1, and 4 μg of each mixed sample were in-solution reduced, alkylated and digested with trypsin. Separation and analysis of the resulting tryptic peptide mixtures was performed by nanoscale reversed-phase-LC-MS/MS. The identification and quantification of proteins was carried out with Protein Pilot software, which detects the heavy/light peak pairs and calculates the heavy/light ratios based on the peak areas. Identifications with a probability score higher than 95% and quantifications with a p value $\leq$0.05 were included in the results list.
Results: Database search (UniprotKB/Swissprot) allowed us the identification of 39 different proteins in the OA chondrocyte secretome and 70 in N chondrocyte secretome. Interestingly, in both cases the most abundant protein was cartilage glycoprotein 39, which has been previously related with OA pathogenesis. For biological and functional analysis we considered only those proteins detected in all replicates with a heavy/light ratio $\geq$1.2 or $\leq$0.8. In OA chondrocytes, chondroitin sulfate mainly improves the anabolic/catabolic balance of the extracellular cartilage matrix, by increasing the level of structural proteins like collagens, decorin, lumican, vimentin and fibronectin. In N chondrocytes stimulated with IL1β, CS appears to act primarily as an anti-inflammatory drug. We show in this work how CS reduces inflammation by two mechanisms: directly, by decreasing the presence of potent inflammatory mediators like IL6 (ratio=0.6), and also indirectly, by increasing proteins such as tumor necrosis factor α-induced protein (TSG6, ratio=3). TSG6 plays a crucial role in extracellular matrix formation, inflammatory cell migration and cell proliferation. It's a key component of a negative feedback loop operating through the protease network which reduces matrix degradation during OA process. The mechanism driven by TSG6 leads to a decrease in proMMPs activation, which might protect cartilage from extensive degradation even in the presence of acute inflammation (represented in our case by a high level of IL1β).
Conclusions: We have carried out the first pharmacoproteomic study using a quantitative proteomics approach (SILAC), based on the metabolic labelling of the cells, to study the effect of CS on chondrocyte secretome. Our findings provide novel information about the mechanisms that may exert the *in vivo* beneficial effects of CS on the OA disease process. This work also illustrates that chondrocyte secreted proteins are an attractive sub-proteome for the discovery of new targets of CS in OA therapy.

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QUANTITATIVE VALIDATION OF PROTEINS IDENTIFIED IN THE CARTILAGE SECRETOME IN AN EXPLANT MODEL OF EARLY OSTEOARTHRITIS

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Purpose: Previous in-house proteomic work identified several secreted proteins from equine cartilage explants in response to recombinant equine interleukin-1 beta (IL-1β) with or without the non-steroidal anti-inflammatory drug, carprofen. The aim of this study was to validate the presence of six of these proteins by western blotting. We also used quantitative densitometry to determine the effects of IL-1β, with or without carprofen treatment, on levels of these proteins in the cartilage secretome compared to control explants.
Methods: Cartilage explants were obtained from weight-bearing regions of metacarpophalangeal joints of horses euthanized for purposes other than for research. Explants were either incubated alone (control: C), with IL-1β (10ng/ml), or in combination with IL-1β and carprofen (IL-1β+ CA, 10ng/ml and 100μg/ml respectively) at 37°C for 5 days. Culture medium supernatants were collected and each sample divided into two aliquots. One aliquot underwent tryptic digestion and high-throughput proteomic analysis by ESI (Electrospray Ionisation) mass spectrometry using a Bruker HCT PTM discovery ion trap instrument. Comparative proteomic analysis of the supernatants identified a number of potentially relevant proteins. The remaining corresponding aliquots were resolved on 1-D gels and either silver stained to compare their electrophoretic profiles or used for western blotting to validate protein expression. Six of the most commonly identified proteins were selected for quantitative validation by western blotting; cartilage oligomeric matrix protein (COMP), thrombospondin-1 (TSP-1), clusterin (mature and precursor forms), cartilage intermediate layer protein-1 (CILP-1) and the matrix metalloproteinases MMP-1 and MMP-3.
Results: SDS-PAGE and silver staining revealed qualitative differences between the electrophoretic profiles of samples exposed to the different treatments. Western blotting confirmed the presence of COMP, TSP-1, clusterin, clusterin precursor, CILP-1, MMP-1 and MMP-3 in explant supernatants. Quantitative densitometry indicated that TSP-1, MMP-1 and MMP-3 levels were increased in IL-1β and IL-1β+CA samples compared to controls. Carprofen reduced MMP-1 and MMP-3 levels in IL-1β+CA compared to IL-1β treatment alone. CILP-1 and clusterin levels remained unchanged in all treatments, although the clusterin precursor was decreased in IL-1β samples.

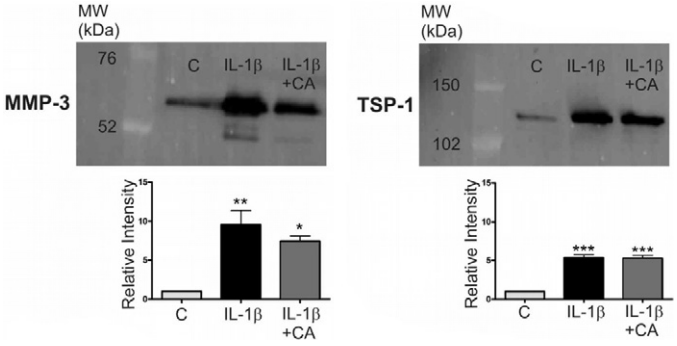

Figure 1

Conclusions: The authors' previous proteomic work has identified several relevant extracellular matrix proteins in explant supernatants stimulated with IL-1β. This study confirmed the presence of six of these proteins by quantitative western blotting and densitometry. Many of the identified proteins have well-known matrix functions including participation in cell-matrix and matrix-matrix interactions (i.e. TSP-1, COMP, CILP), matrix turnover (MMP-1, MMP-3) and extracellular molecular chaperone activity (clusterin). The validation described in this study suggests that this high-throughput proteomic system provides a useful tool to identify candidate proteins from the cartilage secretome for further quantitative analysis using western blotting.
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COMPREHENSIVE ANALYSIS OF THE INTERLEUKIN-1-BETA-MEDIATED MODULATION OF CHONDROCYTE INTRACELLULAR AND EXTRACELLULAR PROTEOMES BY METABOLIC LABELLING

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Purpose: The aim of this study is to standardize the stable isotope labelling by amino acids in human chondrocytes cell culture (SILAC) technique, and to apply this novel strategy for the study of osteoarthritis (OA) pathophysiology. To attain this objective, we have used an *in vitro* model of inflammation based on the stimulation of human articular chondrocytes with the cytokine Interleukin-1β (IL1β), a key OA mediator.
Methods: Cartilage obtained from patients with no history of joint disease