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 ([13C6], and isotope-labelled L-arginine ([13C6,15N4]) were used. When complete incorporation of the heavy isotope was achieved in the cells (2-3 weeks), normal (N) chondrocytes were treated with CS 200 μg/mL and then stimulated with IL-1β (10 ng/mL), while osteoarthritic (OA) chondrocytes were treated with CS 200 μg/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 ≤ 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 > 1.2 or > 0.8. In OA chondrocytes, chondroitin sulfates mainly improve 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 IL-1β, 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 mechanism 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 metacarpalphalangeal joints of horses euthanized for purposes other than for research. Explants were either incubated alone (control: C), with IL-1β (10 ng/ml), or in combination with IL-1β and carprofen (IL-1β+ CA, 10 ng/ml and 100 μg/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 FTICR 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-ΒETA-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 osteoarthritits (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-1 β (IL-1β), a key OA mediator.

Methods: Cartilage obtained from patients with no history of joint disease...
was provided by the Tissue Bank at CHU A Coruña. The study was approved by the local Ethics Committee. The SILAC technique is based on the incorporation of stable isotope labelled amino acids into the whole proteome, including its secretome (cell-conditioned medium). Chondrocytes released from cartilage by enzymatic digestion were recovered and plated at low density in SILAC medium (Silantes), lacking lysine and arginine. In the case of light media, we supplemented it with the standard amino acids, while in the heavy media isotope-labelled L-lys(13C6) and L-arg(13C615N4) were employed. Chondrocytes were used at week 2-3 in primary culture, after making them quiescent by incubation in a medium containing 0% FBS for 24h. Titration assays were performed to ensure a 100% labelling of the proteins without chondrocyte de-differentiation. Cells were then washed and incubated in serum-free medium with IL-1β at 5 ng/mL for 48 hours. Then, conditioned media were collected and intracellular proteins were extracted. Heavy and light secretome samples were mixed 1:1, and 4 μg of each mixed sample were directly in-solution reduced, alkylated and digested with trypsin. In the case of intracellular extracts, heavy and light mixed proteins were first fractionated by SDS-PAGE. Then, the whole lane was excised into 16 bands which were subsequently processed for in-gel tryptic digestion. In both cases, separation of the resulting tryptic peptide mixtures was performed by nanoscale reversed-phase-LC-MS/MS. The identification and quantification of proteins (by calculating the heavy:light ratios for each peptide) was carried out with Protein Pilot software, considering significant changes those with a ratio ≥1.2 or ≤0.8, and a p value <0.05.

Results: Collection and LC/MS analysis of the chondrocyte secreted proteins allowed the identification of 76 different proteins. 65 of them could also be quantified and 32 presented statistically significant differences between band and IL1β samples: 17 were increased and 15 decreased. Most of the identified proteins are cartilage ECM components (27%), such as collagens, laminin, proteoglycans or the cartilage oligomeric matrix protein, which clearly demonstrates the usefulness of chondrocyte secretome analysis for the study of cartilage ECM metabolism. We could also detect a high number of growth factors (19%), matrix remodelling proteins (14%), structural proteins (11%) and cytokines (8%). On the other hand, the analysis of chondrocyte intracellular proteins allowed the identification of 347 different proteins. All of them were quantified (except 4), and 40 proteins were characterized as significantly modulated by IL1β: 50% were increased and 50% decreased by the treatment. Interestingly, almost 30% of the downregulated proteins are related with cytoskeletal remodelling, indicating the changes in chondrocyte morphology caused by IL1β. The proteins increased are related with a number of cellular processes like metabolism, stress response, defence or signal transduction.

Conclusions: We have standardized the SILAC technique for the proteomic analysis of human primary cartilage cells, and we have applied this technology to study the effect of the proinflammatory cytokine IL-1β, a key molecule in the OA process, on the chondrocyte secretome and proteome. The obtained information will increase knowledge about OA pathogenesis, and already points out a number of possible markers of the disease.

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OSTEOARTHRITIS OF THE KNEE: EXPLANATIONS FOR THE EFFECTS OF EXERCISE THERAPY ON PAIN AND FUNCTIONING

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Purpose: Exercise therapy is a conservative treatment for osteoarthritis of the knee (OAK) of which the effectiveness is well substantiated: exercise significantly diminishes the pain and improves the patient’s functioning. No clear explanation is given for this health improving effect of exercise. The present systematic literature review aims to provide an overview of the theoretical models that are put forward to explain the treatment effects of exercise in OA as well as their scientific foundation.

Methods: We included all studies collected in Cochrane reviews emphasizing on exercise therapy for OAK. Therefore we used the search engine of the Cochrane Library website (http://www.cochrane.org/; keywords: osteoarthritis, exercise, knee). We also collected guidelines that are included in the Physiotherapy Evidence Database (http://www.pedro.org.au/) that were published since 2000 and that are related to the treatment of OAK (keywords: osteoarthritis, practice guideline, body part: lower leg or knee). If exercise therapy is recommended in these guidelines, the studies that were used to empower this advice, were included in this work. The introduction and discussion parts of the included studies were subsequently screened for plausible explanations supporting influences of exercise on OA.

Conclusions: Several theoretical models have been found in the literature to explain the health improving effects of exercise in patients with osteoarthritis of the knee. The most cited explanation is the improved stability of the osteoartritic knee was mentioned in 8 papers as a possible explanation. One author suggests that the improved energy absorbing quality of the peri-articular muscles of the knee have been proposed as mechanisms that decrease tear and shear stresses of the articular cartilage, finally resulting in pain reduction. Improved stability of the osteoarthritic knee was mentioned in 8 papers as a possible explanation. One author suggests that the improved energy absorbing quality of the muscles of the knee after exercise may contribute to a decreased amount of energy that is transmitted through the subchondral bone and articular cartilage. Weight loss following exercise is hypothesized in 3 studies as a reason for the treatment effects of exercise in OA. The observation that exercise can be related to an improved mental state was proposed as an explanation for the subjective health improving effect of exercise in 4 papers. In 3 papers higher cartilage proteoglycan content and improved joint fluid viscosity is put forward to explain the improvements in pain and functioning.

Results: Three Cochrane reviews and 7 guidelines were identified. After eliminating double references, 37 full papers were included in the study. Potential explanations for the effects of exercise for OAK were mentioned in 14 papers. A better knee stability, as well as weight loss and the improved energy absorbing quality of the peri-articular muscles of the knee have been proposed as mechanisms that decrease tear and shear stresses of the articular cartilage, finally resulting in pain reduction. Improved stability of the osteoarthritic knee was mentioned in 8 papers as a possible explanation. One author suggests that the improved energy absorbing quality of the muscles of the knee after exercise may contribute to a decreased amount of energy that is transmitted through the subchondral bone and articular cartilage. Weight loss following exercise is hypothesized in 3 studies as a reason for the treatment effects of exercise in OA. The observation that exercise can be related to an improved mental state was proposed as an explanation for the subjective health improving effect of exercise in 4 papers. In 3 papers higher cartilage proteoglycan content and improved joint fluid viscosity is put forward to explain the improvements in pain and functioning.