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CELLULAR AND MOLECULAR ACTIVITY OF A STANDARDIZED SMALL SEA FISH EXTRACT IN AN EXPERIMENTAL MODEL OF PRIMARY HUMAN CARTILAGE CELLS

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Abstract

The inflammatory, degenerative and rheumatic diseases are triggered and evolving at osteoarticular level due to the disruption of functionality of constitutive cells and extracellular microenvironment correlated with systemic responses as synthesis of progressively degradative cytokines.

Objectives. The in vitro bioactive efficacy of standardized small sea fish extract has therapeutic relevance on the strength of optimal physiological and structural biologic system rendered by primary cells isolated from human cartilage (HCH, PromoCell) unlike standardized cell lines that may have genetic and functional changes and beside the primary cells directly isolated from animal tissue that do not reproduce an authentic biological response because of metabolic stress adaptation to growing conditions in vitro. The main therapeutic targets in osteoarticular disorders are regeneration of affected cartilage and attenuation of local inflammatory processes.

Materials and methods. The in vitro articular matrix reconstruction action was evaluated by molecular tests with relative quantification of gene expression (qPCR) for aggrecan, the predominant component of human cartilage, and for the enzyme responsible for degradation of aggrecan, aggrecanase (ADAMTS4) whose activities were phenotypically measured by ELISA (enzyme-linked immunosorbent assay) test. The anti-inflammatory profile was proved by techniques of quantitative evaluation of mRNA level and detection of extracellular soluble proteins by flow-cytometry (BD™ Cytometric Bead Array) for pro-inflammatory cytokines IL-6, IL-8 and gene expression for nuclear factor NF-κB.

Results and discussion. The results prove the matrix regenerator effect of standardized small fish extract by stimulation at gene expression level of aggrecan synthesis in early stages of chondrocytes differentiation, directing cells to a mature functional status, as well as by inhibition of aggrecan degradation by action on gene expression and extracellular activity of ADAMTS4. The standardized small sea fish extract has an anti-inflammatory effect proven by extracellular and transcriptional inhibition of pro-inflammatory cytokines, IL-6, IL-8 and the nuclear factor NF-κB gene.

Conclusions. The in vitro anti-inflammatory and regenerative action of standardized small fish extract reflects a significant therapeutic potential for osteoarticular disease sustained both through the effects at gene and phenotypic expression levels and through the reproducible characteristics of dynamics of in vivo chondrocytes evolution evidenced by primary cell lines.

Keywords: osteoarthritis, inflammation, extracellular matrix, small marine sea fish concentrate, aggrecan, aggrecanase, interleukin

ABBREVIATIONS

FC – standardized small marine fish concentrate
OA – osteoarthritis
ECM – extracellular marix
ADAMTS4 – A Disintegrin And Metalloproteinase with ThromboSpondin motifs4
ADAMTS5 – A Disintegrin And Metalloproteinase with ThromboSpondin motifs5
IL-1β – interleukin 1β
IL-6 – interleukin 6
IL-8 – interleukin 8
NF-κB – nuclear factor-kappa B
HCH – human chondrocytes

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INTRODUCTION

Osteoarthritis (OA) is the most common type of arthritis. It is a crippling, late-onset and degenerative disease characterized by the loss of articular cartilage and synovial inflammation, leading to joint stiffness, swelling, pain and loss of mobility. The widely held view was increased pressure or overload on weight-bearing joints, anatomical joint incongruence and fragility of articular cartilage tissue were the key predisposing factors. Nowadays, thanks to the advent of molecular biology and key discoveries in the field, OA is being redefined as a very complex and multifactorial disease (1,2). Therefore, there is great interest in identifying and characterizing the proteases and inflammatory cytokines responsible for cartilage degradation and disease progression as potential targets in the development of therapeutics that prevent joint destruction in arthritis.

Articular cartilage is composed of chondrocytes embedded in an ECM, which provides the biomechanical characteristics that are essential for articular movement. One of the major ECM components of cartilage is aggrecan – a large proteoglycan that provides cartilage with the ability to resist compressive forces. Aggrecan fills the interstices of the collagen meshwork by forming large aggregated complexes interacting with hyaluronan and link proteins (3). Degradation of aggrecan is an important manifestation of OA. Aggrecan depletion in osteoarthritic cartilage can be ascribed to increased proteolytic cleavage of the core protein and is mediated by various matrix proteinases. *In vitro* aggrecanolysis by matrix metalloproteinases (MMPs) has been widely studied; however, it is now well recognized that aggrecanases are the principal proteinases responsible for aggrecan degradation *in situ* in articular cartilage. In diseases like OA, degradation of ECM macromolecules exceeds their synthesis, resulting in a net decrease in the amount of cartilage matrix, eventually leading to total or partial erosion of cartilage. Two recently identified aggrecanase isoforms (ADAMTS-4 and ADAMTS-5) are members of the 'A Disintegrin And Metalloproteinase with ThromboSpondin motifs' (ADAMTS) gene family, and there has been much interest in the possible role of these isoforms as therapeutic targets in OA (4,5). In human chondrocytes, ADAMTS4 is inducible by treatment with cytokines such as interleukin 1β (IL-1β), but the expression of ADAMTS5 is constitutive. Recent studies that ADAMTS4 is a major aggrecanase in human osteoarthritic cartilage (6,7).

Inflammation is increasingly being regarded as an important part of OA. Inflammation can occur locally, within the synovium, and systemically, with inflammatory agents circulating in the blood. In the pathophysiology of OA, proinflammatory cytokines have been shown to play important roles in the destruction of cartilage, synovitis, and pain. The severity and form of inflammation appears to change with disease progression, with different cytokine signatures being present in early and advanced stages of the disease (8,9). IL-6 also plays a major pro-inflammatory role in OA. Alongside these, chemotactic cytokines, such as IL-8 have been shown to influence inflammation in OA through their ability to influence the number of immune cells in the vicinity of the joint. They also stimulate IL-6 production and proteoglycan depletion (10,11). The nuclear factor NF-κB pathway has long been considered a prototypical proinflammatory signaling pathway, largely based on the role of NF-κB in the expression of proinflammatory genes including cytokines, chemokines, and adhesion molecules. The promoter region of the IL-8 and IL-6 genes contains binding sites for the transcription factors, nuclear factor-kappa B (NF-κB) (11,18,19). The transcription factor NF-κB binds the IL-8 promoter as dimer and also NF-kappa B is an important mediator for activation of the IL-6 gene (11,20,21).

The maintenance of composition and organization of the extracellular matrix in articular cartilage relies on specific programs for the synthesis and turnover of each matrix component (12,13,14). Identifying genes and regulatory pathways involved as OA cartilage becomes damaged may provide new targets for treatment to delay or reverse the damage (15,16,17,22). Such as, the rationale behind this study was the monitoring of matrix turnover under the action of a standardized small marine fish concentrate (FC) by analysis of gene expression for the main ECM component, aggrecan and their proteinase, aggrecanase (ADAMTS-4) and also the phenotypic expression of these on primary cells culture HCH (human chondrocytes). Furthermore, inflammatory status under the action of FC was evaluated by phenotypic screening of pro-inflammatory cytokines IL-6, IL-8 and by relative quantification of gene expression for nuclear factor NF-kB and cytokines IL-6, IL-8 on primary cells culture HCH (human chondrocytes).

MATERIALS AND METHODS

Small marine fish concentrate (FC) was obtained by an original patented method of extraction from small marine fish. The small marine fish concentrate (FC) is a standardized extract by a rigorous control of extraction stage and also of physical-chemical characteristics in accordance with European pharmaceutical legislation. The standardized small marine fish concentrate (FC) is active pharmaceutical ingredient in injectable solution Alflutop®.

Cell Culture

The primary cells line: human articular chondrocytes (HCH) was purchased from PromoCell (C-12710). Primary Human Chondrocytes (HCH) are isolated from normal human articular cartilage from the knee and hip joints (lot specific source information is available on request). These chondrocytes have the capacity to produce and maintain the extracellular matrix of cartilage, i.e. collagen (mostly type II) and proteoglycans (primarily aggrecan). The advantages of using these primary chondrocytes are superior to those isolated directly from tissue due to metabolic and functional stabilization at the first passage immediately after thawing, whereas cells isolated directly from tissue need a long time to adapt to in vitro growth conditions and to restore the full functionality. The cells were cultured in PromoCell Growth Medium (C-27101) supplemented with 10% Chondrocytes Growth medium supplement mix (C-39635) and 1% antibiotics and antimycotics (A5955, Sigma Aldrich) and the cells culture vessels were placed in an incubator (37^0C, 5% CO_2). The cells were used in our experimental models at passages 3-5 and at a density of adherent cells of 1 x10^4-2 x10^4 cells /cm^2.

Study design

The *in vitro* experimental model consist of two groups of cells: cells treated with FC (concentrations in culture medium were 0.2%, 0,1%, 0,02%), and human cytokine IL-1β 10ng/ml (Peprotech, 200-01B) for 48h in culture, having also an untreated cell control with FC but treated only with IL-1β 10ng/ml; and the second group of cells is treated only with FC without IL-1β cytokine treatment and also an untreated cell control. The cytokine IL-1β was used as inflammatory stimulus due to targeting of pro-inflammatory cytokines production and stimulation of proteinases at cellular level. After 48h of treatment in culture, the culture medium was removed and used for aggrecan, aggrecanase activity assay (ELISA) and soluble pro-inflammatory cytokines (IL-6, IL-8) analysis by flow cytometry. The cells were detached using 100 µl Trypsin/ EDTA Solution per cm^2, inactivated with FBS (fetal bovine serum), resuspended in cold phosphate buffered saline (PBS), counted in a hemocytometer and used for gene expression by RT-PCR.

Gene Expression Analysis

Isolation of total RNA, revers transcription and RT-PCR from primary HCH was perfomed using the TaqMan Gene Expression Cells-to-CT kit (A1728, Ambion) following the manufacturer's recommendations. Lysates were stored at -80^0C until reverse transcription.

Reverse transcription was performed by assembling an RT master mix (25µL RT Buffer (2x); 2,5µL RT Enzyme Mix (20x); nuclease-free water 12,5µL) and adding lysate (10µL) and also no-template control (contain all the RT components except the cell lysate). The reaction was run by a Real-Time PCR instrument (Step One Plus, Applied Biosystem).

Real-Time PCR (RT-PCR) was carried out using TaqMan Gene Expression Master Mix (TaqMan Gene Expression Cells-to-CT kit, A1728, Ambion) which contains ROX passive reference dye and TaqMan Gene Expression Assay labelled with FAM-MGB (aggrecan gene symbol- ACAN, assay ID: Hs00202971_m1; aggrecanase gene symbol: ADAMTS4 cod Hs00192708_m1; gene symbol: IL-6, assay ID: Hs 00985639_m1; gene symbol: IL-8, assay ID: Hs00174103_m1; gene symbol: NF-kB, assay ID: Hs00765730_m1; Thermo Fischer Scientific). Relative expression levels were normalised with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated with the $2^{-\Delta CT}$ method using QuantStudio Design&Analysis Software on a RT-PCR instrument QuantStudio5, Applied Biosystem. The data were obtained from three biological experiments and triplicates/experiment.

Aggrecanase Activity ELISA

The quantitative determination of aggrecanase activity in cell culture medium samples was carried out using Sensitive Aggrecanase Activity ELISA kit (M046009, Mdbioproducts), following the manufac-

turer's recommendations. Sample aggrecanase is evaluated by two methods: the concentration of active aggrecanase in samples is calculated from the standard curve obtained with purified aggrecanase; and the amount of product ARGSVIL-peptide-s produced by aggrecanase is calculated from the standard curve of ARGSVIL-peptide-s.

Flow-cytometry analysis

Soluble pro-inflammatory cytokines IL-6 and IL-8 levels from cell culture medium samples were determined using Human IL-6 Flex Set BD CBA (558276), Human IL-8 Flex Set BD CBA (558277) and Human Soluble Protein Master Buffer Kit BD CBA (558265), BD Biosciences. The bead population is resolved in two fluorescence channels of a flow cytometer (BD FACSCanto II). After acquiring samples on a flow cytometer, use FCAP Array™ software to generate results in graphical and tabular format. We analysed samples in triplicates from each biological experiment and from three different biological experiments.

Human Aggrecan ELISA

The human aggrecan from cell culture medium samples was analysed using Human Aggrecan ELISA kit (ab213754, Abcam), following the manufacturer's recommendations. The density of obtained colour is proportional to the human aggrecan amount of sample captured in plate in a microplate spectrophotometer (Tristar LB 941, Berthold Technologies).

Statistical analysis

Differences between unstimulated/ IL-1β stimulated cells and treated with FC in comparison with unstimulated/ IL-1β stimulated control cells were tested using the t-test two tailed test by Graph Pad Prism 5 software.

RESULTS

The qPCR analysis of gene expression for the major structural component of extracellular matrix ACAN (aggrecan) showed that small marine fish concentrate (FC) induced a 4,75 fold increase ($p<0.001$) in comparison with control cells for basal conditions of cells (unstimulated) and a 1,4 fold increase ($p<0.001$) in comparison with control cells for inflammatory conditions of cells culture by treatment for 48h with osteoarticular degradative cytokine IL-1β (10ng/ml). The aggrecan turn-over in cartilage cells is mainly controled by aggrecanase activity (ADAMTS4) that gene expression was significantly decreased by FC ($p<0.001$) for basal and degradative in vitro simulation by IL-1β (Fig. 1, Table 1).

The inflammatory profile was evaluated by relative quantification of genes for pro-inflammatory cytokines IL-6 and IL-8 and also for the nuclear factor NF-kB as an important regulator of transcriptional rate of the inflammatory molecules. In primary chondrocytes HCH, FC decreased ($p<0.001$) the rate of transcription for genes coding the cytokines IL-6

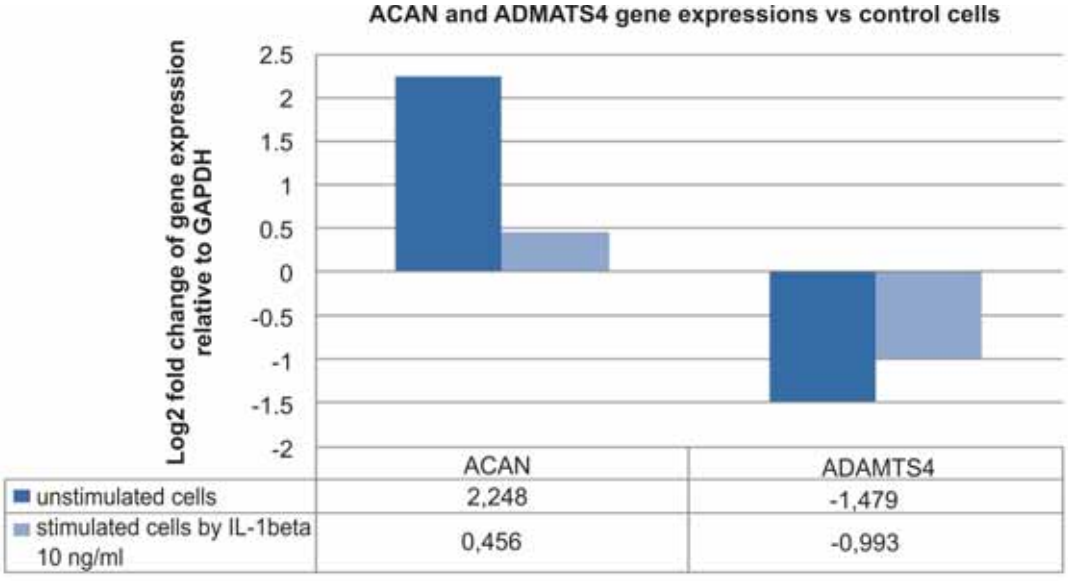

FIGURE 1. *The gene expression of ACAN and ADAMTS4 relative to GAPDH (endogenous control) vs control cells as reference sample (log2 fold change=0)*

and IL-8. The results showed a remarkable downregulation of IL-8 gene by FC in cells under the aggressive action of IL-1β as promoter of osteoarticular inflammation (Table 1, Fig.2).

The gene expression for nuclear factor NF-kB was significantly reduced ($p<0.001$) in stimulated cells by IL-1β 10ng/ml which was correlated with the decrease of gene expression for pro-inflammatory cytokines.

The ELISA analysis of extracellular levels for aggrecan and aggrecanase on FC action in primary chondrocytes revealed a notable increase of aggrecan (1,3 ng/ml in IL-1β stimulated cells vs 0,81 ng/ml in control, $p<0.0002$) and a correlated decrease of extracellular aggrecanase (53,02 pM in IL-1β stimulated cells vs 72.34 pM in control, $p<0.0001$) that attribute an articular renewable effect in osteoarticular disorders (Fig. 3, Table 2).

The fish extract acts through a combined, significant effect of increased the synthesis of this proteoglycan, coupled with decreased its degradation promoted by aggrecanases, a process that results in aggrecan restore in extracellular fluids. FC augmented the aggrecan synthesis even in basal (1,77 ng/ml vs 1,01 ng/ml in control, $p<0.0001$), unstimulated conditions, preparing the chondrocytes functional status and producing resources for further inflammatory damages that could occure. Even in unstimulated conditions was revealed a rise of extracellular aggrecanase (45,69 pM in unstimulated cells vs 35,87 pM in control, $p=0.0008$) induced by FC, there are cellular intrinsic mechanisms acting for protein homeostasis restoring, ex. physiological activation of ADAMTS when a proteic excedent arise (Fig. 3, Table 2).

The flow cytometry analysis of extracellular soluble pro-inflammatory cytokines IL-6 and IL-8, which were secreted by primary chondrocytes, evidenced that FC significantly decreased (6699.83 pg/ml IL-8 vs 12110.46 pg/ml control, $p<0.0001$; 4225.81 pg/ml IL-6 vs 5591.29 pg/ml control) the inflammatory markers, especially in IL-1β stimulated cells (Fig. 4, Table 3).

TABLE 1. *The mean values of log2 fold chance of gene expression for aggrecan (ACAN), aggrecanase (ADAMTS4), nuclear factor (NF-kB), pro-inflammatory cytokines (IL-6, IL-8) in comparison with control cells as reference sample:*

Gene	The mean values of log2 fold chance of gene expression	
	Unstimulated cells	Stimulated cells (IL-1β 10 ng/ml)
ACAN	2.239 ± 0.03738 a) p < 0.0001 → ***	0.4563 ± 0.06909 a) p < 0.0001 → ***
ADAMTS4	-1.479 ±0.3274 a) p < 0.0001 → ***	-0.9933 ± 0.1305 a) p < 0.0001 → ***
IL-6	-0.5963 ± 0.1319 a) p < 0.0001 → ***	-0.4987 ± 0.1236 a) p < 0.0001 → ***
IL-8	-0.5133 ±0.1252 a) p < 0.0001 → ***	-0.8820 ± 0.2478 a) p < 0.0001 → ***
NF-KB	-0.3807 ± 0.03367 a) p < 0.0001 → ***	-0.6803 ± 0.3265) a) p < 0.0001 → ***

a) sample vs. control cells

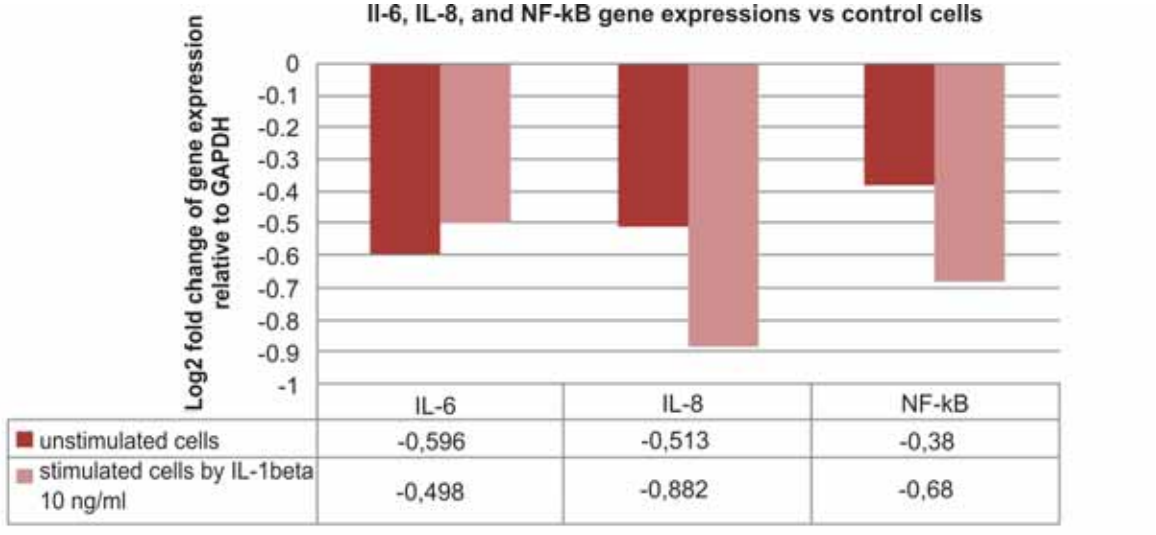

FIGURE 2. *The gene expression of IL-6, IL-8 and NF-kB relative to GAPDH (endogenous control) vs control cells as reference sample (log2 fold change=0)*

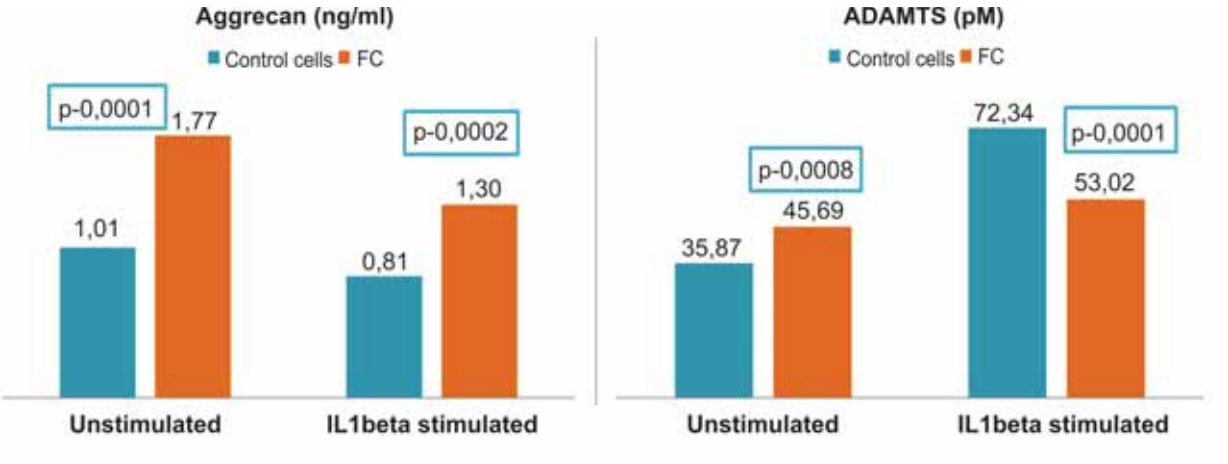

FIGURE 3. *The modulation by FC of extracellular aggrecan/ aggrecanase status*

TABLE 2. *The extracellular aggrecan and aggrecanase by ELISA analysis:*

Samples	Aggrecan (ng/ml)		ADAMTS (pM)	
	unstimulated	IL1beta stimulation	unstimulated	IL1beta stimulation
Control cells	1.013 ± 0.06947	0.8133 ± 0.02251 b) p = 0.0001→ ****	35.87 ± 1.389	72.34 ± 0.8998 b) p = 0.0001→ ****
FC	1.767 ± 0.1297 a) p = 0.0001→ ****,	1.297 ± 0.1048 a) p = 0.0002→ ***,b) p = 0.0001→ ****	45.69 ± 2.347 a) p = 0.0008→ ****,	53.02 ± 1.516 a) p = 0.0001→ ****, b) p = 0.0001→ ****

a) sample vs. control cells; b) stimulated vs unstimulated

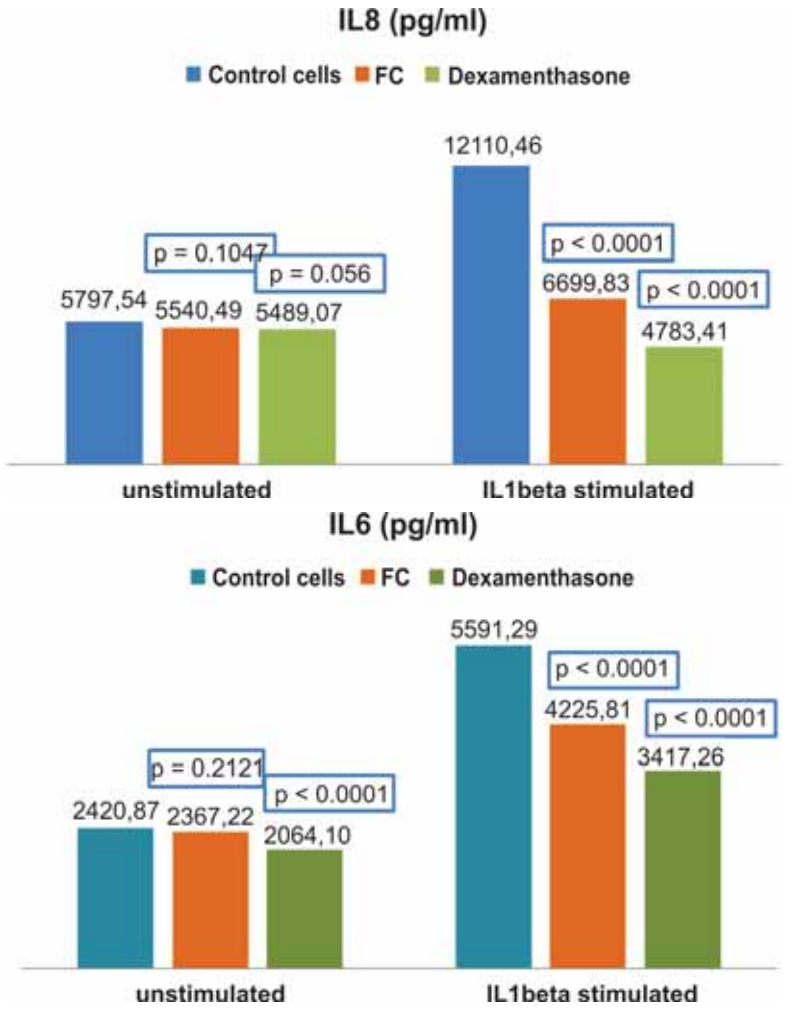

Figure 4. *Extracellular secretion rates of IL-6 and IL-8 by primary chondrocytes cells (HCH) under the action of FC*

TABLE 3. *The variations of IL-6 and IL-8 extracellular levels on fish concentrate action*

Samples	IL8 (pg/ml)		IL6 (pg/ml)	
	unstimulated	IL1beta stimulation	unstimulated	IL1beta stimulation
Control cells	5798 ± 118.9	12110 ± 290.3 b) p = 0.0001→ ****	2421 ± 69.1	5591 ± 35.26 b) p = 0.0001→ ****
FC	5540 ± 232.2 a) P = 0.1047 →ns	6700 ± 134.3 a) p < 0.0001→ ****, b) p < 0.0001→ ****	2367 ± 50.89 a) p = 0.2121 → ns	4226 ± 37.61 a) p < 0.0001→ ****, b) p < 0.0001→ ****
Dexametazona 200 ng/ml	5489 ± 59.15 a) p = 0.0056 → **	4783 ± 82.08 a) p < 0.0001→ ****, b) p < 0.0001→ ****	2064 ± 64.77 a) p < 0.0001→ ****,	3417 ± 26.78 a) p < 0.0001→ ****, b) p < 0.0001→ ****

a) sample vs. control cells; b) stimulated vs unstimulated

The fish concentrate shows a significant inhibition of extracellular release of IL-6 and IL-8, the main modulators of acute phase inflammation and signaling cascades promoters, was almost similar to dexamethasone, a potent anti-inflammatory drug used as positive control but with in vivo serious adverse reaction.

DISCUSSION

Summarizing our results, we observed that standardized small marine fish concentrate (FC) sustain the articular matrix regeneration and attenuation of inflammatory processes by action on genotypic and phenotypic level for main molecules involved in progression of osteoarticular diseases.

The osteoarthritic joint is characterized by catabolic processes that degrade the aggrecan molecules, so impairing aggrecan function and predisposing the articular cartilage to erosion. Such degradation is associated with aggrecanase activity and inflammatory status (23,24). The genetic and cellular modulation of aggrecan synthesis by FC in primary cells isolated from human cartilage (HCH, PromoCell) was highlighted by a significant increase of gene expression in comparison with control cells which was positive correlated with a phenotypic response at extracellular level. The correspondence between gene transcription and extracellular aggrecan support the efficacy of standardized FC in restoration of the main structural molecule from articular cartilage.

Aggrecan degradation facilitated by ADAMTS enzymes is a process that occurs within normal and arthritic cartilage, signifying a role for these proteases in normal turnover as well as in arthritis. Inflammatory cytokines such as IL-1β expressed locally in the articular joint cause inflammation, stimulating the production of ADAMTS enzymes (25,26). ADAMTS4 is mainly expressed in an active form in osteoarthritic cartilage, and suggest that ADAMTS4 may play an important role in the degradation of aggrecan in human osteoarthritic cartilage, while ADAMTS5 was constitutively expressed in osteoarthritic and normal cartilage (27). The standardized small marine fish concentrate (FC) in stimulated chondrocytes by IL-1β 10ng/ml significantly inhibits the mRNA expression levels of ADAMTS4 gene and extracellular activity of aggrecanase. Thus, the acceleration by fish concentrate of aggrecan production in IL-1β aggressed articular cartilage cells was efficiently balanced by inhibition of enzymatic degradation of aggrecan by targeting extracellular ADAMTS. This mechanism of action suggests that fish concentrate could be an efficient regenerator of extracellular matrix in osteoarticular diseases by equilibration between synthesis and destruction of the main structural molecule in osteoarthritic joint.

In the pathophysiology of OA, proinflammatory cytokines have been shown to play important roles in the destruction of cartilage, synovitis, and pain. The severity and form of inflammation appears to change with disease progression, with different cytokine signatures being present in early and advanced stages of the disease. A number of proinflammatory cytokines have been, and continue to be, studied as potential biochemical markers with possible candidates being found for burden of disease assessment, prognostics and diagnostics. IL-1β and TNF-α are among the key players in terms of proinflammatory cytokines involved in OA. IL-6 also plays a major pro-inflammatory role in OA (28,29, 30). IL-8, produced by human OA chondrocytes, is an important mediator in the pathophysiology of OA including promotion of a number of pathogenic processes such as: release of matrix metalloproteinases, neutrophil accumulation and activation and leukocyte homing to the synovium (28). Our results showed that fish concentrate remarkably decreased the genotypic expression of pro-inflammatory cytokines IL-6 and IL-8 which are in a strength relationship with inhibition of nuclear factor NF-kB at

transcriptional level. The limitation of mRNA synthesis of NF-kB by fish extract could suggest a correlative inhibition of the IL-8 and IL-6 genes by quantitative reduction the main nuclear transcription factor that acts by binding to sites in the promoter region of inflammatory cytokines. The molecular results were sustained by phenotypic analysis of extracellular inflammatory manifestations. The nuclear status of inflammatory profile was translated into reduced IL-6 and IL-8 extracellular levels which converge towards an anti-inflammatory effect of fish concentrate on IL-1β stimulated chondrocytes.

The in vitro anti-inflammatory and regenerative action of standardized small fish extract reflects a significant therapeutic potential for osteoarticular disease sustained both through the effects at gene and phenotypic expression levels and through the reproducible characteristics of dynamics of in vivo chondrocytes evolution evidenced by primary cell lines. Bioactive efficacy of standardized small sea fish extract has therapeutic relevance on the strength of optimal physiological and structural biologic system rendered by primary cells isolated from human cartilage (HCH) unlike standardized cell lines that may have genetic and functional changes and beside the primary cells directly isolated from animal tissue that do not reproduce an authentic biological response because of metabolic stress adaptation to growing conditions *in vitro*.

CONCLUSION

Standardized small marine fish extract has an important therapeutic potential in osteoarticular disease supported by the matrix regenerator effect by stimulation at gene expression level of aggrecan synthesis in early stages of chondrocytes differentiation, directing cells to a mature functional status, as well as by inhibition of aggrecan degradation by action on gene expression and extracellular activity of ADAMTS4. Also, the standardized small sea fish extract has a competitive anti-inflammatory effect proven by extracellular and transcriptional inhibition of pro-inflammatory cytokines, IL-6, IL-8 and the nuclear factor NF-κB gene.

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