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The pharmaceutic capitalization of a natural, marine active principle through *in vitro* anti-citokinic and proliferative mechanisms of HC-OA osteoarthritic chondrocytes

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Abstract

Osteoarthritis (OA) as a multi-factorial disease that features interrelated mechanisms (senescence/ apoptosis, proliferation, decreased synthesis of matrix proteins, activation of degrading enzymes, inflammation) and a diversity of cells involved in bone and cartilage metabolism: chondrocytes, osteoblasts, osteoclasts etc. A complete pharmaceutical solution remains an unsolved question in this interdisciplinary field of research.

We focused our approach on osteoarthritic primary chondrocytes HC-OA (Cell Application), exploring on this primary cell line the small sea fish extract's potential to improve the proliferation rate and to stop the cytokines pro-inflammatory signaling. Previous research results have shown an important therapeutic potential of the small sea fish extract, restoring the intrinsic functionality of the cartilage through its correlative effects: antioxidant and anti-inflammatory action, stimulatory activity of cell proliferation and matriceal proteins homeostasis. In the context of pro-inflammatory and degradative stimulation with IL1β, the small sea fish extract prove a counteracting "in vitro" effect, restoring the proliferative capacity of HC-OA cells and inhibiting the IL8 release, with implication in several cartilage degradation pathways: MMP13 activation, neutrophils' accumulation, synovium leucocytes activation, as well as the hypertrophic and differentiation processes of chondrocytes.

Keywords: small sea fish extract, osteoarthritic chondrocytes, proliferation, pro-inflammatory cytokines, degenerative osteoarthicular diseases

INTRODUCTION

The increased degradation of cartilage tissue in the joint is caused by the overproduction of enzymes degrading the extracellular matrix, one of the main feature of osteoarthritis, accompaigned by cell proliferation decline and the progression of inflammation. Despite the initial proliferation and activation of chondrocytes, they are not able to efficiently repair the degrading cartilage (1). Abnormal mechanical stress on cartilage induces catabolic and inflammation-related events via intracellular signaling pathways that are similar to those activated by oxidative stress, inflammatory cytokines, and products of matrix damage (2,3). Chondrocytes

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express cytokines and their receptors, as well as MMP-13 and type II collagen cleavage epitopes in regions of matrix depletion in osteoarthritic cartilage. Inflammatory stimuli can also increase either ADAMTS4 or 5 or both, in different experimental models (4,5). Inflammatory cytokines may also induce chemokines, including IL-8, and other cytokines such as IL-6, leukemia inhibitory factor, IL-17, and IL-18. Many of these factors synergize with one another in promoting chondrocyte catabolic responses (6,7,8).

Osteoarthritis (OA) constitutes a widespread health problem and a complete pharmaceutical solution, acting on the main biological mechanisms (chondrocyte senescence or apoptosis, proliferation, decreased synthesis of cartilage matrix proteins, inflammation), remains an unsolved question in the interdisciplinary field of cartilage and bone tissue research.

Alflutop® is a Romanian original drug, having as active principle a standardised small sea fish extract, with chondro-restitutive action through the synergysm of its active components. Previous research results have shown an important therapeutic potential of the small sea fish extract, restoring the intrinsic functionality of the cartilage through its correlative efects: antioxidant and anti-inflammatory action, stimulatory activity of cell proliferation and matriceal proteins homeostasis (9,10).

MATERIALS AND METHODS

Research conducted in SC Biotehnos SA identified new molecular and cellular targets of Alflutop®'s action, relevant in degenerative articular pathologies (11,12,13), but studies has to be continued due to the huge diversity of cells involved in bone diseases: chondrocytes, osteoblasts, osteoclasts etc.

We focused this approach on osteoarthritic primary chondrocytes HC-OA (Cell Application), exploring on this primary cell line the small sea fish extract potential to improve the proliferation rate and to stop the cytokines pro-inflammatory signaling.

***Cell culture* – Human Chondrocytes Osteoarthritis – HC-OA (Cell Application)** – derived from human articular cartilage of donors with OA. HC-OA provides a useful model to explore changes in chondrocyte metabolism in response to abnormal environment of the osteoarthritic joint. Cells were cultivated in Human Chondrocyte Growth Medium at 37°C, in 95% humidified air and 5% CO_2 incubator. Cells were treated for 48h with different concentrations of small sea fish extract (codified CP) and detached through trypsinization (Trypsin/EDTA 0.1 g% – Sigma).

Chemical Reagents:

- BD Cytometric Bead Array (CBA)- Human Inflammatory Cytokines kit *(BD Pharmingen)*
- Cell Cycle sequentiation (BD Cycletest Plus DNA kit - 340242);

Methods:

Cell cycle sequentiation, especially the evaluation of mitotic phases: S- DNA synthesis phase and **G2/M** – the entry in mitosis. The identification of DNA replication cycle is done applying nuclei fluorescence labeling with propidium iodide, followed by flow-cytometric quantification.

Extracellular pro-inflammatory cytokines quantification by flow cytometry (14):

The simultaneous detection of several soluble analytes (inflammatory cytokines) is done through versatile technique that uses a series of discrete fluorescence intensity particles with a capture surface coated with specific antibodies for IL-8, IL-6, IL1 etc. The capture beads, the conjugated detection antibodies and the recombinant standards or assay samples are incubated together to form a sandwich complex that is visualized in APC-A / PE-A coordinates following acquisition of flow cytometry. Analysis of fluorescence histograms and interpolation of values on calibration curves is performed with FCAP Beads Array software.

Equipment*:*

- Flow cytometer FACS CANTO II (Becton-Dickinson) with DIVA 6.1., FCS Express and FCAP Array softwares.
- Experiments were performed in triplicate and mean values ± standard deviation were considered. Graphic representations include the standard deviations between experiments.

RESULTS

The experimental design is oriented to evaluate the proliferative status of HC-OA cells, in unstimulated conditions, compared with a pro-inflammatory and pro-degradative context induced through IL-1beta stimulation (10 ng/ml, 24 h). Cells were cultivated 48 h prior the addition of the standardized small sea fish extract (codified CP) and another 48 h with the extract and the corresponding solvent control.

TABLE 1. *HC-OA proliferative status express as cell cycle phases – passage 2*

2nd passage; 48 h treatment	2 days treatment without stimulation					Stimulation ILlbeta 10 ng/mll 24 h				
	%G0/G1	%S	%G2/M	%S+%G2/M	Variation S ±G2/M	%G0/G1	%S	%G2/M	%S±%G2/M	Variation S ±G2/M
Control cells	91.3	4.76	3.94	8.7 ± 0.245	100	91.74	4.81	3.45	8.26 ±0.118	100
Solvent control 0.2%	92.09	4.65	3.27	7.92 ± 0.2	83.9	91.04	5.04	3.92	8.96 ±0.151	108.47
Solvent control 0.02%	91.55	4.49	3.96	8.45 ± 0.094	89.89	92.69	4.03	3.29	7.32 ±0.163	88.62
CP 0.2%	92.06	4.44	3.5	7.94 ±0.167	84.47	92.82	3.54	3.64	7.18 ±0.033	86.92
CP 0.02%	93.28	3.2	3.52	6.72 ±0.184	71.49	92.27	3.96	3.77	7.73 ±0.167	93.58

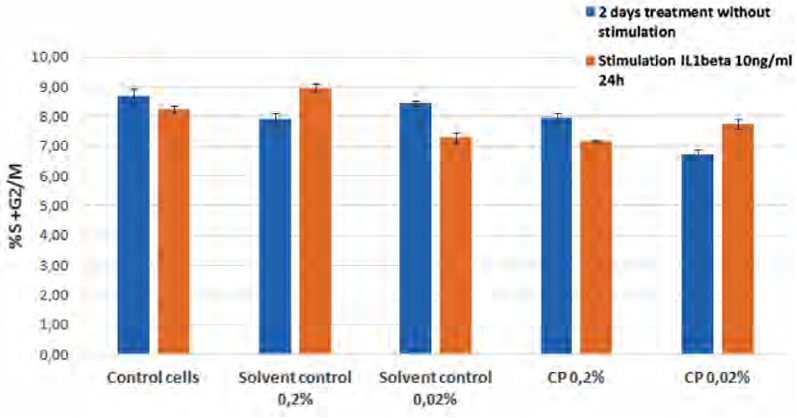

FIGURE 1. *Mitotic phases' evolution modulated by the fish extract (CP) – passage 2*

We had to mention that IL-1β is one of the main promoters of the degradation of cartilage, inducing metalloproteinases activation and pro-inflammatory response in osteoarthritis Results are presented in the tables and figures below. The effects were appreciated as % of variation compared with the cellular control.

Differences of cellular behavior upon IL1-beta stimulation were highlighted in fig. 4, regarding the variation of mitotic progression (cellular % in S and G2/M cell cycle phases). We had to mention that IL-1β is one of the main promoters of the degradation of cartilage, inducing metalloproteinases activation and pro-inflammatory response in osteoarthritis.

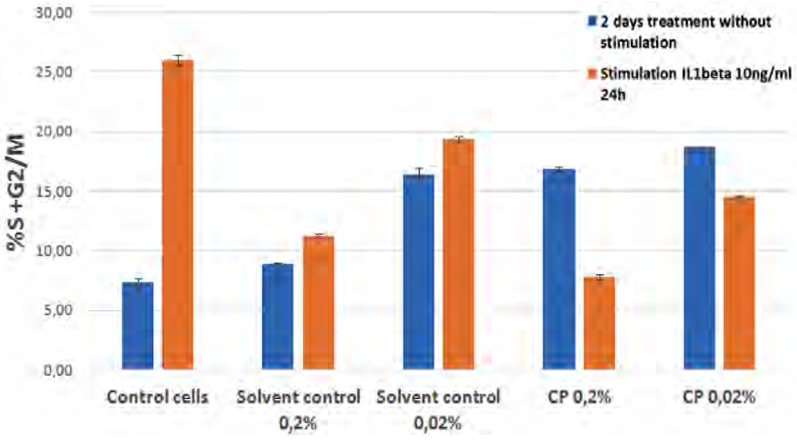

FIGURE 2. *Mitotic phases' evolution modulated by the fish extract (CP) – passage3*

TABLE 2. *HC-OA proliferative status express as cell cycle phases - passage 3*

3nd passage; 48 h treatment	2 days treatment without stimulation					Stimulation ILlbeta 10 ng/mll 24 h				
	%G0/G1	%S	%G2/M	%S+%G2/M	Variation S ±G2/M	%G0/G1	%S	%G2/M	%S±%G2/M	Variation S ±G2/M
Control cells	92.81	4.04	3.51	7.55±0.368	100	74.03	22.21	3.76	25.97±0.396	100
Solvent control 0.2%	91.09	5.71	3.2	8.91±0.073	118.013	88.75	6.41	4.85	11.26±0.159	43.36
Solvent control 0.02%	83.57	11.82	4.61	16.43±0.469	217.616	80.62	15.78	3.6	19.38±0.237	74.62
CP 0.2%	83.14	12.99	3.87	16.86±0.159	223.311	92.18	3.59	4.24	7.83±0.233	30.15
CP 0.02%	81.33	15.45	3.22	18.67±0.082	247.285	85.52	9.64	4.84	14.48±0.155	55.76

TABLE 3. *HC-OA proliferative status express as cell cycle phases- passage 6*

6nd passage; 48 h treatment	2 days treatment without stimulation					Stimulation ILlbeta 10 ng/mll 24 h				
	%G0/G1	%S	%G2/M	%S+%G2/M	Variation S ±G2/M	%G0/G1	%S	%G2/M	%S±%G2/M	Variation S ±G2/M
Control cells	86.53	10.05	3.42	13.47±0.163	100	88.92	6.48	4.61	11.09±0.245	100
Solvent control 0.2%	91.33	6.37	2.3	8.67±0.245	61.02	87.5	8.72	3.78	12.5± 0.041	112.71
Solvent control 0.02%	87.05	9.28	3.67	12.95±0.122	68.89	89.47	6.33	4.2	10.53±0.139	94.95
CP 0.2%	74.7	18.81	6.49	25.3± 0.327	139.64	71.61	19.811	8.59	28.4±0.245	256.09
CP 0.02%	74.02	17.01	8.97	25.98±0.057	162.93	72.24	17.43	10.33	27.76±0.163	250.32

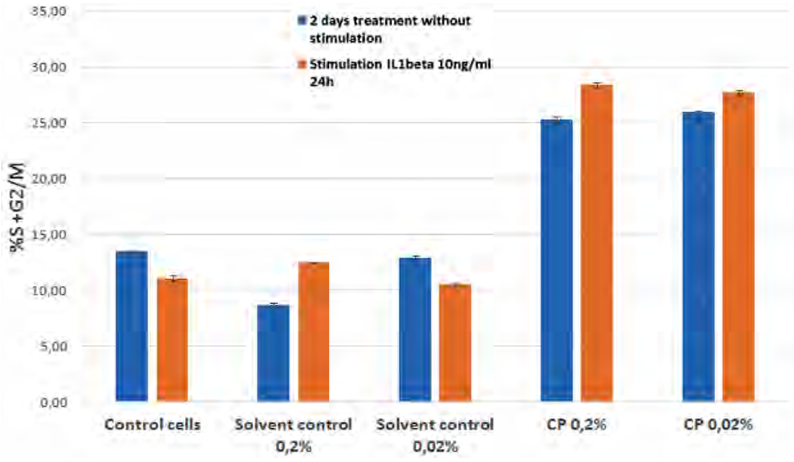

FIGURE 3. *Mitotic phases' evolution modulated by the fish extract (CP) – passage 6*

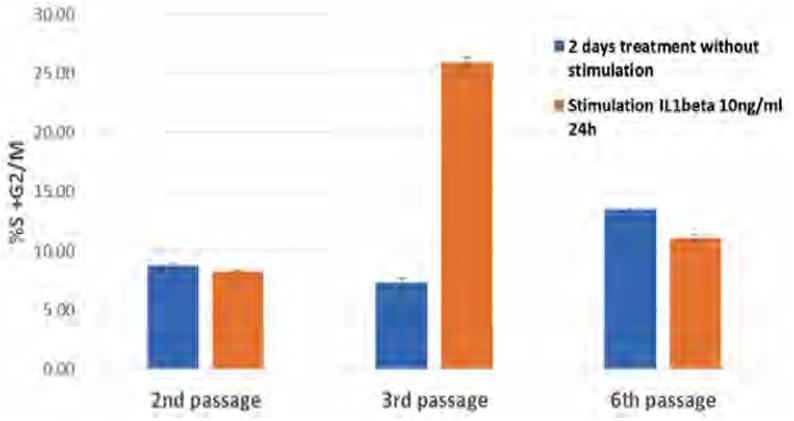

FIGURE 4. *HC-OA chondrocytes ageing correlated with the proliferative response to IL1-beta stimulation*

The pro-inflammatory response of IL1-beta stimulation was quantified in the terms of IL6, Il8, IL1-alpha and IL1-beta extracellular release, as main mediators of osteoarthicular inflammation. Cytokines are transiently produced by most cells, including chondrocytes, in response to cellular activation signals. They produce effects in the paracrine manner on the adjacent tissue or cells, or on the cells from which they were secreted, in the autocrine manner. TNF-α and IL-1 do not act isolated, but as a result of IL6 and IL8 interaction, producing chain reactions (metalloproteinase production, activation of reactive oxygen species etc.), resulting cartilage degradation (15).

IL6, besides the role of active participant in degenerative joint processes, has pluripotent functions in immunomodulation, regulation of cell growth and differentiation, induction of inflammation (16).

Studies of extracellular pro-inflammatory cytokines have been performed on the osteoarthritic chondrocyte cell line at different passages. The results obtained are presented in the tables below, with a special mention for IL1β. In series stimulated with IL1-beta 10ng/ml we cal-

TABEL 4. *HC-OA Cytokines profile at passage 2*

2nd passage; 48 h treatment	IL8 (pg/ml)			IL6 (pg/ml)			Hlalfa (pg/ml)			IL1 beta (pg/ml)		
	unstimu-lated	stimulated	% variation	unstimu-lated	stimulated	% variation	unstimu-lated	stimu-lated	% variation	unstimu-lated	stimulated	% variation
Control cells	7254.57	41231.48±8.16	100.00	552.51	8497.46±11.08	100.00	513.58	681.02±16.33	100.00	1323.13	1493.13±31.13	100.00
Solvent control 0.2%	7404.02	41651.98±9.80	101.00	639.89	8569.87±16.33	100.90	844.58	633.35±24.21	93.00	1563.76	1459.17±23.75	97.70
Solvent control 0.02%	8969.92	10315.69±12.17	978.00	763.87	8517.23±15.51	100.20	696.55	600.52±16.33	88.20	1436.76	1414.41±15.92	94.70
CP 0.2%	7027.96	19518.73± 4.70	95.80	639.89	88883.41±24.49	104.50	711.92	665.32±13.06	97.70	1414.41	1504.6±14.40	100.80
CP 0.02%	7404.02	18315.69±10.61	92.90	569.89	8490.87±17.95	99.90	681.02	649.43±40.82	95.40	1436.76	1436.76±27.88	96.20

TABEL 5. *HC-OA Cytokines profile at passage 3*

3nd passage; 48 h treatment	IL8 (pg/ml)			IL6 (pg/ml)			IL1 alia (pg/ml)			IL1 beta (pg/ml)		
	unstimul-ated	stimulated	% variati	unstimu-lated	stimulated	% variati	unstimu-lated	stimu-lated	% variati	unstimu-lated	stimu-lated	% variatic
Control cells	6798.37	41784.72±61.24	100	517.92	8925.56±26.96	100	633.35±40.27	649.32	100	1459.17	1436.76±25.07	100
Solvent control 0.2%	6875.24	41737.3±32.21	99.9	817.44	8766.29±50.92	98.2	416.25±22.61	617.05	95	1436.76	1369.4±40.82	95.3
Solvent control 0.02%	8204.11	41547.33±94.14	99.4	970.19	8746.72±38.26	98	764.6±22.54	681.02	104.9	1481.75	1601.32±25.89	111
CP 0.2%	8764.26	30906.67 ±46.28	74	1386.33	8831.42±36.84	98.9	815.84±46.65	657.17	101.2	1747.23	1504.6±22.13	104.7
CP 0.02%	7478.27	37975.82±61.24	90.9	763.87	8909.36±27.55	99.8	522.65±16.08	566.68	87.3	1425.58	1425.58±25.30	99.2

TABLE 6. *HC-OA Cytokines profile at passage 6*

6nd passage; 48 h treatment	IL8 (pg/ml)			IL6 (pg/ml)			ILlalfa (pg/ml)			IL1 beta (pg/ml)		
	unstimu-lated	stimulated	% variation	unstimu-lated	stimulated	% variation	unstimu-lated	stimulated	% variatlor	unstimu-lated	stimulated	% variatior
Control cells	8969.92	41499.66+43.79	100.00	1440.59	9009.67+43.41	100.00	649.43	711.92±18.47	100.00	1504.60	1459.17±50.78	100.00
Solvent control 0.2%	7772.18	42819.94+28.50	103.20	871.22	8707.53±26.32	96.60	696.55	717.05±32.79	100.70	1459.17	1436.76+40.38	98.50
Solvent control 0.02%	7103.83	40766.88+56.60	98.20	1042.40	8424.84+28.77	93.50	772.00	681.02±39.56	95.70	1551.63	1576.07+31.92	108.00
CP 0.2%	7917.33	40453.64+45.76	97.50	1268.65	8736.92±29.49	97.00	711.92	742.22±32.06	104.30	1576.07	1504.6+17.20	103.10
CP 0.02%	8345.79	40864.18+52.81	98.50	1205.26	8746.72±46.96	97.10	583.74	681.02±32.20	95.70	1369.40	1481.75±36.6	101.50

culated this cytokine concentration subtracting this value from the final result.

DISCUSSION

IL-1β is one of the most important cytokine involved in osteoarthritis pathogenesis and it is widely used for in vitro inflammation models, to screen drugs or natural compounds. IL-1 is capable of inducing chondrocytes and synovial cells to synthesize MMPs, suppresses the synthesis of type II collagen and proteoglycans, and inhibits transforming growth factor-ß stimulated chondrocyte proliferation. It acts through an in situ mechanism, in a paracrine or in an autocrine way (17).

Our study on the primary line of osteoarthritic chondrocytes reveals different effects of IL-1 stimulation, dependent of the cell evolution, from the beginning of cell maturity (passage 2), to the functional status decline (passage 6).

The HC-OA cell line is form of primary cells from osteoarthritic patients and has a limited time of preserving the normal physiological status, including the proliferative one.(fig.no.1). This is the reason of the decrease of responsivity at the higher passages. At passage 2, the IL1-β stimulation doesn't disturb the mitotic rate. The stimulation effect is counteracted at this level by the cellular intrinsic regulatory mechanisms. At passage 3, the IL1-beta stimulation accelerates the DNA synthesis, leading to hypertrophic chondrocytes. In this case, the fish extract (CP) stimulates the cells division only in the unstimulated conditions, and decrease the proliferation in IL-1β activated cells, preventing the abnormal bone formation. At the 6th passage, when the proliferation rate is lower and metabolic status decline, CP activates the osteoarthritic chondrocytes turn-over in both stimulated and unstimulated experimental series. This is a significant action, considering that osteoarthritis is charac-

terized by the endochondral ossification and by a "senescence-associated secretory phenotype "characterized by the secretion of interleukin-1 (IL-1) and interleukin-6 (IL-6), as well as matrix metalloproteinase-3 (MMP-3) and-13 (MMP-13), and the regenerative capacity of OA chondrocytes is lost (18). We must remark that the fish extract doesn't stimulates the mature chondrocytes that have a normal division cycle, but only the cells that are in a decline state.

IL-1β production was detected in medial and lateral primary chondrocyte cultures; however, the concentration of this cytokine was highest in the cultures established from the region of greatest pathology. (19) It was demonstrated that IL-1beta is the initiator of several cascades of pro- inflammatory cytokines, IL-6 and IL8 being the most significant. IL8 expression in human osteoarthritic chondrocytes is a clinical predictive marker and a possible target, in the treatment of arthritic diseases (20).

We decided to monitor in our experimental design IL6 and IL8 release, as essential promoters of the inflammatory response, as well as the two IL1cytokines, IL1α and IL1β There are scientific evidences that indicate increased IL-1α in cartilage is associated with early degenerative changes, suggesting an autocrine/paracrine role for this cytokine in osteoarthritic pathogenesis (21). The other similar protein, IL1β is the more potent form of the pro-degradative cytokine IL1, both having synergistic effect in cartilage injuries (22).

Our "in vitro" study reveals only a slow response in terms of IL1 autocrine stimulation in the metabolic active status of the cells (passages 2 and 3), but a very high release of IL6 and IL8 after a 24h pro-inflammatory conditions (IL1 paracrine stimulation of cytokines) in every stage of osteoarthritic chondrocytes development. The fish extract is not active at passage 2, or at the ending of osteoarthritic chondrocytes development (passage 6). It inhibits IL8 release from HC-OA cells cultivated at passage 3, a mature stage of cell development, an importing effect having in mind the role of IL8 chemokine in MMP13activation, neutrophils' accumulation, synovium leucocytes activation, as well as the hypertrophic and differentiation processes of chondrocytes.

These effects could be compared with the others reported by the scientific literature for compounds used as anti-cytokines agents in osteoarthritic therapy: glucosamine (23), schisandrin A (24), caffeic acid (25), diacerein (26) etc.

CONCLUSIONS

The osteoarthritic chondrocytes – HC-OA, studied as cellular model for active principles interactions in disease, has proven biological relevance as responsiveness to pro-inflammatory stimulation and IL6 and IL8 release, as well as proliferative behavior function the stage of cellular development (passage 2 compared to passage 6).

In the context of pro-inflammatory and degradative stimulation with IL1β, the small sea fish extract prove a counteracting "in vitro" effect, restoring the proliferative capacity of HC-OA cells and inhibiting the IL8 release. These experimental data must be projected as an addition of a new cellular model with biological relevance in the background of already existing evidences on several standardized lines and primary cells (CHON-001, HCH-human normal chondrocytes). Results confirm on osteoarthritic chondrocytes the small sea fish extract's effects previously proved, especially the regeneration capacity and the anti-cytokine action.

Future experiments should explore the other interrelated metabolic pathways of osteoarthritic chondrocytes in order to explain and sustain the pharmacological action of the standardized fish extract on this cellular model.

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