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"*In vitro*" chondro-restitutive capacity of Alflutop® proved on chondrocytes cultures

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Abstract

Cartilage is characterized by a limited intrinsic repair capacity, thereby finding new therapeutic agents that stimulate the cellular anabolism and inhibit protein breakdown is a scientific priority. The effect of Alflutop® on cartilage cell regeneration was studied using CHON-001 cells (human bone cartilage chondrocytes), a standardized "in vitro" model for functional investigations, as well as on primary cells isolated from rabbit cartilage. The main explored mechanisms were: proliferative status (cell cycle sequence and proliferation) - representative for dynamic cell regeneration and extracellular release of TGF-β - key protein in extracellular matrix homeostasis. The results support the in vitro action of Alflutop® on chondrocyte turn-over, stimulating the cell proliferation and modulating extracellular TGF-β release. The action on the above mentioned cellular metabolic pathways suggests the therapeutic efficacy of the product in degenerative osteoarticular diseases.

Keywords: cartilage repair, chondrocyte, proliferation, Alflutop®

1. Introduction

Osteoarthritis is a degenerative pathology, characterized by a gradual loss of articular cartilage integrity. The functional limitations are manifested as a consequence of progressive cartilage injuries, thickening of the subchondral plate and proliferation of osteophytes (BUCKWALTER & al., 1998[1]). The advanced stages of the disease imply the vascular invasion and articular cartilage calcification, leading to bone remodeling. The inflammation arises in peri-articular tissue and has a mild intensity, compared with the rheumatoid arthritis. The remodeling process of the cartilage is initiated by an abnormal extracellular stimulation cascade, including autocrine and paracrine factors, synovial stimuli and protein components from damaged matrix. The proteoglycans homeostasis is seriously disturbed due to abnormal cellular responses concerning the anabolic and catabolic activity. The chondrocyte number decreases as a consequence of dysregulated apoptosis and proliferation, while cells became pre-senescent, losing their functionality (SILVER& al., 2001[2]). Moreover, osteoarthritis is not simply a local disease caused by mechanical stress, but also a generalized disorder in which various interrelated humoral, metabolic, cellular and molecular mediators contribute to the progressive degradation of articular cartilage (ALCARAZ& al., 2013[3]).

Cell proliferation is critical for every tissue homeostasis and repair, but progressive hypocellularity of articular cartilage is a well-recognized hallmark of osteoarthrosis, associated with a reduced proliferative capacity in articular chondrocytes. These observations indicate

that articular chondrocytes lack the requisite proliferative capability to effectively maintain cell numbers in response to age-related pathological changes. The cellular turnover is slow and generates a lower response capacity to injuries (VENEZIAN& al., 1998[4]). Stimulation of chondrocyte proliferation could be considered as a therapeutical solution for osteoarticular diseases. TGF-β signaling is involved in a wide array of cellular activities in both physiological and pathological conditions, regulating gene expression through several signaling pathways. In cultured chondrocytes, TGF-β1 stimulates cell proliferation and extracellular matrix formation. Treatment with TGF-β increases cartilage matrix synthesis, especially aggrecan. TGF-β increases total glycosaminoglycan synthesis in immature cartilage, but not in mature cartilage, maintaining the matrix components of cartilage in an immature state (SMITH& al., 2000[5]). Through a Smad-dependent pathway, TGF-β induces aggrecan expression in chondrogenic cell lines. In response to TGF-β, Smad2 is rapidly phosphorylated, leading to the initial activation of the aggrecan gene. TGF-β simultaneously upregulates the expression of aggrecanase, and thus accelerates the turnover of cartilage matrix. While TGF-β enhances chondrocyte proliferation, it inhibits the terminal differentiation of chondrocytes and helps chondrocytes remain in the prehypertrophic stage (LI& al., 2005[6]). TGF-β plays an important role in all chondrogenesis phases, mesenchymal condensation, chondrocytes proliferation, extracellular matrix restructuring (aggrecan and type II collagen synthesis), and terminal differentiation, being a key promoter of chondrogenesis. Cellular condensation is strongly stimulated by TGF-β, this also amplifying cellular adhesion. After mechanic stimulation, chondrocytes secrete TGF-β through an autocrine signaling mechanism. Integrins, binding proteins from the extracellular matrix, are responsive to this signal. TGF-β - cellular surface receptors complex generates certain signaling cascades (ex. TGF-β - SMAD), mentioning the terminal differentiation involved in the begining and progression of cartilage breakdown (DeLISE& al., 2000[7]). The long term efficacy in osteoarthritis impose a multi-targeted therapy based on the signaling factors and their implication in specific metabolic pathways or particular mechanisms, from proliferative processes to architectural matrix foundation. Alflutop® is a product based on a small marine fish extract, used for the treatment of degenerative osteoarticular diseases. Our experimental investigations target several cellular processes (proliferation and extracellular signaling of TGF-β), to highlight the molecular mechanisms of action for Alflutop®.

2. Materials and Methods

Cell cultures:

CHON-001 (ATCC® CRL-2846™) are human normal chondrocytes from long bone cartilage. Cells were cultivated in high glucose DMEM (ATCC - Catalog No. 30-2002) with 10% fetal bovine serum, 0.1mg/ml G-418 antibiotic solution, at 37°C, in 95% humidified air and 5% CO_2 incubator. Cells were treated for 48h with different concentrations of Alflutop® and 15 μM ascorbic acid (as positive control) and detached through trypsinization (Trypsin/EDTA 0.1g% - Sigma).

Chondrocytes isolated from rabbit cartilage (primary culture) – Chondrocytes were isolated from cartilage fragments dissected from long bones of 2 years old female rabbits through enzymatic digestion with collagenase II (BRITTBERG & al., 2001[8], ZHONGHUA& al., 2005 [9]). The primary chondrocytes obtained were seeded in high glucose DMEM (ATCC - Catalog No. 30-2002) with 10% fetal bovine serum, supplemented with antibiotic-antimycotic solution, at 37°C, in 95% humidified air and 5% CO_2. At the second passage, cells were distributed in 6 wells plates at a density of 100000 cells/well, kept in culture for 48h, and then treated with different concentrations of Alflutop® for another 48h.

Chemicals and reagents: Cycle TEST PLUS DNA Reagent (BD PHARMINGEN); Cell Trace CFSE Cell Proliferation Kit (Invitrogen); BD Cytometric Bead Array (CBA) for soluble factors detection: *Human TGF-β Single Plex Flex Set & Protein Master Buffer Kit* (BD Pharmingen).

Equipment: Flow cytometer FACS CANTO II (Becton-Dickinson) with DIVA 6.1., FCS Express and FCAP Array softwares.

Methods:

ASSESMENT OF CELL PROLIFERATIVE STATUS – CELL CYCLE AND SUCCESIVE GENERATION PROGRESSION

SUCCESIVE GENERATION PROGRESSION - evaluated by fluorescence quantification of CFSE (carboxyfluorescein diacetate succinimidyl ester) staining, a cell permeant fluorescein-based dye which covalently attaches to cytoplasmic components of cells, resulting in uniform bright fluorescence (LYONS, 2000 [10]). Upon cell division, the dye is equally distributed between daughter cells, allowing the resolution of up to eight cycles of cell division by flow cytometry.

CELL CYCLE SEQUENCE - The isolation and label of the nuclei in cell suspensions is done using Cycle TEST PLUS DNA Reagent (BD PHARMINGEN). DNA amount measurements by flow cytometry are possible due to the specific labeling of the DNA with propidium iodide (PI) fluorochrome. Fluorescence intensity is proportional to the dye quantity, corresponding to the DNA quantity to which it had been bound. In the distribution histogram for DNA quantity/normal population, the following are plotted: a first peak corresponding to the cell fraction which is found in the phase G0/G1, having a diploid DNA quantity (2N); a second peak, found at a double distance from the first one, which represents the cell fraction in phases G2+M, having a tetraploid DNA quantity (4N); area included between the two peaks, representing the cells in the synthesis phase S, having an intermediate content of nucleic acids (RABINOVITCH, [11], Darzynkiewicz& al., 2010 [15]).

TGF-β EVALUATION- through BD™ Cytometric Bead Array (CBA), a flow cytometry application that allows users to quantify soluble proteins. The system uses the broad dynamic range of fluorescence detection offered by flow cytometry and antibody-coated beads to efficiently capture analytes. The detection reagent provided in the kit is a mixture of phycoerythrin (PE)–conjugated antibodies, which provides a fluorescent signal proportional to the amount of bound analyte (in our particular case TGF-β).

The analysis of the results (standard curve for cytokine and concentration calculation) is done with FCAP Beads Array software.

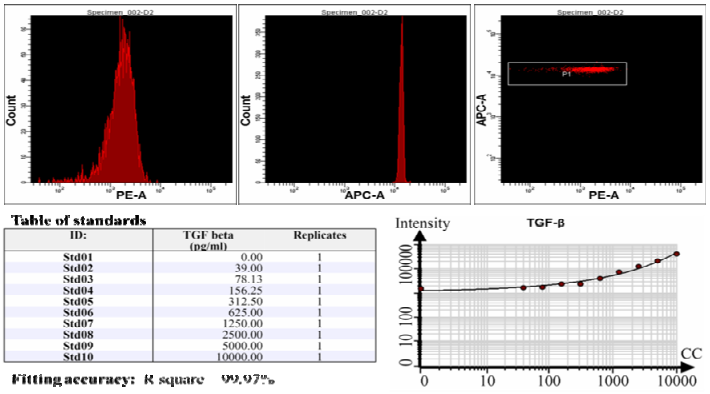

Figure 1: Methodology for TGF-β evaluation as extracellular soluble protein - fluorescent capture beads technique

When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (captured bead + analyte + detection reagent) are formed. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector. Detection was done using APC-A/PE-A coordinates (Figure1).

Statistics

Experiments were performed in triplicate and mean values were considered. Graphic representations include the standard deviations between experiments. Statistics were done using t-test, where * means p-value<0.05, ** means p-value<0.01 and *** means p-value<0.005.

3. Results and Conclusions

The experimental models were designed to demonstrate the action of Alflutop® as a chondro-protective agent, highlighting the chondrocyte proliferative status and structural protein homeostasis modulated by TGF-β. One of the cellular models used was a standardized cell line of human normal bone chondrocytes, CHON-001. The other one was a primary culture of freshly isolated cells from rabbit cartilage that have the advantages of better maintaining the physiological features of the primary source, the cartilage. Ascorbic acid 15 μM was considered as positive control, being a chondrocyte proliferation activator (KIMLL& al., 2003, [12]).

A. The chondro-protective effect of Alflutop® evaluated on CHON-001 standardized cell line.

The proliferative status was explored by two complementary methods: the division rate and the proliferative generation succession – quantified through the proliferative index (PI) and cell cycle phases distribution, especially evaluated through S (DNA synthesis) and G2/M (the start of mitosis) phases. It is important to estimate the total percent of cells in DNA synthesis and mitosis stages (%S+%G2/M) that decide the proliferative index and offer a right image of the cellular multiplication process (GRIMES& al., 1997, [13]).

The results are presented in Fig.1 (the proliferation index variation) and Fig.3 (%S+%G2/M and DNA histograms for control /Alflutop®). Experiments include also ascorbic acid as a well-known stimulator of proliferation.

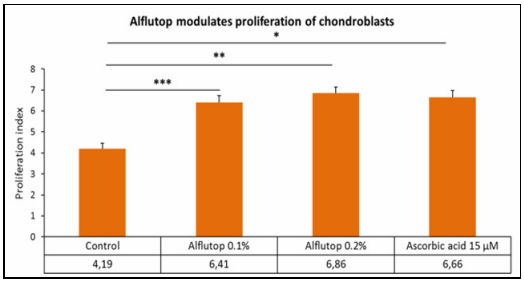

Fig. 2. The succession of proliferative generation estimated through PI-proliferation index.

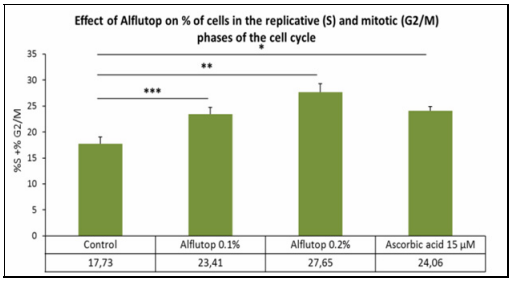

Fig. 3. The chondrocytes CHON-001cell cycle dynamics modulated by Alflutop®

Alflutop® product improves the proliferative status of the chondrocytes, PI rising in a dose-dependent manner and significantly with 53% (for Alflutop 0.1%), respectively 64% (for Alflutop 0.2%) compared to the cellular control (Fig.2).

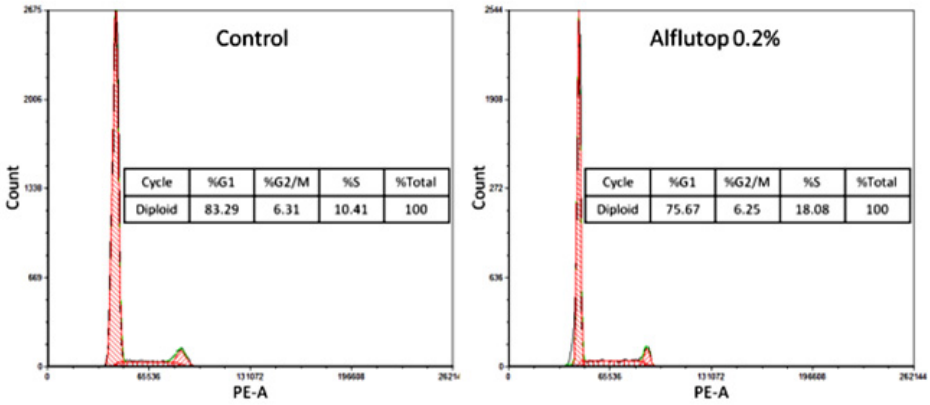

Fig. 4. DNA histograms showing the effect of Alflutop® on chondrocyte cell cycle modulation

The other complementary method used, based on cell cycle distribution, shows significantly increased DNA synthesis and more cells starting mitosis, with 32% for 0.1% Alflutop and 56% for 0.2% Alflutop compared with the cellular control (Fig.3, 4). This process is reflected in a rehabilitation of chondrocyte number and functionality that help maintain and improve the balance between articular cartilage components.

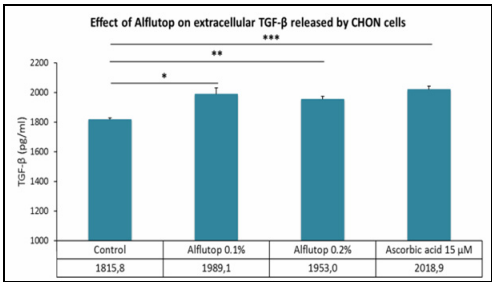

Fig. 5. TGF-β extracellular release (CBA technique) in CHON-001 cell line treated with Alflutop®

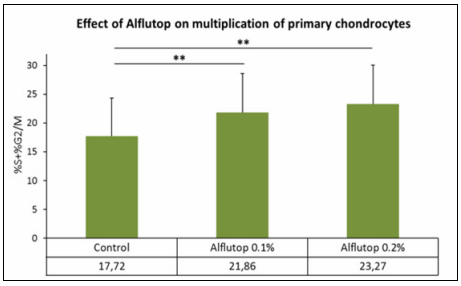

Fig. 6. Modulation of primary chondrocytes multiplication (%S and %G2/M cell cycle phases)

TGF-β extracellular release was detected to verify the hypothesis that Alflutop® can regulate the extracellular matrix homeostasis through balancing protein anabolic/catabolic processes. Fig.5 shows the results from successive experiments, proving a significant 7% activation of TGF-β induced by Alflutop® compared with the cellular control. Ascorbic acid, tested as a cellular metabolism activator, elevated extracellular TGF-β with 10% compared with the cellular control. The minor but significant activation of TGF-β should be considered in respect to the general homeostasis of the cell. A sudden release of a high pool of extracellular TGF-β could destroy the fine balance of protein synthesis/degradation and lead to abnormal ossification.

B. The chondro-protective effect of Alflutop® evaluated on primary cells from rabbit cartilage

The chondro-restitutive effect of Alflutop® on standardized cell line CHON-001 was evaluated on primary cells isolated from rabbit cartilage to confirm the previously observed

action. Thus, the sequence of cell cycle phases was evaluated at the final check point after 48h of incubation with Alflutop®. Table 1 and Fig.6 present cell cycle events modulated by Alflutop® and the percent of variation of the two proliferative cell cycle phases: S (DNA synthesis) and G2/M (the beginning of mitosis).

Table 1. Cell cycle distribution modulated by Alflutop®

	%G0/G1 (average)	%S (average)	%G2/M (average)	%S+%G2/M	Variation index %
Control	81.97	5.78	11.94	17.72	-
Alflutop 0.1%	78.13	7.92	13.94	21.86	25.16
Alflutop 0.2%	76.73	8.54	14.73	23.27	33.77

Alflutop® proved to have an effect also in primary chondrocyte cell cycle regulation. It stimulates in a dose-dependent manner with 25%- 33% the DNA synthesis and the progression in mitosis. These observations, correlated with those obtained from the standardized cell line CHON-001, indicate that Alflutop® could be an effective agent in the stimulation of chondrocyte proliferative capability, which may have a significant impact in pathological changes of cartilage (mechanical or age-related). The results presented in this study together with our previous findings about the *in vitro* modulation of important mediators of inflammation (IL6, IL8, VEGF) induced by Alflutop® (OLARIU& al., 2015, [14]), support the chondro-restitutive capacity of this product and define its multi-targeted cellular action in osteoarticular dysfunction.

Conclusions

The chondrocyte cell line CHON-001 represent an efficient, reproducible *in vitro* model, relevant for structural and functional osteoarthritic changes, as proved by comparative and confirmatory results obtained on primary chondrocytes. Degenerative joint disease comprises a complex pathology with intrinsic and external modulators, one of the most representative mechanisms of progression being the slow division rate of chondrocytes, the main cellular components responsible for cartilage homeostasis. Alflutop® induces the stimulation of chondrocyte proliferation, both on a standardized human cell line and on freshly isolated cells from rabbit cartilage, proving its positive impact on an essential process of cartilage injuries. Moreover, results from two different techniques showing correlative processes converge to Alflutop® positive influence on cellular proliferation. Additionally we observed that Alflutop® treatment of CHON-001 cells modulates a suitable TGF-β release, the basis of extracellular signaling for structural proteins synthesis. All these mechanisms converge towards the chondro-restitutive action of this drug, stimulating both the cellular multiplication and extracellular network support.

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