Academy of Romanian Scientists *Volume 6, No. 1, 2017, pp. 82 - 99*
Annals Series on Biological Sciences ISSN 2285 – 4177

ALFLUTOP® MODULATES "IN VITRO" RELEVANT MECHANISMS OF OSTEOARTHRITIC PATHOLOGY

Laura OLARIU[1, 2], Brindusa DUMITRIU[1,*], Diana Manuela ENE[1], Alexey PAVLOV[3], Natalya PYATIGORSKAYA[3], Natalia ROSOIU[1,2]

[1] S.C. Biotehnos S.A., 3-5 Gorunului Street, 075100-Otopeni, Ilfov, Romania, Phone: +40317102402, Fax: +40317102400

[*] Corresponding author: Brindusa DUMITRIU, e-mail: dbrandusa@biotehnos.com

[2] Academy of Romanian Scientists, 54 Splaiul Independentei 050094, Bucharest, Romania

[3] I.M. Sechenov First Moscow State Medical University, Moscow, Russia

Abstract

The osteoarticular injuries have a large complexity, and could be counteract on two main direction: inducing the reconstitutive and regenerative effects at cellular level and proteic core and /or through the antioxidant and antiinflammatory effect. Alflutop® is a standardised injectable solution based on small fish concentrate, with therapeutical indication in degenerative rheumatic diseases. Its pharmacological action is founded on the synergy of the active principles that are present in the formulation of the product. The objectives of this study comprise the identification of more cellular and molecular targets of Alflutop®'s action, relevant for articular degenerative pathologies. Our results reveals an important therapeutic potential in the rising of the intrinsic recovery capacity of the organism, restoring biomechanical stability of viscoelastic matrix of cartilage tissue, leading to chondro-protective action, restoration of inter- and intracellular signaling pathways in the cartilage matrix and thus the improvement of the joint compressive strength and the decrease of inflammation.

Key words: Alflutop®, chondrocytes cell culture, osteoarthritis, cartilage inflammation, chondrogenic therapies

Introduction

The degenerative articular pathologies are chronic disabilities that degrade the quality of life, having as main causes a cumulus of local or systemic risk factors, with high impact on the disease evolution: joint injuries, alignment, bone metabolism, obesity, etc. Advancements in molecular biology reveals osteoarthritis as a very complex, multifactorial disease, characterized by "low-grade inflammation" in cartilage and synovium, resulting in the loss of joint structure and progressive deterioration of cartilage. (1, 14)

Healthy tissue is represented by normal cartilage without any fissures, with no signs of synovial inflammation. Osteoarthritic pathology is characterised by early local degenerate lesion and 'fibrillated' cartilage, as well as remodelling of bone, leading to bony outgrowth and subchondral sclerosis episodically inflammation of synovium, fryed tendons. (2)

The cartilage degradations could have several pathological correspondences, as the main ones: arthrosis, osteoarthritis, rheumatoid arthritis. All of them are progressive degenerative pathology, with an inflammatory component at the synovial membrane level, and is characterized by the degradation of cartilage, osteophyte formation, but are accompanied by a different intensity of the inflammatory components. In degenerative articular pathologies appear cellular and molecular modifications, as degraded extracellular matrix, fragmented proteoglycan network, pre-senescent chondrocyte. Articular chondrocytes exhibit a dose- and time-dependent response to shear stress that results in cytokine release, matrix metalloproteinase expression and activation of intracellular signaling pathways. The release of soluble mediators and extracellular matrix macromolecules as response to mechanical stimulation contributes to the maintenance of articular cartilage homeostasis in vivo. (3).

Osteoarthritic chondrocytes are constantly abnormally extracellularly stimulated by autocrine and paracrine factors, synovial stimuli and protein fragments from the degraded matrix inducing multiple cellular responses at the anabolism, catabolism and phenotype levels. Chondrocyte number decreases by proliferation and apoptosis perturbations. Cells become pre-senescent losing a lot of their functions. OA presents multiple pro-inflammatory extracellular signaling factors like pro-inflammatory cytokines (IL6, IL8, IL1b), matrix metalloproteinases (collagenases), aggrecanases, hyaluronidases, growth factors (TGFb), neuronal mediators. Besides these, free radicals (ROS) hasten progression of this disease together with autoimmune factors.

The strategies of therapeutical approaches should have in mind the biochemical complexity of intra /extra cellular interactions directed by key factors, haveing as final target the treatment of the diseases’ cause, not of the effect. For a complete “in vitro” screening we have to cover all the possible targets of this main pathologies (Fig.1)

The experimental studies performed since the first development of this drug, allow molecular targets identification for Alflutop® and also the optimum active configuration. This paper will present an up to date review of the main “in vitro” effects explored.

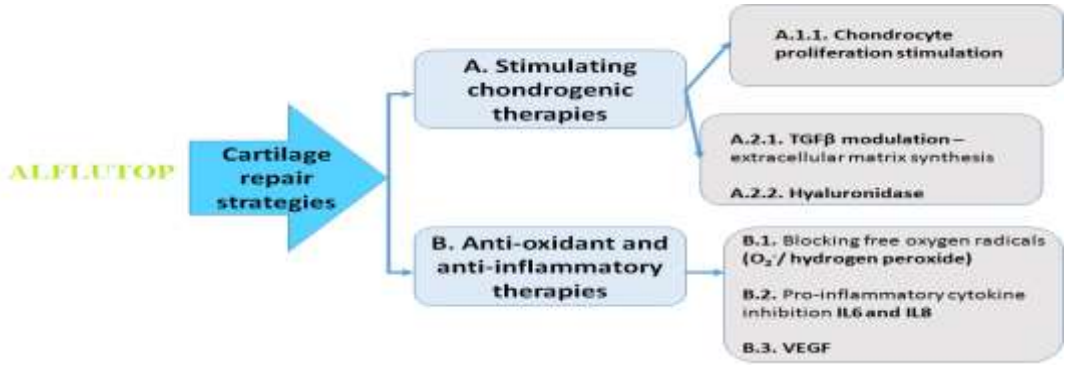


Fig.1: Therapeutic targets for an osteoarthritis treatment, focused in Alflutop® drug development ("in vitro" screening)

Materials and Methods

Cells cultures:

CHON-001 (ATCC® CRL-2846™), human normal chondrocytes from long bone cartilage bring the *advantages of a standardised and reproducible cell model.*

Cultivation: high glucose DMEM media, 10% fetal bovine serum, 0.1mg/ml G-418 antibiotic solution, at 37°C, in 95% humidified air and 5% CO_2 incubator.

Chondrocytes isolated from rabbit cartilage (primary culture) - *advantages of better maintaining the physiological features of the primary source, the cartilage.* Chondrocytes were isolated from cartilage fragments dissected from long bones of 2 years old female rabbits through enzymatic digestion with collagenase II.

Cultivation: high glucose DMEM, 10% fetal bovine serum, supplemented with antibiotic-antimycotic solution, at 37°C, in 95% humidified air and 5% CO_2. They were used within the first two passages.

***In vitro* methods to test the biologic action of Alflutop®:**

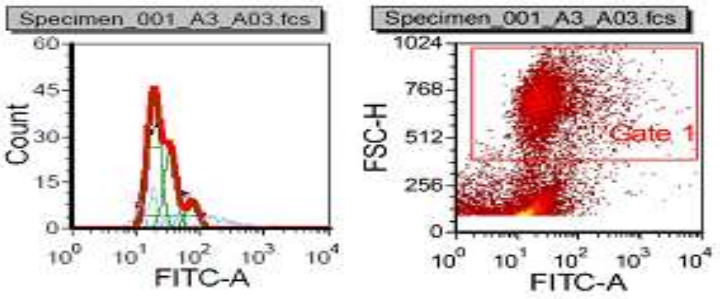
CELL CYCLE AND CELL DIVISION by flow cytometry detection:

Cell cycle: - specific labeling of the DNA with propidium iodide (PI) fluorochrome. (Cycle Test Plus DNA Reagent – BD Pharmingen)

Successive generation proliferation (Cell Trace CFSE Cell Proliferation Kit-Invitrogen): - 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. 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. (Fig. 2)

Soluble, key-proteins from extracellular environment (VEGF, TGF-β, pro-inflammatory cytokines: IL6, IL8) quantified through flow cytometry - BD™ Cytometric Bead Array (CBA) - BD Pharmingen - 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 protein. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture 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 shown by APC-A / PE-A coordinates) The analysis of the results (standard curve for each cytokine and concentration calculation) is done with FCAP Beads Array software.



Generation	Generation #	Log Std Dev	# of Cells	% of Orig Cells
Undivided Cells	0.00	35.73	502.00	10.32
Generation 1	1.00	35.73	1561.00	32.09
Generation 2	2.00	0.00	0.00	0.00
Generation 3	3.00	35.73	2756.00	56.66

Division Index	Proliferation Index	% Divided	# of Original Cells	RMS Error	R-Chi Square
3.84	2.96	69.15	1627.00	2.95	10.95

Fig.2: A flow cytometry model of cell proliferation experimental data acquired (Data analysis with FACS Express software)

HYALURONIDASE INHIBITION to estimate the extracellular matrix maintenance

Measurement of hyaluronidase reaction rate was based on the Reissing method, which

determines the concentration of reducing β-N-acetyl- D-glucosamine ends generated from hyaluronan acid hydrolysis. N-acetyl-D-glucosamine was used as a standard. For the evaluation of N-acetyl-D-glucosamine quantity formed as consequence of hyaluronidase activity it is necessary to do calibration curves which correlete the N-acetyl-D-glucosamine quantity with the probes absorbance at 585 nm (the wavelenght value where the compound formed by N-acetyl-D-glucosamine and 4-dimethylaminobenzaldehyde has its maximum of absorbance). The experimental procedure using DMAB is detailed in our previous paper(4)

OXIDATIVE BALANCE MODULATION - Intracellular CATALASE (CAT) and SUPEROXIDE DISMUTASE (SOD) assessment

To assess the enzyme activity of CAT and SOD, after detach adherent cells with trypsine / EDTA, cells were washed once with cold PBS and suspended in 300 µl of Cell Lysis Solution per 1 - 5 x 10^6 cells. The suspension was transferred to a 1.5 mL tube and centrifuge for 5 minutes at 12,000 - 14,000 x g at 2 - 8° C. The supernatant contain extracted cellular catalase and superoxide dismutase. Superoxide dismutase (SOD) is metallo-enzyme that catalyzes the dismutation of superoxide anion into oxygen and hydrogen peroxide. We have been used the spectrometric procedures described by Sigma Aldrich to determine the SOD activity in samples(5) method is based on the spectrophotometric evaluation (550nm absorbtion spectra) of the inhibition rate of cytochrome C reduction by competing for the superoxide radical with superoxide dismutase. Catalase (CAT) was assayed according to the method of Aebi (6) The estimation was done spectrophotometrically measuring the decrease in absorbance at 240nm. The reaction mixture contained 0.01M phosphate buffer (pH 7.0), 2mM H_2O_2 and cell lysates. The specific activity of catalase is expressed in terms of units/mg protein. A unit is defined as the velocity constant per second.

OXIDATIVE BALANCE MODULATION - Flow cytometry for reactive oxygen species –ROS – quantification – DCFH-DA (for HYDROGEN

Cellular oxidative stress through intracellular activation of superoxide anionand and hydrogen peroxide is quantified by simultaneous measurement of intracellular levels of H_2O_2 and O_2^- - DCFH-DA (dichlorofluoresceine diacetat), and HE (hydroxiethidium) staining and flow cytometry analysis. DCFH-DA is

embedded in lipid hydrophobic region of membrane where hydrolytic enzymes clive the diacetat residues, releasing the membrane permeant configuration which is oxidized in the cytoplasm by the intercellular hydrogen peroxide, producing FITC-A fluorescence (530nm emission). HE permeates the cell membrane and is oxidized by superoxide anion ethidium bromide which tight bond DNA and emits at 620nm (PE-A). Hydrogen peroxide and superoxide anion quantities are proportional with the variation of mean fluorescence channel: FITC–A mean – for Hydrogen peroxide and PE-A mean – for superoxide anion. Flow cytometry diagrams shows cellular subpopulation (green and blue) producing hydrogen peroxide (FITC-A positive) and superoxide anion (PE-A positive)- fig.3

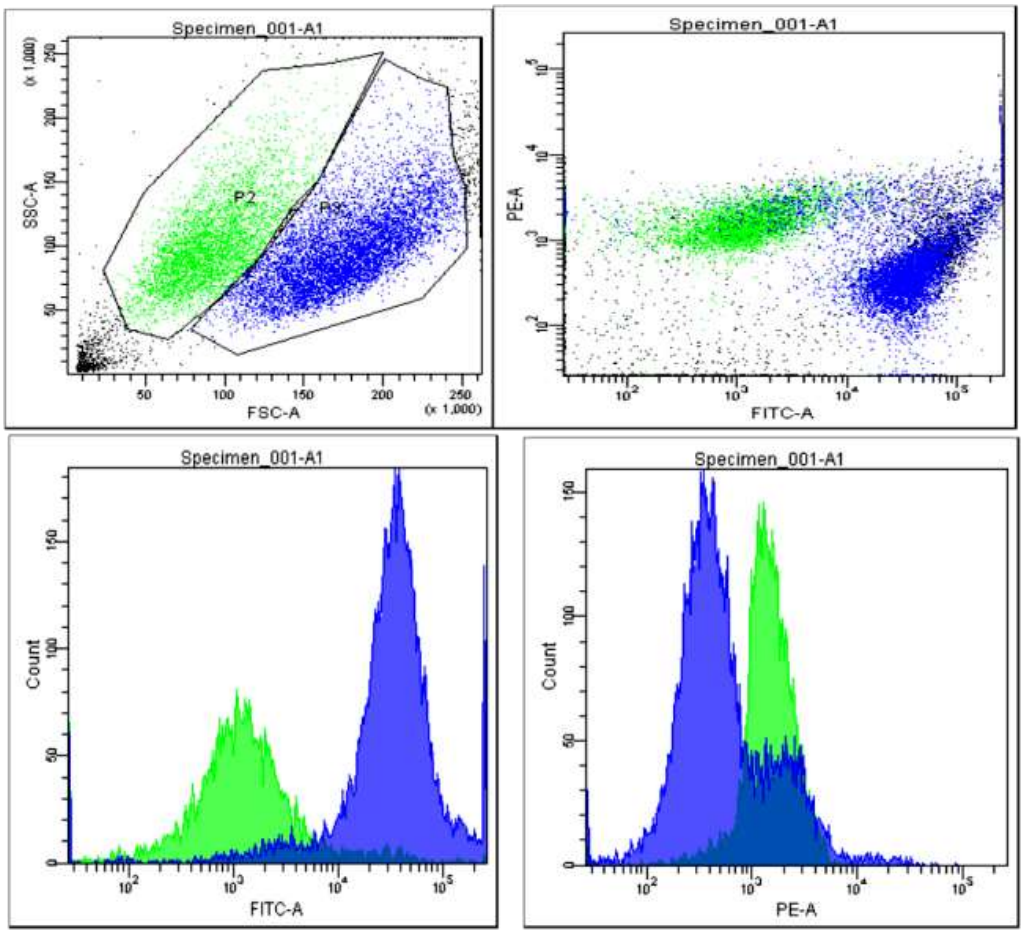


Fig.3: Flow cytometry diagrams showing H_2O_2 and O^{2-} intracellular level

Models of cell stimulation: IL1β and TNFα , dominants cytokines in the inflammatory cascade, but acting with a few differences:

- TNFα - primary inflammatory agent with systemic impact, activates NF-kβ and induce apoptosis
- IL1β triggers production of pro-inflammatory cytokines, stimulates production of stromelysin and collagenase and osteoclast differentiation.

Positive controls:

- Ascobic acid was used as a potent anti-oxidant control, as well as a positive control for condrocytes proliferation
- glycchyrizic acid (Gly) was used as a potent control for hyaluronidase inhibition
- N-acetylcysteine were used as a potent anti-oxidant
- Eicosapentaenoic acid (EPA) is used as a anti-oxidant control
- Dexametasone is a well-established anti-inflammatory agent

Results and Discussion

In vitro models to test the biological action of Alflutop® were choose considering a suitable complete screening of the main pathways of osteo-arthicular pathologies, as described in fig.1.

A.Stimulating chondrogenic therapies (as we presented in fig.1)

A.1.1. Cellular component – proliferative status amplification

The reaction patterns of chondrocytes in osteoarthritis can be summarized in five categories: proliferation and cell death (apoptosis); changes in synthetic activity and degradation; phenotypic modulation of the articular chondrocytes; and formation of osteophytes. (7) Chondrocytes act as the main actor for the maintenance, organization and composition of the cartilage matrix. Additionally, there is a low percentage of chondrocytes (1-5% of the total volume of the cartilage) and they have a slow rate of multiplication, thus stimulating them is an extremely important target for a drug. (8)

Our previous studies shows that Alflutop® significantly increases the % of cells in replicative (S) and mitosis (G2/M) phases of cell cycle. As well as, Alflutop® significantly increases the proliferation index of chondroblasts CHON 001 and primary rabbit chondrocytes (9)

A.2.1. Extracellular matrix maintenance through TGFβ modulation

Transforming growth factor beta (TGF-beta) is an ubiquitous regulator of cellular growth and differentiation. TGF-beta markedly stimulated DNA synthesis in a dose-dependent manner, showing increasing mitogenicity with increasing cellular maturation, with maximal stimulation in the proliferating and early hypertrophic cells, suggesting a potentially important autocrine function for TGF-beta in modulating chondrocyte proliferation and matrix synthesis in endochondral calcification. (10) When TGF- beta bind to chondrocytes cell surface receptor signaling cascades are triggered, among which the TGF- beta - Smad pathway is the most important. TGF- beta also activates protein kinases, including MAPK, PKA and PKC, and modulates gene expression via its delicate interaction with other signaling pathways. Improving the research of mechanisms underlying TGF- beta -mediated signaling pathways and their effects may greatly impact the treatment of many common orthopaedic diseases. (11)

Previous published data (9) showed a significant 7% activation of TGF-β induced by Alflutop® compared with the cellular control. This activation of TGF-β should be considered in respect to the general homeostasis of the cell, haveing in mind that TGF-β regulates the fine balance of protein synthesis/degradation and a high pool of this signal protein could lead to abnormal ossification.

A.2. 2. Extracellular matrix maintenance through Hyaluronidase inhibition

The intracellular matrix is a complex gel containing water, electrolytes, metabolites, vitamins, enzymes, carbohydrates, lipids and proteins. The viscousity of these matrix solution is due to the plenty of macromolecules: acid mucopolysaccharides with long chain strengthen at microscopic level by a three-dimension network of collagen fibres. An important feature of the intracellular substances is its very high viscosity and cohesion. This feature depends on the chemical integrity of the large molecules. High molecular size hyaluronan, hyaluronic acid (HA) occurs in normal tissues and has anti-angiogenic, anti-inflammatory, and anti-immunogenic properties. Fragmented HA, in contrast, is highly angiogenic, very inflammatory and immuno-stimulatory, a reflection of tissues under stress (12). A major function of high molecular mass HA in the synovial joint is to maintain proper viscoelasticity of synovial fluid. This enables the HA to serve as a shock absor-ber and also to ensure smooth joint movements The viscosity can be reduced and structural integrity destroyed by the depolymerizing action of the enzyme called hyaluronidase. In the degenerative

processes, the hyaluronic acid are intensely depolymerized by hyaluronidase, the matrix-ground substance of the connective and cartilaginous tissues being destroyed. Due to the reduction of molecules dimensions, the viscosity of hyaluronic acid is reduced. (13, 16). Since the development of many diseases can be linked to activity of this enzyme, currently many pharmaceutical research focuses on the study of specific inhibitors and potential hyaluronidase.(15) The enzymatic activity of hyaluronidase was determined in the presence of Alflutop and glycyrrhizic acid and results are presented in Fig 4.

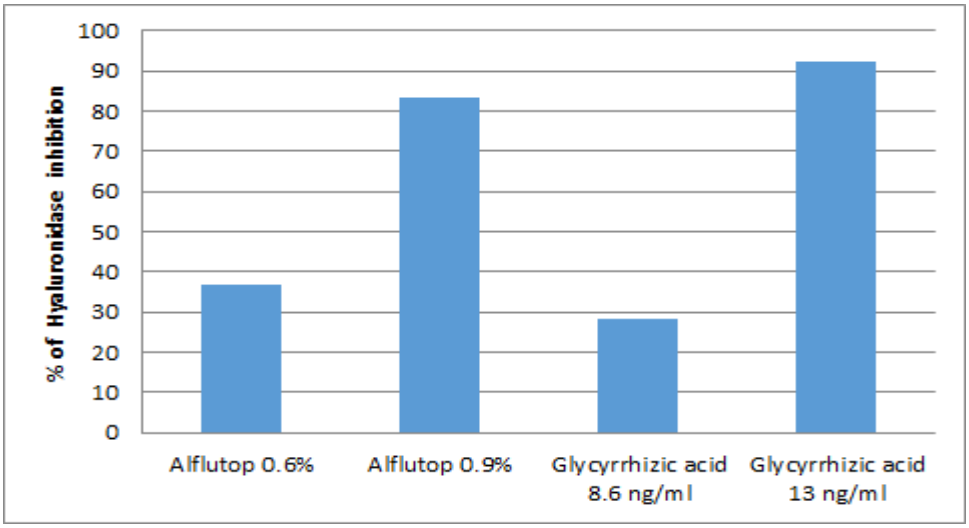


Fig. 4. Hyaluronidase enzymatic activity in the presence of Alflutop and glycyrrhizic acid

Alflutop® shows an inhibition of the hyaluronidase activity in a dose-dependent manner, similar with the positive control, glycyrrhizic acid.

B. Anti-oxidant and anti-inflammatory effects (as we presented in fig.1)

B.1. Oxidative balance modulation

Reactive oxygen species (ROS) can contribute to the onset and progression of degenerative arthritic pathologies by inducing indispensable chondrocyte death and matrix degradation. However, ROS are also key components of many normal physiological processes, and at moderate levels, they act as indispensable second messengers. (16) As a first step in antioxidant/antiradical screening the cellular catalase (CAT) and superoxide – dismutase (SOD) activity was measure,

correlated with further intracellular hydrogen-peroxide and superoxide anion monitoring through flow cytometry.

B.1.1. Catalase and Superoxid dismutase assay from rabbit primary chondrocytes culture

After 48h treatment of rabbit primary chondrocytes in the presence or absence of IL1β stimulation, cells were lysed and enzymatic activity was assessed from the lysate by 1) indirectly measuring quercetin auto-oxidation linearly correlated with SOD activity and with 2) hydrogen peroxide degradation as a direct measure for catalase activity. Results are presented in the graphics below (Fig.5)

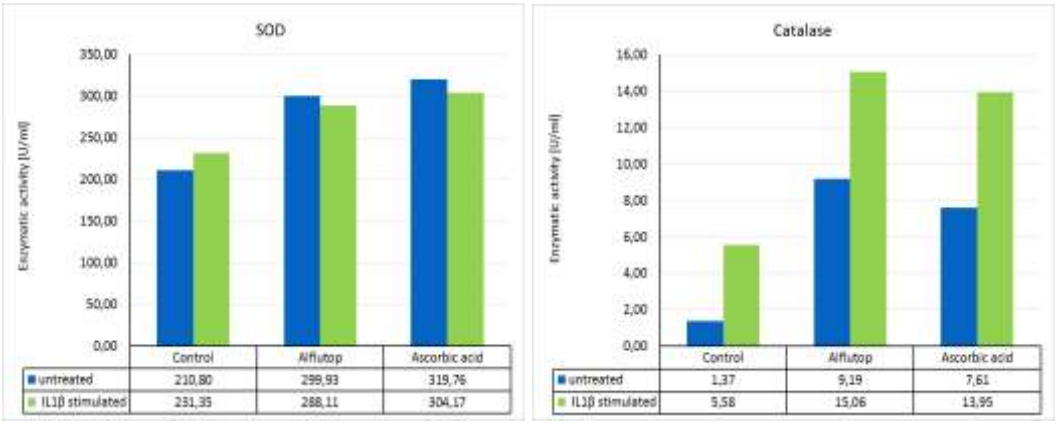


Fig. 5: Intracellular Superoxide dismutase (SOD) and catalase activity modulation by Alflutop® and ascorbic acid - positive control

Alflutop® treatment of primary rabbit chondrocytes stimulates SOD and catalase activity, in both unstimulated conditions and pro-inflammatory degradative injuries mimicked by IL1β stimulation. The rise of the catalytic activity of these antioxidant enzymes induced by Alflutop® leads to a protective effect against oxidative stress, transforming superoxide anion and oxygen peroxide, aggresive reactive oxygen species.

B.1.2. Antioxidant activity evaluation through intracellular reactive oxygen species monitoring from rabbit primary chondrocytes culture

IL1β stimulation generates a cellular oxidative stress expressed at ROS level through the mean rise of the hydrogen peroxide fluorescence (143% compared with the unstimulated control) and of superoxide anion with only 13%. The

massive release of hydrogen peroxide is therefore a characteristic of particular type of stimulation haveing as final result generalised injuries of the articular tissue. In these special conditions, Alflutop® product reduces the hydrogen peroxide with 33% compared with the stimulated control, similar with the positive control, 30% for ω3 fatty acid (eicosapentaenoic acid – EPA 10μM). The intracellular superoxide anion is reduced with 21% by Alflutop® and with 20% by EPA 10 μM. (Figure 6) IL1β stimulation generates a cellular oxidative stress expressed at ROS level through the mean rise of the hydrogen peroxide fluorescence (143% compared with the unstimulated control) and of superoxide anion with only 13%. The massive release of hydrogen peroxide is therefore a characteristic of particular type of stimulation haveing as final result generalised injuries of the articular tissue. In these special conditions, Alflutop® product reduces the hydrogen peroxide with 33% compared with the stimulated control, similar with the positive control, 30% for ω3 fatty acid (eicosapentaenoic acid – EPA 10μM). The intracellular superoxide anion is reduced with 21% by Alflutop® and with 20% by EPA 10 μM. (Fig. 6)

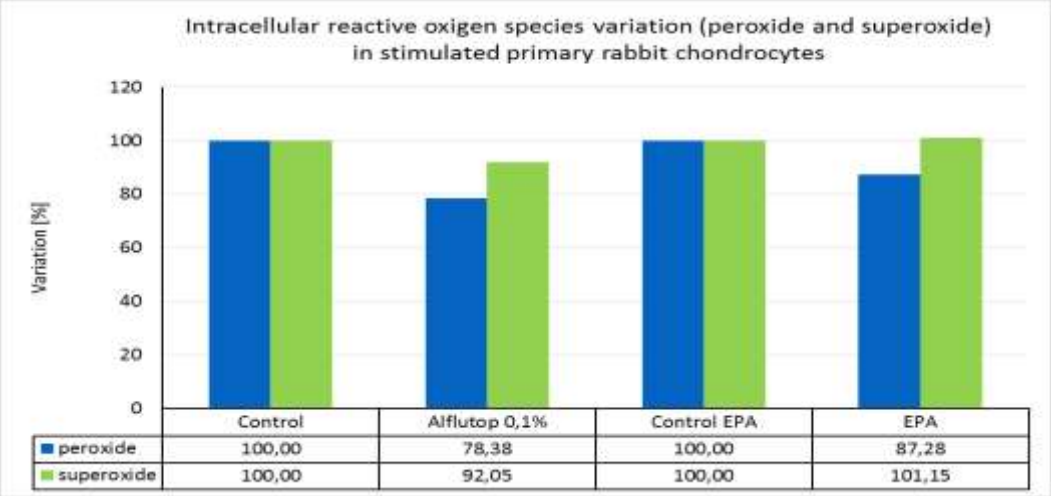


Figure 6: ROS evolution (hydrogen peroxide and superoxide anion) in rabbit primary chondrocytes stimulated with IL1β and treated with Alflutop® and EPA

B.1.3. Antioxidant activity evaluation through intracellular reactive oxygen species detection from human normal chondrocytes –CHON-OO1 standardised cell line

The human chondrocytes from standardised cell line CHON-001 were stimulated 24h. with TNFα 15ng/ml and PMA 0.1μM, the induced oxidative stress being appreciated through the rise of fluorescence with 38% for the hydrogen peroxide and 61% for the superoxid anion. The action of Alflutop® was compared with N- Acetil-Cysteine 5mM and ascorbic acid 45μM, two well known antioxidants. The results are presented in the figure 6, as % of variation of the specific fluorescence channel (H_2O_2 - FITC-A; (O_2^-) – PE-A) compared with the stimulated control.

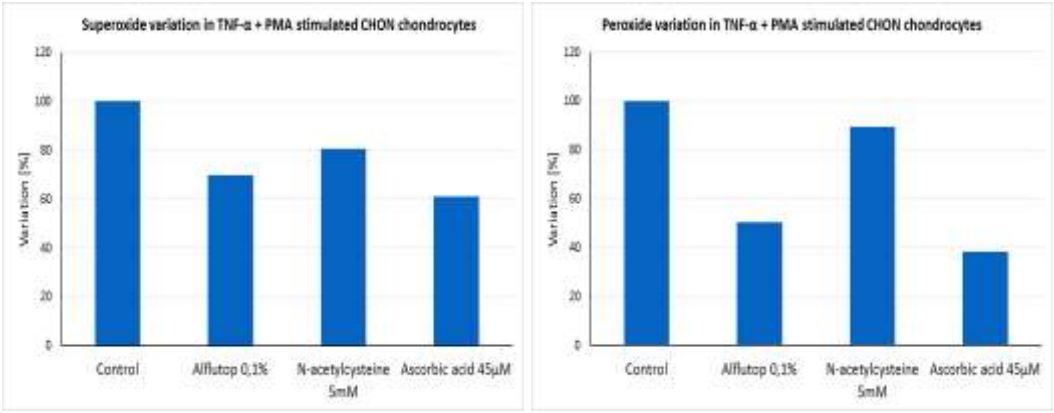


Figure 7: ROS evolution (hydrogen peroxide and superoxide anion) in human chondrocytes –CHON-001, stimulated with TNFα+PMA and treated with Alflutop®, Ascorbic acid and N-Acetil-Cysteine

Alflutop® induces the inhibition of both oxygen reactive species, an important step in ROS mediated perturbancies. The intracellular hydrogen peroxide decrease in the presence of Alflutop® with 50% compared with the stimulated control, the other positive controls effects being of 11% for N-Acetil-Cysteine, and 62% respectively for Ascorbic acid. The action on intracellular superoxide anion is lower, Alflutop® reduce it with 31%, N-Acetil-Cysteine with 20%, and Ascorbic acid with 39% (Fig. 7)

Considerations regarding the antioxidant activity of Alflutop®:

In osteoarthrithic pathology the anion superoxide and hydrogen peroxide production is dramatically stimulated, inducing a marked expression of reactive oxygen species (ROS), in synergy with the rise of pro-inflammatory cytokines and

proteasic activity. SOD improves the pathological events of osteoarthritis. The catalase association amplify the protective effect, both through the quicker elimination of hydrogen peroxide and blocking the SOD degradation by it. The rise of SOD in the presence of Alflutop® induces the oxidative stress inhibition, with superoxide anion implication, considering its role in the chain of oxidative stress aggressive compounds triggering. The rise of SOD prove the inhibition of superoxide anion accumulation, and the experimental data sustain through complementary methods the product's implication in this antioxidant mechanisms. It could be noted the implication of Alflutop® especially in the enzimatic change system of intracellular hydrogen peroxide, neutralizing the production of H_2O_2. This is one of the most aggressive species of oxygen, that inhibits SOD and produces superoxide anion accumulation, initiates pathways that converge to lipids peroxidation and leads to cells injuries and death, block gluthation-peroxidase stopping this way the synthesis of gluthation as intrinsic antioxidant. Decreasing the intracellular H_2O_2, Alflutop® contributes to prevent the escalation of all these processes. Being an activator of SOD and Catalase, Alflutop® decrease the inflammation mediators release in their propagation flow.

B.2. Modulation of pro-inflammatory cytokines

Osteoarthritic disease in cartilage is driven by both mechanical and inflammatory signals. Specific signaling pathways linked to altered physiological states that drive stressed articular chondrocytes to proliferate or differentiate, are related to inappropriate activation of NF-κB signaling. Inflammation and stress-induced responses orchestrated by canonical NF-κB signaling may impact both directly and indirectly on OA disease onset and/or progression. (16). Different molecular aspects involved in the pathogenesis of osteoarthritis are controlled by inflammatory mediators, their cascade signaling further contributing to the highly catabolic state, chondrocyte apoptosis, and the resultant progressive degeneration of articular cartilage (17). Cytokines produced by inflammatory cells induces synovial inflammation and joint destruction in rheumatoid arthritis.(18,19) Pro-inflammatory cytokines are able to induce apoptosis, whereas IL-4 as an anti-inflammatory cytokine can inhibit the effect of IL-1α and TNF-α on NO production and proliferation of chondrocytes. (20). The catabolism of osteoarthritic cartilage involves the action of proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α). Interleukin-1 down-regulates extracellular matrix (ECM) synthesis and up-regulates metalloprotease synthesis through production of nitric oxide (NO) in chondrocytes. Human

synoviocytes spontaneously release IL-6 in a manner that is increased by IL-1 and TNF-α. Interleukin 6 levels have been correlated with pain in the temporomandibular joint, IL-6 synergizes with IL-1 to promote collagen degradation in cartilage. (21)

Alflutop reduces especially the IL6 release in human chondrocytes stimulated with IL1β, that will prevent or slow the inflammatory cascade progression. When human chondrocytes are stimulated with TNFα, Alflutop® inhibits both IL6 and IL8 interleukins release, the main modulators of inflammatory acute phase progression, proving significant anti-inflammatory effect on these classical pathways. "In vitro" modulation of these important mediators of inflammation sustains Alflutop® contribution in the rehabilitation of the cartilage physiology through anti-cytokines action. (22)

B.3. Regulation of VEGF

Angiogenesis is another event of synovial tissue inflammation. It starts early in the firsts stages of disease and could be asymptomatic, IL8, VEGF or FGFβ acting as pro-angiogenic markers. An increasing number of observations suggest that VEGF, for a long time considered to be endothelium specific on the basis of its receptor localization, might instead have effects also on non endothelial cell types, holding active signal transduction.

There is consistent evidence for VEGF being involved in cartilage pathological neovascularization, with factor increase in synovial fluids deriving from rheumatoid arthritis. The presence of VEGF receptor and functional signal transduction in hypertrophic chondrocytes was considered in the light of a possible additional differentiating or morphogen effect of VEGF in endochondral bone formation. Expression of VEGF activates the chondrocytes autocrinally for producing MMP-1, -3, and -13. TIMP-1 and -2, the inhibitors of MMPs, are reduced by mechanical overload. The increase of MMP and the decrease of TIMP contribute to the destruction of the articular cartilage. (24)

ALFLUTOP® inhibits VEGF, an important angiogenesis factor with a recent discovered impact as a biochemical mediator in destructive processes of osteoarthritis (results previously published). (23)

In adidtion with all these effects of Alflutop®, we could also mention: the inhibition of the expression of proteases responsible for the degradation cascade of the core-protein of aggrecan (mRNA expression of ADAMTS-4); the increasing aggrecan and hyaluronan synthesis (activation of hyaluronan synthase mRNA expression - HAS-1); and the activation of a pivotal transcriptional

regulator essential for articular cartilage formation and hypertrophic maturation, SOX 9 (24), in order to prevent the hypertrophy and extracellular matrix decline(24).

Conclusions

Overview of molecular and cellular effects from the *in vitro* studies to determine mechanisms of action for Alflutop®:

This product, through its complex of complementary biological components, shows an integrated algorithm of *in vitro* action specific for osteoarticular diseases. The product influences the mechanisms that restore and configure the architectural matrix at the cartilage level, allowing the recovery of the functional pathways.

- Alflutop® reduces/stops cartilage destruction, inhibiting the expression of proteases responsible for the degradation cascade of the core-protein of aggrecan, improving cellular response in catabolic processes by increasing aggrecan and hyaluronan synthesis, and in the same time inhibiting the action of hyaluronidase.
- Alflutop® helps recover the structural integrity of cartilage, stimulating chondrocyte proliferation.
- Alflutop® restores cellular turn-over and maintains the protein-core, through stimulation of DNA synthesis in chondrocytes and the moderate stimulation of TGFβ, a molecule responsible for the depletion / restoring dynamics of the balance between the synthesis and degradation of the matrix.
- Alflutop® works in numerous ways to reduce inflammation processes and also diminishes oxidative stress having an antioxidant effect demonstrated by scavenging free radicals and inducing the activation of the enzymes implicated in oxidative cascades.

All these effects allow Alflutop® inhibit the physiologic degeneration modulated by intrinsic and external factors, individual variability. The in vitro preclinical action of ALFLUTOP® is a synergism confirmed through convergent cellular mechanisms.

References:

1. Musumeci G., Aiello F.C., Szychlinska M.A., Di Rosa M., Castrogiovanni P., Mobasheri A., (2015) Osteoarthritis in the XXIst Century: Risk Factors and Behaviours that Influence Disease Onset and Progression, *Int. J. Mol. Sci*., **16**, pp 6093-6112
2. Felson D.T., Lawrence R.C., Dieppe P.A., Hirsch R., Helmick C.G.., Jordan J.M., Kington R.S., Lane N.E., Nevitt M.C., Zhang Y., Sowers M., McAlindon T., Spector T.D., Poole A.R., Yanovski S.Z., Ateshian G., Sharma L., Buckwalter J.A., Brandt K.D., Fries J.F., (2000) Osteoarthritis: new insights. Part 1: the disease and its risk factors, *Ann Intern Med*.; **133**, pp. 635-46
3. Lane S.R., Trindade M.C., Ikenoue T., Mohtai M., Das P., Carter D.R., Goodman S.B., Schurman D.J., (2000), Effects of shear stress on articular chondrocyte metabolism, *Biorheology,* **37**(1-2), pp. 95-107
4. Crăciun M.L., Văcaru A.M., Ene M.D., Văcaru A.M., Olariu L., Roşoiu N., (2016)pH influence on different bovine testicular hyaluronidase determination assays, *Academy of Romanian Scientists, Annals Series on Biological Sciences,* 5(1)
5. http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-superoxide-dismutase.html
6. Aebi H.E., (1978), Catalase in Bergmeyer, HU, Methods in enzymatic analysis., *Weinheim: Verlag Chemie*, pp. 273-286
7. Sandell L.J., Aigner T., (2001), Articular cartilage and changes in Arthritis: Cell biology of osteoarthritis, *Arthritis Research & Therapy*, **3**, pp. 107
8. Tallheden T., Bengtsson C., Brantsing C., Sjögren-Jansson E., Carlsson L., Peterson L., Brittberg M, Lindahl A., (2005), Proliferation and differentiation potential of chondrocytes from osteoarthritic patients, *Arthritis Research & Therapy*, **7**, R560
9. Olariu L., Pyatigorskaya N., Dumitriu B., Pavlov A., Vacaru A. M., Vacaru A.,(2016), In vitro chondro-restitutive capacity of Alflutop® proved on chondrocytes cultures, *Romanian Biotechnological Letters*, **22**(6), pp 12047 – 12053

10. Rosier R.N., O'Keefe R.J., Crabb I.D., Puzas J.E., (1989), Transforming growth factor beta: an autocrine regulator of chondrocytes, *Connect Tissue Res.*;**20**(1-4), pp. 295-301
11. Li T.F., O'Keefe R.J., Chen D., (2005), TGF-beta signaling in chondrocytes, *Front Biosci.,* **10**, pp. 681-8
12. Stern R., Asari A.A., Sugahara K.N., (2006), Hyaluronan fragments: an information-rich system., *Eur J Cell Biol*, **85**(8), pp. 699–715
13. Juranek I., Stern R., Šoltes L., (2014), Hyaluronan peroxidation is required for normal synovial function: An hypothesis, *Medical Hypotheses* 82, pp. 662–666
14. Rosoiu N, Lascarache Gh., A natural extraction drug used for the treatment of degenerative diseases, (1994), *Rev.Roum.Biochim.*, Ed. Academiei Române, 31, 1, pp. 57-63
15. Rosoiu N., Nita R., Ene M.D., Constantinovici M., Olariu L., (2010), Certain bioactive effects of complexes rich in glycosaminoglycans obtained from small sea fish, *Roumanian Biotechnological Letters,* **15** (5), pp. 5566-5575
16. Li D, Wang W, (2012), Reactive Oxygen Species: The 2-Edged Sword of Osteoarthritis, *Am J Med Sci*, 344, Issue 6, 486–490
17. Goldring M. B., Otero M., Plumb D. A., Dragomir C., Favero M., Hachem K. El, Hashimoto Ko, Roach H. I., Olivotto E., Borzì R. M., Marcu K. B., (2011), Roles of inflammatory and anabolic cytokines in cartilage metabolism, *European Cells and Materials*, **21**, pg.202-220
18. Bellucci F., Meini S., Cucchi P., Catalani C., Nizzardo A., Riva A., Guidelli G.M., Ferrata P., Fioravantik A., Maggi C.A., (2013), Synovial fluid levels of bradykinin correlate with biochemical markers for cartilage degradation and inflammation in knee osteoarthritis; *Osteoarthritis and Cartilage*, **21**, pp. 1774-1780
19. Schuerwegh A. J., Dombrecht E. J., Stevens W. J., Van Offel J. F., Bridts C. H., De Clerck L. S., (2003), Influence of pro-inflammatory (IL-1α, IL-6, TNF-α, IFN-γ) and anti-inflammatory (IL-4) cytokines on chondrocyte function, *OsteoArthritis and Cartilage, Biomechanics and Cartilage Inflammation***, 11**, 681–687)
20. Guilak F., Fermor B., Keefe F.J., Kraus V. B., Olson S. A., Pisetsky D.S., Setton L. A., Weinberg J. B., (2004), The Role of Biomechanics and Inflammation in Cartilage Injury and Repair, *Clinical Orthopaedics and Related Research Number,* **423**, *pp. 17–26*

21. Olariu L., Dumitriu B., Buse E., Rosoiu N., (2015), The „in vitro" effect of Alflutop product on some extracellular signaling factors involved in the osteoarthicular pathology inflammation, *Analele AOSR*, **4** (2), pp. 7-18
22. Onyekwelu I,. Goldring M.B., Hidaka C., (2009), Chondrogenesis, joint formation, and articular cartilage regeneration., *J Cell Biochem.*, **107**(3), pp. 383-392
23. Lefebvre V., Smits P., (2005), Transcriptional control of chondrocyte fate and differentiation., *Birth Defects Res C Embryo Today*., **75**(3), pp. 200-212
24. Olariu L., Vacaru A., Pyatigorskaya N., Vacaru A. M., Pavlov A., Dumitriu B., Ene D. M., Craciun L. M., (2016), Original romanian product involved in cellular chondro - modulatory mechanisms, Euroinvent medalia de aur, Catalog EUROINVENT pg. 437.