Academy of Romanian Scientists *Volume 4, No. 2, 2015, pp. 7-17*
Annals Series on Biological Sciences ISSN 2285 – 4177 Online Edition

ORIGINAL PAPER

THE "IN VITRO" EFFECT OF ALFLUTOP ®PRODUCT ON SOME EXTRACELLULAR SIGNALING FACTORS INVOLVED IN THE OSTEOARTHICULAR PATHOLOGY INFLAMMATION

Received for publication, September, 15, 2015.
Accepted, December, 01, 2015

Laura OLARIU[1,2], Brindusa DUMITRIU[1], Emilia BUSE[1], Natalia ROSOIU[1,2],

[1] S.C. Biotehnos S.A., 3-5 Gorunului Street, 075100-Otopeni, Ilfov, Romania, Phone: +40317102402, Fax: +40317102400, e-mail: lolariu@biotehnos.com
[2] Academy of Romanian Scientist, 54 Splaiul Independentei 050094, Bucharest, Romania,e-mail:natalia_rosoiu@yahoo.com.,phone:0722737516;+40241605012

Abstract. The biochemical diversity and the complex relationship between the mechanisms involved in osteoarthritis suggest a targeted therapy on several metabolic pathways, in order to obtain a superior long term efficacy. After the discovery of the cytokines as humoral factors that modulates the connective tissue, it has been opened new therapeutic insights through the stopping of inflammation based on synthetic / natural agents. The aim of our studies is to highlight the effect of Alflutop® product on soluble factors involved in the progression of inflammation (IL6, IL8 and VEGF), correlating the gene response with the phenotypic expression. The inflammation was induced in a human chondrocyte cell line (CHON-001) by three types of pro-inflammatory stimuli: TNFα, IL1β, PMA (phorbol myristat acetat) and the extracellular release of IL6, IL8 cytokines and VEGF pro-angiogenic factor were analyzed. (Flow-cytometry and qRT-PCR).
The results proved the"in vitro" anti-inflammatory effect induced by Alflutop® on chondrocytes through mechanisms involving the cytokine signaling pathway, at genotypic and phenotypic level..

Keywords: Alflutop®, IL6, IL8, VEGF, anti-inflammatory effects.

Introduction

Osteoarthritis (OA) is a joint disease caused by mechanical, biochemical and genetic factors, involving the degeneration of articular cartilage, limited intraarticular inflammation and changes in the subchondral bone. Current pharmacological interventions that address chronic pain are insufficient and no proven disease-modifying therapy is available. Chondrocytes, the unique cellular component of adult articular cartilage, respond to structural changes in the surrounding cartilage matrix, profiling a valuable target for therapeutic intervention. The capacity of the adult articular chondrocyte to regenerate the normal cartilage matrix architecture is limited, however, and the damage becomes irreversible unless the destructive process is interrupted [1]. Identification of methods for early therapy or preserving normal homeostasis is of key importance, blocking or reversing structural damage being a promising therapeutic intervention [2].

Articular degradation is accompagned by cellular and mollecular events that lead to the progression of these disfunction, through direct and indirect mechanisms. Under normal conditions, chondrocytes maintain a dynamic equilibrium between synthesis and degradation of ECM components. In osteoarthritic states, however, there is a disruption of matrix equilibrium leading to progressive loss of cartilage tissue, clonal expansion of cells in the depleted regions, induction of oxidative states in a stressful cellular environment, and eventually, apoptosis of chondrocytes. Chondrocyte metabolism is unbalanced due to excessive production of inflammatory cytokines and matrix-degrading enzymes, in conjunction with a downregulation of anabolic factors, eventually leading to destruction of the extracellular matrix and subsequent cartilage degradation. Oxidative stress elicited by reactive oxygen species (ROS) further disturbs cartilage homeostasis and promotes catabolism via induction of cell death, breakdown of matrix components, upregulation of latent matrix-degrading enzyme production, inhibition of ECM synthesis, and oxidation of intracellular and extracellular molecules [2].

Considering the pro-inflammatory level of osteoarthritis development, there are expressed the following type of extracellular signaling factors: pro-inflammatory cytokines, matrix metalloproteinases, growth factors, neuronal mediators [3].

The cartilage integrity is maintained by a balance between anabolic and catabolic processes directed by cytokines.

The catabolism of osteoarthritic cartilage is thought to involve the action of pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α) [4]. Chondrocytes also secrete a variety of immunocompetent cytokines including IL-1 beta, IL-6, IL-8 that can interact to regulate the cellular

metabolism [5]. Cytokines such as IL-1 and TNF-alpha (tumor necrosis factor alpha) produced by activated synoviocytes, mononuclear cells or by articular cartilage itself significantly up-regulate metalloproteinases (MMP) gene expression [6].

TNFα together with IL6 interact to produce the proteoglycans degradation from the extracellular matrix, mediated by aggrecanase [7]. T lymphocytes are the main cellular activators responsibles for the inflammatory processes triggering, especially through IL2, IL3, IL4, TNFβ, IFNγ. IL4 is an anti-inflamatory cytokine, secreted by T lymphocytes, with a deep impact in internal mechanisms regulation of inflammatory state progression, preventing, alone or with IL10, the cartilage injuries [8].

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 [9].

Expression of many genes encoding pro-inflammatory mediators and matrix degrading enzymes are regulated by the transcription factor, nuclear factor-kappa B (NF-κB).Suppression of the NF-κB activating cascades could effectively down-regulate the expression of pro-inflammatory mediators [10].

Disrupted homeostasis and phenotypic modulation in chondrocytes during initiation and progression of osteoarthritis focused current research to identify new pharmacologic agents that can inhibit pro-inflammatory mediator production with less adverse side-effects [11]. Several non-pharmacologic products targeting the NF-κB transduction pathway and promoting joint health have been reported. Among these products is avocado/soybean unsaponifiables which is used in Europe for osteoarthritis management. This product suppresses gene expression of IL-1β, TNF-α, COX-2, and inducible nitric oxide synthase (iNOS), as well as PGE2 and nitric oxide production in bovine and human joint tissue cells [12].

Among these concerns are the use of compounds as glucosamine, chondroitin sulfate, pentosan polysulfate, plant or animal extracts that alone or in combination could interact with osteoarthritis mediators [13].

The aim of our studies was to reveal the molecular effects of Alflutop® product on anti-inflammatory and anti-angiogenic pathways involving cytokines

signaling. Alflutop® is a widely used product in the treatment of degenerative osteoarthicular diseases, inflammatory rheumatism or post-traumatic bones inflammation.

In order to create an accurate „in vitro" model, we choosed the chondrocyte standardised cell line, CHON-001, derived from long bone, presenting specific properties of articular subchondral tissue and assuring a very good inter-experimental reproductibility. We control the phenotype and the genotype expression through modern techniques as flow cytometry multiplexed beads based assay and qRT-PCR for key molecules genes (*IL6, IL8, IL1β*).

We design the following experimental series, in different stimulation conditions:

- **Unstimulated cells**
- **TNFα stimulation** – systemic stimulus, one of the two major cytokines players in the physiopathology of OA.
- **PMA stimulation** – Stimulation with Phorbol Myrestate Acetate (PMA) is described to activate protein kinase C and upregulate NADPH oxidase, which lead to superoxide anion production, one of the major reactive oxygen species [14]. PMA reproduce "in vitro" the start of angiogenesis in arthicular inflammatory pathology.
- **IL1β stimulation** - the second major cytokines players in the physiopathology of OA, promoting the cartilage degradation.

Materials and Methods

Cell cultures:

CHON-001 - (ATCC® CRL-2846™) – human normal condrocytes from long bone cartilage. Cells were cultured in high glucose DMEM (ATCC - Catalog No. 30-2002) with 10% fetal bovine serum, 0.1mg/ml G-418 antibiotic solution, under standard culture conditions (37°C, 95% humidified air and 5% CO_2), harvested 48h before treatment, 48 h with tested substances.
Cells are harvested through tripsinisation (Tripsin/EDTA 0.1g% - Sigma).

Chemicals and reagents:

- TaqMan® Array Human IL6 Pathway; TaqMan® Array Human IL8; TaqMan® Array Human IL1 α (Thermo-Fischer Scientific)
- BD Cytometric Bead Array (CBA)- Human Inflammatory Cytokines kit (BD Pharmingen) for the simultaneous detection of soluble factors: *IL6 flex set, IL8flex set,VEGF flex set, Protein Master Buffer Kit.*

Equipments:

- Flow cytometer FACS CANTO II (Becton – Dickinson) with DIVA 6.1 and FCS Express softwares.
- Step One Plus - Real Time PCR System (Applied Biosystems)

Methods:

a) **Pro-inflammatory cytokines staining with *BD Cytometric Bead Array (CBA)* technique -** BD™ Cytometric Bead Array (CBA) is a flow cytometry application that allows users to quantify multiple proteins simultaneously. Each capture bead in the kit has been conjugated with a specific antibody. The detection reagent provided in the kit is a mixture of phycoerythrin (PE)–conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte (in our particular case **IL6, IL8 and VEGF**). The analysis of the results (standard curve for each cytokine and concentration calculation) is done with FCAP Beads Array software [15].
b) **RT-PCR System (Real Time PCR)** was applied for the functional analyses of IL6, IL8 and IL1 β genes, using specific TaqMan reagents. This type of reagent use a fluorogenic probe that is accumulated during the amplification cycles, the fluorescence intensity being proportional with the quantity of amplicons produced during the PCR reaction. The higher is the number of copy of DNA sequence, the emitted fluorescence is detected earlier. For the quantitative analyze of the target gene expression (in this particular case **IL6, IL8 and IL1β**) the comparative method Ct (ΔΔCt) is used. It is compared the amplification of the target gene with a house keeping gene (the endogenous control, a gene with an unchanged expression, with a normalization role). The **relative quantification (RQ)** express the quantity of the target gene normalized to that of the house keeping gene for each analyzed sample. The data output is expressed as a fold-difference of expression levels compared with the untreated sample [16].

Results and Discussion

CHON-001 cells were incubated 24h. prior to the treatment with Alflutop® and Dexamethasone, respectively. Dexamethasone 200ng/ml was used as positive control, being an effective anti-inflammatory agent [17]. Considering the stimulation conditions and the inflammatory pathway involved in every particular case, we investigated the effect of Alflutop® on a panel of most important cytokines promoting the osteoarthritic injuries: IL6, IL8 and VEGF. IL6 is an acute phase protein released in osteoarthritis that generate a signaling cascade having as final result the destruction of joint tissue. IL8 is a chemokine that establish the chemotactic flow toward the articular surface and mediate the migration and attachment of neutrophils and lymphocytes to this tissue. VEGF is

an autocrine stimulator of chondrocytes that mediates mainly destructive processes in osteoarthritis.

IL1β and TNFα are the dominant cytokines in the inflammatory cascade, both promoting cartilage degradation by enhancing MMP synthesis and chondrocyte apoptosis, but acting with a few differences:

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

The appropriate time and doses of stimuli were previously tested, the final configuration being as following:

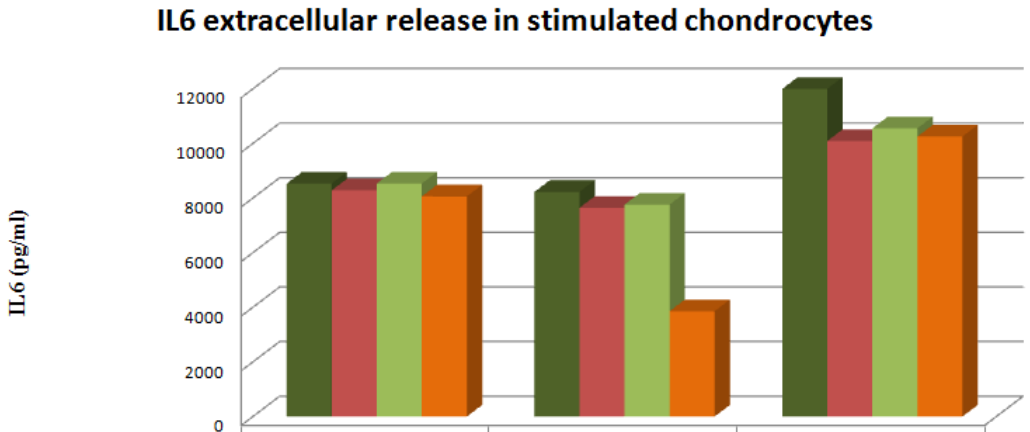
No. of experi-mental version	**Stimulus**	**Time of stimulation**	**Dose of stimuli**	**Response on CHON-001 cells**
1.	IL1β	24h.	10ng/ml	Stimulates IL6 release; IL8 level decrease in extracellular fluids; VEGF level remain unchanged
2.	IL1β	72h.	10ng/ml	Stimulates VEGF release; IL6and IL8 level decrease in extracellular fluids
3.	TNFα	20h	20ng/ml	Stimulates IL6 and IL8 release; VEGF level remain unchanged
4.	PMA	24h	1μM	Stimulates IL6, IL8 and VEGF release

IL1β induce a reversible stimulation on CHON, especially on IL6. The higher extracellular level of IL6 could be noticed after 24h. of stimulation, at 72h. the intrinsic cellular mechanisms counteract IL1β action and even decrease IL6 and IL8 release. VEGF could be measured acurately only after 72h. IL1β stimulation.

Considering the optimum combination of stimulation parameters, the experiments were conducted and the Alflutop® effects were quantified. Results are presented in the figures 1-3.

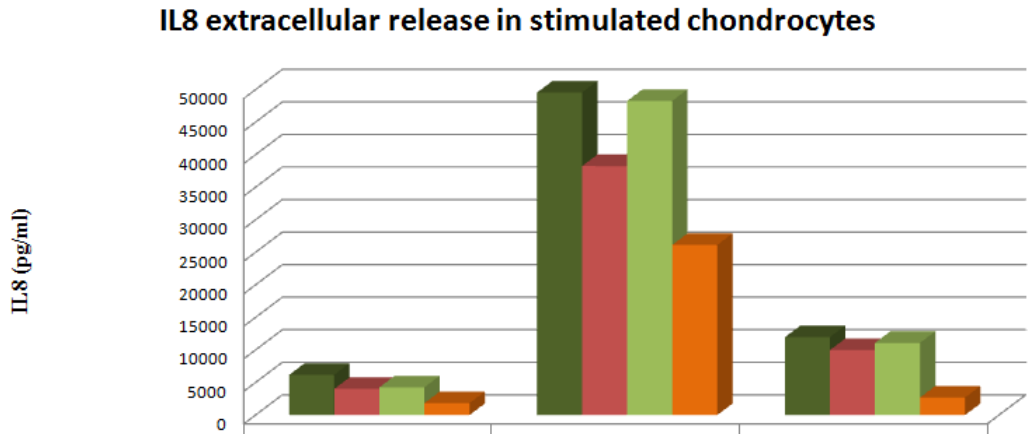
IL6 extracellular release is inhibited by Alflutop® product, especially on PMA and TNFα pathways, that will prevent or slow the inflammatory cascade progression.

IL8 extracellular release is stronger when the chondrocytes were stimulated with PMA, connecting two cellular events: reactive oxygen species activation and neutrophils recruitmant at the inflammatory site. Alflutop® counteracts these cellular events, inhibiting IL8 mediated inflammation.



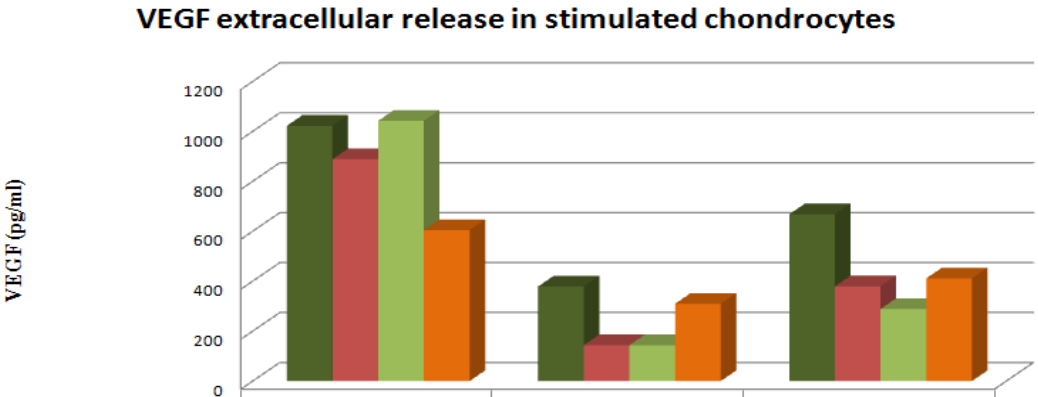
	IL1beta 24h.	PMA 1 uM 24h	20h TNF alfa
Control	8528,05	8226,65	11999,94
Alflutop 0,2%	8282,66	7645,15	10090,77
Alflutop 0,1%	8531,97	7761,8	10553,35
Dexamethasone 200ng/ml	8061,19	3858,02	10253,42

Fig. 1: *Alflutop® effects on IL6 extracellular release in pro-inflammatory stimulated chondrocytes (CHON-001 cell line).*



	IL1beta 24h.	PMA 1 uM 24h	20h TNF alfa
Control	6186,15	49557,12	11958,62
Alflutop 0,2%	4026,2	38259,04	9976,59
Alflutop 0,1%	4261,25	48270,44	11087,22
Dexamethasone 200ng/ml	1827,82	26135,79	2692,65

Fig. 2: *Alflutop® effects on IL8 extracellular release in pro-inflammatory stimulated chondrocytes (CHON-001 cell line).*



	IL1beta 72h.	PMA 1 uM 24h	20h TNF alfa
Control	1022,54	378,86	668,07
Alflutop 0,2%	888,97	143,76	379,54
Alflutop 0,1%	1042,49	143,76	288,84
Dexamethasone 200ng/ml	604,71	309,71	411,51

Fig.3: *Alflutop® effects on VEGF extracellular release in pro-inflammatory stimulated chondrocytes (CHON-001 cell line).*

Alflutop® significantly reduce the extracellular VEGF, suggesting a therapeutical involvment in VEGF mediated destructive processes (ex. MMP stimulation and matrix protein degradation). Dexamethasone, a powerful anti - inflammatory drug doesn't interract as strong as Alflutop® in VEGF release modulation, especially on the PMA and TNFα inflammation pathways.

Genes encoding IL6, IL8 and IL1α was analised for the **molecular model of anti-inflammatory action.** We chose as representative for gene expression model the above mentioned cytokines having in mind their strong implication in the progression of systemic / osteoarthicular inflammation: **IL-6** has a complex role in the pathogenesis of osteoarthritis through the initiation of inflammatory process; **IL-8** – chemotactic factor for cytokines, inducing the chemotaxis of primary neutrophiles and granulocytes causing their migration at the inflammatory situs; **IL-1β** –involved in the initiation and progression of osteoartritis, starting an intracellular events cascade that decide the proteinases activation, establish pro-destructive arthicular sites and reduce the extracellular matrix synthesis. GAPDH (gliceraldehide 3-phosphate dehidrogenase) was used as endogenous control gene for the cytokine gene expression. We choose as pro-inflammatory stimulus TNF-α , an ubiquitar, systemic agent, with significant effects at phenotypic level.

Pro-inflammatory genes expression in unstimulated, basic conditions compared with those under the TNFα stimulation reveals the over-expression effect of the target genes (IL6, IL8 and IL1α), sustaining the TNFα role as a trigger of pro-inflammatory cascade.

The graphic below represents the relative quantification of target genes expression in TNFα stimulated cells treated with 2 doses of Alflutop®, compared with the untreated control (Ratio of RQ- relative quantification of sample /control). (figure 4)

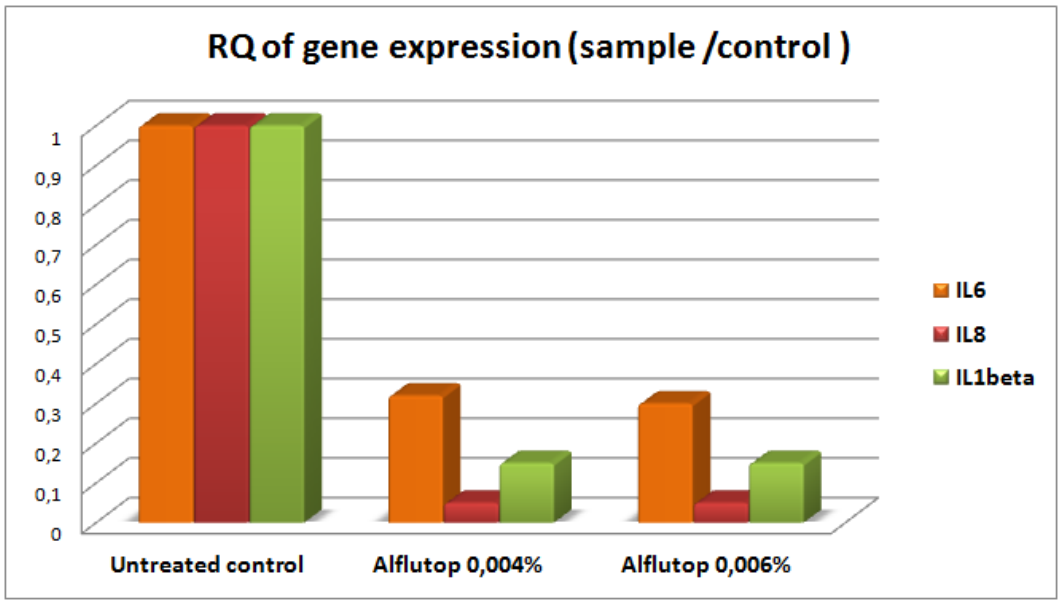


Fig. 4: *Alflutop® effects on IL6, IL8 and IL1β genes over- expressed in chondrocytes (CHON-001 cell line)pro-inflammatory stimulated with TNFα.*

Results confirm the sustained involvement of Alflutop® in pro-inflammatory cytokines signaling pathway, starting with the gene expression control **(down-regulation of IL6, IL8 and IL1β genes).**

Conclusions

Alflutop® inhibits IL6 and IL8 interleukins release, the main modulators of inflammatory acute phase progression, proving significant anti-inflammatory effect on several classical pathways. The bioactive components complexity of Alflutop® action converges towards several stimulation pathways belonging to the osteoarthritis inflammatory spectrum: TNFα (primary inflammatory agent with systemic impact, mediating the catabolic cascade and cell apoptosis), PMA (promotes inflammation generated by reactive oxygen species) and IL1β (promoter of degradative enzymes activation).

As well as, Alflutop® inhibits VEGF, an important angiogenesis factor with a recent discovered impact as a biochemical mediator in destructive processes of osteoarthritis.

"In vitro" modulation of these important mediators of inflammation sustains Alflutop® contribution in the rehabilitation of the cartilage physiology through anti-cytokines action.

References

[1] Goldring M.B.,(2006), Update on the biology of the chondrocyte and new approaches to treating cartilage diseases, Best Pract Res Clin Rheumatol. 20(5):1003-25.
[2] Calich A.L., Domiciano D.S., Fuller R.,(2010), Osteoarthritis: can anti-cytokine therapy play a role in treatment? Clin Rheumatol., 29(5):451-5
[3] Bonnet CS, Walsh DA.,(2005), Osteoarthritis, angiogenesis and inflammation. Rheumatology.;44(1):7-16
[4] 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, 423, pp. 17–26
[5] Henrotin YE, De Groote DD, Labasse AH, Gaspar SE, Zheng SX, Geenen VG, Reginster JY, (1996),Effects of exogenous IL-1 beta, TNF alpha, IL-6, IL-8 and LIF on cytokine production by human articular chondrocytes, Osteoarthritis Cartilage,4(3):163-73
[6] Zafar R, Nahid A, Tariq M.(2010),Advanced Glycation End Products (AGEs)-Induced Expression of IL-6 and IL-8 in Human Osteoarthritis Chondrocytes Correlates with the Induction of Endoplasmic Reticulum Stress. Arthritis Rheum;62 Suppl 10 :632.
[7] Fernandes JC, Martel-Pelletier J, Pelletier JP. (2002) The role of cytokines in osteoarthritis pathophysiology. Biorheology, 39, 237 – 246
[8] van Meegeren ME, Roosendaal G, Jansen NW, Wenting MJ, van Wesel AC, van Roon JA, Lafeber FP., (2012), Osteoarthritis Cartilage. 20(7):764-72.
[9] Carlevaro M F., Cermelli S, Cancedda R, Descalzi Cancedda F, (2000), Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation Journal of Cell Science, 113, 59-69 59
[10] Henrotin Y, Lambert C, Couchourel D, Ripoll C, Chiotelli E (2011) Nutraceuticals: do they represent a new era in the management of osteoarthritis? – A narrative review from the lessons taken with five products,Osteoarthritis Cartilage, 19:1–21.7
[11] Malemud C. J., Goldberg V.M., (1999), Future directions for research and treatment of osteoarthritis, Frontiers in Bioscience, 4, d762-771
[12] Au RY, Al-Talib TK, Au AY, Phan PV, Frondoza CG (2007) Avocado soybean unsaponifiables (ASU) suppress TNF-alpha, IL-1beta, COX-2, iNOS

gene expression, and prostaglandin E(2) and nitric oxide production in articular chondrocytes and monocyte/macrophages, Osteoarthritis Cartilage, 15, 1249–1255

[13] Ownby S L, Fortuno LV, Au AY, Grzanna MW, Rashmir-Raven AM, Frondoza Ownby G, (2014) Expression of pro-inflammatory mediators is inhibited by an avocado/soybean unsaponifiables and epigallocatechin gallate combination, Journal of Inflammation, 11:8

[14] Fay, J; Varoga, D; Kohrs, S; Lemke, AK; Kurz, B; Schuenke, M; Goldring, MB; Pufe, T, The vascular endothelial growth factor (VEGF) and its receptors are induced by reactive oxygen species (ROS) in human chondrocytes and articular cartilage explants, 52nd Annual Meeting of the Orthopaedic Research Society, Paper no. 1373.

[15] Cook E. B., Stahl J.L., Lowe L., Chen R., (2001), Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry, J. Immunol. Methods, 254: 109-118

[16] www.appliedbiosystems.com / support

[17] Hettmannsperger U, Detmar M, Owsianowski M, Tenorio S, Kammler HJ, Orfanos CE., (1992), Cytokine-stimulated human dermal microvascular endothelial cells produce interleukin 6--inhibition by hydrocortisone, dexamethasone, and calcitriol, J Invest Dermatol.,99(5):531-6.