THE *IN VITRO* INFLUENCE OF A PHARMACEUTICALLY ACTIVE SMALL SEA FISH EXTRACT ON APOPTOSIS AND PROLIFERATION MECHANISMS AMPLIFIED BY INFLAMMATORY CONDITIONS

LAURA OLARIU^{1,2}, BRINDUSA DUMITRIU¹, LUIZA CRACIUN¹, EMILIA BUSE¹, NATALIA ROSOIU^{2,3}, MIHAI BOJINCA^{4*}, TOMA PAPACOCEA⁵

¹S.C. Biotehnos S.A., 3-5 Gorunului Street, 075100-Otopeni, Ilfov, Romania

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

³Ovidius University, Faculty of Medicine, Department of Biochemistry, Constanța, Romania;

⁴“Dr. I. Cantacuzino” Hospital, Clinique of Internal Medicine and Rheumatology, Bucharest, Romania

⁵“Carol Davila” University of Medicine and Pharmacy, Neurosurgery Department, “St. Pantelimon” Emergency Hospital, Bucharest, Romania

*corresponding author: vmbojinca@yahoo.com

Manuscript received: November 2017

Abstract

The osteoarthritis pathogenesis is depicted by the start of articular cartilage degradation and dysfunction in chondrocytes metabolism. Many drugs were developed in order to prevent or stop one or several pathways of osteoarthritis progression, but a therapeutically efficient solution is far from been achieved. Our research is focused on apoptotic and cell division mechanisms, dramatically changed in osteoarthritis, highlighting inflammatory conditions mimicked through two types of stimuli: IL1 β and TNF α . The protective action of a small sea fish extract was studied on a human chondrocytes cell line (CHON-001), comparing its action with two pharmaceutically active compounds: chondroitin sulphate and ascorbic acid. The previously demonstrated regenerating effect of the product is completed with anti-apoptotic and pro-mitotic action in pro-inflammatory stimulated chondrocytes, as well as the hyaluronic acid extracellular restoring in IL1 β treated CHON-001.

Rezumat

Patogeneza osteoartritică este definită de debutul degradării osteoarticulare și disfuncții în metabolismul condrocitelor. Deși s-au dezvoltat multe substanțe farmaceutice pentru prevenția sau stoparea anumitor căi de propagare a osteoartritei, încă nu s-a ajuns la o soluție terapeutică eficientă. Cercetările noastre sunt direcționate către mecanisme apoptotice și de diviziune celulară, dramatic perturbate în osteoartritoză, evidențiind condiții inflamatorii mimate de două tipuri de stimuli: IL1 β și TNF α . Acțiunea protectoare a unui extract de pește marin mărunț a fost studiată pe o linie celulară umană de condrocite (CHON-001), comparându-se acțiunea sa cu doi compuși farmaceutici activi: sulfatul condroitină și acidul ascorbic. Efectul regenerativ al produsului este completat cu acțiunea anti-apoptotică și pro-mitotică asupra condrocitelor stimulate pro-inflamator, precum și de refacerea acidului hialuronic din mediul extracelular al celulelor CHON-001 tratate cu IL1 β .

Keywords: apoptosis, cell cycle, hyaluronic acid, chondrocytes, inflammation, small sea fish extract

Introduction

Osteoarthritis (OA) is a disease of joints and bones that may result from multiple pathophysiological mechanisms, one of which is the dysregulation of cellular homeostasis, expressed by apoptotic rate and the sequence of cell cycle. Articular degradation becomes a major cause of disability, especially because cartilage tissue has a reduced self-healing potential being un-vascularised, and chondrocytes have a limited ability to migrate and repair the affected site [19]. Pathological chondrocytes undergo an abnormal extracellular stimulus cascade, including autocrine and paracrine factors, synovial stimuli and protein components in the altered matrix, inducing a cumulation of disrupted cellular responses at the anabolic, catabolic and phenotypic level [7, 10]. Converging *in vivo*

signals generate perturbations in physiology of resting articular chondrocyte, including proliferation, hypertrophic differentiation, and lead to osteoarthritis disease [4]. The morphological changes of chondrocytes in OA cartilage are attributed to apoptosis and/or chondroptosis [21].

Having multiple aetiologies, osteoarthritis is a difficult to treat pathology, usually pharmacological interventions solely addressed to chronic pain fail and do not prevent cartilage damage and the associated destruction of other joint tissues [20]. New pharmaco-therapeutic options are emerging, targeting the dysregulated molecular processes responsible for disease onset. Based on the occurrence of apoptosis in the osteoarthritic pathologies, this process could be one of the valid targets to modulate the cartilage degeneration [8].

Ascorbic acid (vitamin C) is known for its role in osteogenesis, wound healing and the maintenance of healthy gums. Recent studies identified that it reduces apoptosis, reduces the loss of viability and markedly decreased H₂O₂-mediated senescence in cells treated with H₂O₂, decreased the activity of nuclear factor (erythroid-derived 2)-like 2 (NRF2), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP1) and matrix metalloproteinase-3, which is stimulated by H₂O₂ [1, 5, 16]. As well as, ascorbic acid stimulated the proliferation of chondrocytes and maintained the chondrogenic properties of the cells in alginate beads culture, promoted the differentiation, stimulating the formation of collagenous matrix [17].

As "*in vitro*" models, chondrocytes are more responsive to the chondroprotective agents, glucosamine and chondroitin sulphate [11]. They improve chondrocytes' ability to repair and regenerate. *In vitro* previous observations suggest that these compounds function as biological response modifiers, agents which improve the natural protective responses of tissues [12, 18]. As a clinical treatment, glucosamine and chondroitin are nutritional supplements that act as chondro-protectors or/and as "disease-modifying osteoarthritis drugs". Although many studies have showed a significant treatment effect and remarkable safety, their relative effectiveness compared with placebo is controversial [9]. The common osteoarthritic therapy with nonsteroidal anti-inflammatory drugs (NSAIDs), is accompanied by the treatment with chondro-protectors, or nutrients such as antioxidants and omega-3 fatty acids [3, 6].

Our studies targeted the pro-inflammatory stimulation of chondrocytes in order to start the cellular degradation and the monitoring of apoptosis and the sequence of cell cycle as the representative parameters for the functional status. In addition, we quantified in chondrocytes the extracellular hyaluronic acid as a key macromolecule responsible for the extracellular matrix homeostasis, before and after proteolysis initiation by IL1β. The biological action of three pharmaceutical compounds was evaluated and compared: the small sea fish extract, ascorbic acid and chondroitin sulphate. The cellular effects of the small sea fish extract have been sustained especially on matrix preservation, oxidative stress and inflammation inhibition by previous research [13, 14]. We designed our studies in order to complete the *in vitro* efficacy spectrum through apoptotic and cell division mechanisms, dramatically changed in inflammatory conditions mimicked through two types of stimuli: IL1β and TNFα.

Materials and Methods

Cell cultures

Chondrocyte cell line CHON-001 (ATCC® CRL-2846™), human normal chondrocytes from long bone cartilage. Cultivation was performed in high glucose DMEM media, 10% foetal bovine serum, 0.1 mg/mL G-418 antibiotic solution, at 37°C, in 95% humidified air and 5% CO₂ incubator. After 24 h, time for adhesion on plates, cells were treated with a small sea fish extract (CP), CS - chondroitin sulphate sodium salt/water soluble (CRS-EDQM - reference substance 0000593) and vitamin C/water soluble (L-ascorbic acid Sigma Aldrich A92902). After a previous cytotoxicity screening, the following concentrations were selected: CP 0.2% and 0.3% (V/V in culture medium); CS 3 µg/mL and 200 µg/mL and vitamin C 45 µM. In order to mimic *in vitro* the degradative, pro-inflammatory conditions in osteoarthritis, cells were stimulated for 48 h and 72 h with IL1β 10 ng/mL and 24 h with TNFα 15 ng/mL. Results were compared with the untreated cells (control), in a dynamic manner, after 48 h and 72 h of treatment.

Chemicals and reagents: CycleTEST™ PLUS DNA Reagent (BD Pharmingen); Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen); Human Hyaluronic Acid ELISA kit (Cusabio); **Equipments:** Flow cytometer FACS CANTO II with DIVA 6.1 and FCS Express software. Berthold TRISTAR.

Apoptosis through phosphatidyl serine (PS) translocation [15]

The Annexin V-FITC, detected through *flow cytometry* as green fluorescence (FITC-A), is binded to PS in the membrane of cells which are beginning the apoptotic process; in cells where the membrane has been compromised propidium iodide stain the DNA and is detected as red fluorescence (PE-A).

DNA staining for flow cytometry cell cycle analysis

The isolation and label of the nuclei in cell suspensions is done using Cycle TEST PLUS DNA Reagent (BD Pharmingen) [2]. PI staining of nuclear DNA shows a histogram representation of G0/G1, S and G2/M cell cycle phases. Cell cycle progression is visualized by flow cytometry (Figure 1).

Extracellular hyaluronic acid was quantified through a high sensitive sandwich enzyme immunoassay using antibodies specific for hyaluronic acid (Human Hyaluronic Acid ELISA kit-CUSABIO CSB – E04805h).

Statistics

Data are expressed as mean ± standard deviation (SD), averaged over at least three independent experiments for normally distributed data. We consider * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, using Repeated Measures ANOVA, Dunnett's Multiple Comparison Test.

Figure 1.

Flow cytometry cell cycle monitoring

Results and Discussion

The experiments evaluated and compared the effects of small sea fish extract (CP), chondroitin sulphate (CS) and vitamin C. In two special stimulation conditions, specific for osteoarthritic pathology, using TNF α 15 ng/mL - systemic stimuli, the main generator of pro-inflammatory cascades and IL1 β 10 ng/mL, as promoter for cartilage degradation through proteolytic

enzymes activation. The extra-cellular matrix decline after the IL1 β specific stimulation was checked by hyaluronic acid quantification from the supernatant of cells treated with the fish extract and vitamin C. Results showed the decrease of extracellular hyaluronic acid in stimulated chondrocytes, followed by the significant restoration of this molecule in cells treated with CP and vitamin C (Table I).

Table IExtracellular hyaluronic acid content in IL1 β stimulated cells; the impact of treatment with CP and vitamin C

Samples/Hyaluronic acid (ng/mL)	Unstimulated cells	IL1 β stimulated cells
Control	3.5 $\pm$ 0.521	1.4 $\pm$ 0.1
CP 0.2%	5.7 $\pm$ 0.12**	6.88 $\pm$ 0.54***
Vitamin C 45 μ M	4.6 $\pm$ 0.21**	5.48 $\pm$ 0.25***

* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001

The evaluation of time - dependent apoptotic process reveals that it is installed even at 48 h for both

stimulation conditions, more evident in IL1 β activated chondrocytes (Figure 2, Table II).

Figure 2. Apoptosis induction on TNF α and IL1 β stimulated CHON-001 chondrocytes

Chondrocytes act as the main trigger for the maintenance, organization and composition of the cartilage matrix, but there is a low percentage (1 - 5% of the total volume of the cartilage) and they have a slow rate of multiplication. Stimulating them is of extreme importance for cartilage restoration, this process

being a pharmaceutical target. The small changes raising the cell proliferation should be considered in respect to the general homeostasis of the cell (Table II). Results of time-dependent studies (48 h and 72 h) are presented in Figure 3.

Table II

Apoptotic and proliferative status at 48 h and 72 h of cultivation

	Apoptosis evolution (after 48 h)		Cell cycle sequentiation after 48 h (mitotic phases)
Unstimulated cells	Early apoptosis	Late apoptosis	%S + %G2/M
Control	8.917 ± 0.6285	2.107 ± 0.4314	18.67 ± 1.564
CP 0.2%	6.987 ± 0.431 ^{***}	1.333 ± 0.1528 [*]	28.23 ± 6.45 ^{ns}
CP 0.3%	8.7 ± 0.7365 ^{ns}	2.073 ± 0.2084 ^{ns}	32.36 ± 15.34 ^{ns}
Vitamin C 45 µM	7.46 ± 0.3732 ^{***}	4.88 ± 0.7279 ^{***}	21.49 ± 10.98 ^{ns}
CS 3 µg/mL	8.52 ± 0.5444 ^{ns}	1.983 ± 0.1069 ^{ns}	22.51 ± 12.55 ^{ns}
CS 200 µg/mL	8.177 ± 0.3742 [*]	2.17 ± 0.3799 ^{ns}	16.89 ± 3.957 ^{ns}
IL1beta stimulation			
Control	11.55 ± 1.096	7.567 ± 0.27140	26.5 ± 0.9852
CP 0.2%	11.3 ± 1.104 ^{ns}	4.117 ± 0.08505 ^{***}	34.92 ± 2.672 ^{***}
CP 0.3%	8.217 ± 0.2538 ^{**}	2.233 ± 0.4944 ^{***}	33.98 ± 1.775 ^{***}
Vitamin C 45 µM	9.043 ± 0.3204 [*]	5.847 ± 0.3711 ^{***}	22.86 ± 2.215 ^{ns}
CS 3 µg/mL	7.378 ± 0.9732 ^{***}	8.903 ± 0.3496 ^{***}	39.17 ± 1.556 ^{***}
CS 200 µg/mL	6.319 ± 1.309 ^{***}	5.103 ± 0.4644 ^{***}	31.17 ± 0.9283 [*]
TNF alfa stimulation			
Control	11.48 ± 0.09165	2.1 ± 0.1735	27.21 ± 1.54
CP 0.2%	8.267 ± 0.38 ^{***}	3.007 ± 0.522 [*]	24.92 ± 1.614 ^{ns}
CP 0.3%	8.27 ± 0.1473 ^{***}	2.74 ± 0.233 ^{ns}	31.86 ± 1.592 ^{**}
Vitamin C 45 µM	10.51 ± 0.5977 ^{**}	6.74 ± 0.8528 ^{***}	24.06 ± 1.861 ^{ns}
CS 3 µg/mL	9.64 ± 0.3124 ^{***}	2.983 ± 0.1106 [*]	25.43 ± 0.8523 ^{ns}
CS 200 µg/mL	12.28 ± 0.5444 [*]	4.167 ± 0.2706 ^{***}	27.52 ± 2.36 ^{ns}
	Apoptosis evolution (after 72 h)		Cell cycle sequentiation after 72 h (mitotic phases)
Unstimulated cells	Early apoptosis	Late apoptosis	%S + %G2/M
Control	6.633 ± 3.932	2.07 ± 0.6807	19.24 ± 1.146
CP 0.2%	6.133 ± 1.358 ^{ns}	1.50 ± 0.3 ^{ns}	26.81 ± 0.7641 ^{***}
CP 0.3%	7.767 ± 1.804 ^{ns}	1.73 ± 0.8145 ^{ns}	25.42 ± 1.682 ^{***}
Vitamin C 45 µM	5.8 ± 2.261 ^{ns}	4.17 ± 2.318 ^{ns}	11.65 ± 1.123 ^{***}
CS 3 µg/mL	7.967 ± 1.102 ^{ns}	3.73 ± 3.004 ^{ns}	11.69 ± 0.8065 ^{***}
CS 200 µg/mL	6.6 ± 2.291 ^{ns}	1.80 ± 1.058 ^{ns}	13.79 ± 1.489 ^{**}
IL1 beta stimulation			
Control	9.33 ± 0.8737 ^{ns}	5.77 ± 3.591	16.95 ± 1.424
CP 0.2%	9.10 ± 4.967 ^{ns}	3.10 ± 1.345 ^{ns}	26.63 ± 1.831 ^{***}
CP 0.3%	6.43 ± 2.538 ^{ns}	2.10 ± 0.9644 ^{**}	31.00 ± 1.213 ^{***}
Vitamin C 45 µM	6.93 ± 3.443 ^{ns}	4.60 ± 2.433 ^{ns}	15.79 ± 1.341 ^{ns}
CS 3 µg/mL	10.00 ± 3.329 ^{ns}	7.77 ± 2.003 ^{ns}	19.77 ± 1.115 ^{ns}
CS 200 µg/mL	8.63 ± 4.038 ^{ns}	4.70 ± 0.8185 ^{ns}	16.31 ± 1.075 ^{ns}
TNF alfa stimulation			
Control	12.27 ± 3.349	3.17 ± 1.704	13.90 ± 1.28
CP 0.2%	6.33 ± 2.631 ^{**}	2.53 ± 1.193 ^{ns}	27.77 ± 1.37 ^{***}
CP 0.3%	6.53 ± 2.139 ^{**}	2.17 ± 1.115 ^{ns}	26.73 ± 2.11 ^{***}
Vitamin C 45 µM	11.07 ± 1.106 ^{ns}	5.80 ± 2.869 [*]	14.36 ± 1.12 ^{ns}
CS 3 µg/mL	10.60 ± 1.992 ^{ns}	3.40 ± 1.562 ^{ns}	15.62 ± 0.63 ^{ns}
CS 200 µg/mL	11.63 ± 2.212 ^{ns}	4.57 ± 2.732 ^{ns}	16.35 ± 1.51 ^{ns}

* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, ns not significant

Even the IL1 β impact is mandatory for the start of the apoptotic process, and enzymatic degradation of extracellular matrix, chondrocytes developed protective mechanisms in order to accelerate the cellular multiplication (the mitotic rate is augmented). As well as, in the case of TNF α stimulation that acts on different pathways of inflammation progression, chondrocytes become apoptotic but 48 h of treatment are not enough to disturb the cell cycle sequence. The apoptotic process is significantly modulated by the bioactive compounds (CP, vitamin C, CS) at 48 h in both pro-inflammatory conditions. After 72 h of *in*

vitro treatment, there are more apoptotic chondrocytes and the cell division declines. The CP compound stimulates proliferation in a significant manner in both pro-inflammatory states; in correlation with the decrease of apoptosis (late apoptosis in IL1 β stimulatory condition and early apoptosis in TNF α stimulated cells) indicates a protective role against degradation in osteoarthritis in response to pro-inflammatory agents. The other compounds (CS and vitamin C) did not significantly change the proliferative nor the apoptotic status at 72 h.

Figure 3.

a. Mitotic phases evolution on 48 h stimulated chondrocytes treated with bioactive compounds; b. Apoptosis evolution on 48 h stimulated chondrocytes treated with bioactive compounds; c. Mitotic phases evolution on 72 h stimulated chondrocytes treated with bioactive compounds; d. Apoptosis evolution on 72 h stimulated chondrocytes treated with bioactive compounds

Conclusions

Considering the fact that the common biomolecular mechanisms of osteoarthritis is complicated and not yet revealed, we consider our studies as a step forward in the understanding of apoptotic and proliferative mechanisms related with chondrocytes inflammation, more specific with IL1 β directed cascades and TNF α systemic stimulation. After 48 hours of stimulation, intrinsic protective mechanisms act to stimulate proliferation, but over time (72 h) they are outdated by long-term pro-inflammatory signalling in the sense of decreasing the mitotic rate. In the particular conditions of IL1 β and TNF α stimulation, it was highlighted the pharmaceutical potency of natural raw materials, in our case pointing out the small sea fish extract that effectively counteracted the chondrocytes decline, both through apoptosis inhibition and stimulation of the proliferation rate. Vitamin C inhibits apoptosis only on IL1 β stimulated cells, chondroitin sulphate stimulates the mitotic phases only when the systemic inflammation is installed (TNF α treated chondrocytes). Together with its stimulatory effect on hyaluronic acid production and our previous results describing the antioxidant, regenerative and matrix-preserving action, we can conclude that through the modulation of multiple regulatory pathways, the small sea fish extract efficiently protects human chondrocytes against damage induced by osteo-articular inflammation.

Disclosure

The producers of Alflutop® (S.C. Biotehnos S.A.) did not interfere with this study (data collection or processing, concluding results or editing endeavours).

References

1. Chang Z, Huo L, Li P, Wu Y, Zhang P, Ascorbic acid provides protection for human chondrocytes against oxidative stress. *Mol Med Rep.*, 2015; 12(5): 7086-7092.
2. Dumitriu B, Olariu L, Ene MD, Zglimbea L, Rosoiu N, The photo-protective activity of Crataegus monogyna extract proved by complementary mechanisms of skin inflammation, damage of extracellular matrix, cellular survival and proliferation disorders. *Int J Biotech Wellness Ind.*, 2012; 1: 177-188.
3. EULAR Recommendations 2003: an evidence based approach to the management of knee osteoarthritis: Report of a Task Force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCISIT). *Ann Rheum Dis.*, 2003; 62: 1145-1155.
4. Goldring MB, Otero M, Plumb DA, Dragomir C, Favero M, Hachem KE, Hashimoto K, Roach HI, Olivetto E, Borzi RM, Marcu B, Roles of inflammatory and anabolic cytokines in cartilage metabolism. *Eur Cell Mater.*, 2011; 21: 202-220.
5. Gonhyung K, Masahiro O, Darko B, Ishiguro T, Park CH, Kadosawa T, Fujinaga T, Effects of ascorbic acid on proliferation and biological properties of bovine chondrocytes in alginate beads. *Japanese Journal of Veterinary Research*, 2006; 51(2): 83-94.

6. Hochberg MC, Altman RD, April KT, American College of Rheumatology 2012 recommendations for the use of nonpharmacologic and pharmacologic therapies in osteoarthritis of the hand, hip, and knee. *Arthritis Care Res.*, 2012; 64(4): 465-474.
7. Husea M, Liu-Bryan Ru, Terkeltaub R, Shifting HIFs in osteoarthritis. *Nature Medicine*, 2010; 16: 641-644.
8. Hyun SH, Hyun AK, Chondrocyte apoptosis in the pathogenesis of osteoarthritis. *Int J Mol Sci.*, 2015; 16(11): 26035-26054.
9. Jerosch J, effects of glucosamine and chondroitin sulfate on cartilage metabolism in OA: outlook on other nutrient partners especially omega-3 fatty acids. *Int J Rheumatology*, 2011; 2011: 1-17.
10. Kondo M, Yamaoka K, Tanaka Y, Acquiring chondrocyte phenotype from human mesenchymal stem cells under inflammatory conditions. *Int J Mol Sci.*, 2014; 15(11): 21270-21285.
11. Lippiello L, Glucosamine and chondroitin sulfate: biological response modifiers of chondrocytes under simulated conditions of joint stress. *Osteoarthritis Cartilage*, 2003; 11(5): 335-342.
12. Mihai L, Mitrea N, Papacoea R, Badarau AI, Dynamic activity of some antioxidant enzymes in primary cultures of neurons under hypoxia and ischemia. *Farmacia*, 2012; 60(1): 49-57.
13. Olariu L, Pyatigorskaya N, Dumitriu B, Pavlov A, Vacaru AM, Vacaru A, "In vitro" chondro-restitutive capacity of Alflutop® proved on chondrocytes cultures. *Romanian Biotechnological Letters*, 2016; 22(16): 12047-12053.
14. Olariu L, Dumitriu B, Ene DM, Pavlov A, Pyatigorskaya N, Rosoiu N, Alflutop® modulates "in vitro" relevant mechanisms of osteoarthritic pathology. *Academy of Romanian Scientists, Annals Series on Biological Sciences*, 2017, 6(1): 100-113.
15. Olariu L, Dumitriu B, Ene DM, Buse E, Rosoiu N, Advanced methods for antitumoral screening applied on pharmaceutical product development. *Academy of Romanian Scientists, Annals Series on Biological Sciences*, 2013; 2(2): 51-82.
16. Pavel IZ, Pârvu AE, Dehelean CA, Vlase L, Csuk R, Muntean DM, Assessment of the antioxidant effect of a maslinic acid derivative in an experimental model of acute inflammation. *Farmacia*, 2017; 65(3): 390-395.
17. Temu TM, Wu KY, Gruppuso PA, Phornphutkul C, The mechanism of ascorbic acid-induced differentiation of ATDC5 chondrogenic cells. *Am J Physiol Endocrinol Metab.*, 2010; 299(2): 325-334.
18. Vasiliadis HS, Tsikopoulos K, Glucosamine and chondroitin for the treatment of osteoarthritis. *World J Orthop.*, 2017, 8(1): 1-11.
19. Venezian R, Shenker BJ, Datar S, Leboy PS, Modulation of chondrocyte proliferation by ascorbic acid and BMP-2. *J Cell Physiol.*, 1998; 174(3): 331-341.
20. Wieland HA, Michaelis M, Kirschbaum BJ, Rudolphi KA, Osteoarthritis - an untreatable disease?. *Nature Reviews Drug Discovery*, 2005; 4: 331-344.
21. Zamli Z, Sharif M, Chondrocyte apoptosis: a cause or consequence of osteoarthritis. *Int J Rheum Dis.*, 2011; 14(2): 159-166.