Potential of Raloxifene in reversing osteoarthritis-like alterations in rat chondrocytes: An *in vitro* model study

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The aim of this study was to investigate the effects of Raloxifene (Ral) on degeneration-related changes in osteoarthritis (OA)-like chondrocytes using two- and three-dimensional models. Five-azacytidine (Aza-C) was used to induce OA-like alterations in rat articular chondrocytes and the model was verified at molecular and macrolevels. Chondrocytes were treated with Ral (1, 5 and 10 μM) for 10 days. Caspase-3 activity, gene expressions of aggrecan, collagen II, alkaline phosphatase (ALP), collagen X, matrix metalloproteinases (MMP-13, MMP-3 and MMP-2), and MMP-13, MMP-3 and MMP-2 protein expressions were studied in two-dimensional model. Matrix deposition and mechanical properties of agarose-chondrocyte discs were evaluated in three-dimensional model. One μM Ral reduced expression of OA-related genes, decreased apoptosis, and MMP-13 and MMP-3 protein expressions. It also increased aggrecan and collagen II gene expressions relative to untreated OA-like chondrocytes. In three-dimensional model, 1 μM Ral treatment resulted in increased collagen deposition and improved mechanical properties, although a significant increase for sGAG was not observed. In summation, 1 μM Ral improved matrix-related activities, whereas dose increment reversed these effects except ALP gene expression and sGAG deposition. These results provide evidence that low-dose Ral has the potential to cease or reduce the matrix degeneration in OA.

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1. Introduction

Osteoarthritis (OA) is a destructive disease of the joints comprising articular cartilage, synovium and subchondral bone (Sandell and Aigner 2001; Lorenz and Richter 2006). In OA, catabolic processes predominate in cartilage metabolism (Nesic *et al.* 2006) causing degradation of articular cartilage. The degeneration of articular cartilage leads to chronic pain and functional restrictions in the affected joints (Lorenz and Richter 2006). Increase in prevalence of OA and absence of effective treatment methods have led to a growing interest in the development of new treatment strategies (Richy *et al.* 2003; Sumantran *et al.* 2007; Jiang *et al.* 2011; Kavas *et al.* 2011).

Raloxifene (Ral) is a selective estrogen receptor modulator (SERM) that can bind to estrogen receptor (ER) α and ERβ, although with less affinity than estradiol. Structurally, Ral does not resemble estradiol; rather, it is benzothiophene with a basic side chain (Dadiboyena 2012). Since Ral has estrogen-like activity in the bones, and anti-esterogen activity in breast and uterine tissues, it is recommended for the treatment of postmenopausal osteoporosis, as well as for the

Keywords. Agarose; cartilage; chondroprotective; *in vitro* models; osteoarthritis; Raloxifene

Abbreviations used: 2-D, two-dimensional; 3-D, three-dimensional; ALP, alkaline phosphatase; Aza-C, 5-azacytidine; C, control chondrocytes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Mipep, mitochondrial intermediate peptidase; MMP, matrix metalloproteinase; OA, osteoarthritis; OC, osteoarthritis-like chondrocytes; OC+1 μM Ral, osteoarthritis-like chondrocytes treated with 1 μM Raloxifene; RA, rheumatoid arthritis; Ral, Raloxifene; SERM, selective estrogen receptor modulator; sGAG, sulphated glycosaminoglycan

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prevention of postmenopausal breast cancer in women (Jochems *et al.* 2007; Bolognese 2010). Jochems *et al.* (2007) reported that a Ral analog had protective effects on cartilage and bone, and immunosuppressive action. The protective effect of Ral on joints was previously shown by its reducing effect on *in vitro* proliferation of synoviocytes in patients with rheumatoid arthritis (RA) (Guiducci *et al.* 2005). Administration of Ral over a period of 12 months to women suffering from OA in the knee revealed its possible anti-osteoarthritic effect (Badurski *et al.* 2005). It was reported that Ral provided a decrease in the degradation of cartilage shown by improved WOMAC and Lequesne indexes. In an *in vitro* study, it was shown that Raloxifene protected human OA chondrocytes from IL-1β-induced damage by increasing proteoglycans and inhibiting IL-1β-induced MMP-3 and NO release (Tinti *et al*. 2011). Hattori *et al.* (2012) have also shown that Raloxifene prevented caspase-3-dependent apoptosis in human chondrocytes.

In the current study, the effect of different doses of Ral on matrix (sGAG and collagen) deposition and degeneration-related changes in OA-like chondrocytes were studied. The chondrocytes were cultured either as monolayer or embedded in agarose to establish 2-D and 3-D *in vitro* OA models, respectively. For the 3-D *in vitro* OA model, agarose was chosen for providing an ideal environment to keep chondrocytes in a differentiated state (Benya and Shaffer 1982). Dynamic hydrostatic pressure was applied to the agarose-chondrocyte discs to mimic the physiological environment of the cartilage (Mauck *et al.* 2000; Hansen *et al.* 2001; Sharma *et al.* 2007) and to enhance nutrient transfer to the chondrocytes (O'Hara *et al*. 1990; Bonassar *et al.* 2001). OA-like changes were induced by exposing chondrocytes to Aza-C (Ho *et al.* 2006; Kavas *et al*. 2011). Upon establishment of *in vitro* OA models, the chondrocytes were treated with Ral (1, 5 and 10 μM) during 10 days of incubation. The 2-D *in vitro* model was used to investigate the dose-dependent effects of Ral on apoptosis, gene expressions of aggrecan, collagen II, ALP, collagen X, MMP-13, MMP-3 and MMP-2, and protein expressions of MMP-13, MMP-3 and MMP-2. Dose-dependent effects of Ral on matrix (sGAG and collagen) deposition by OA-like chondrocytes were investigated in 3-D *in vitro* OA model. Unconfined stress relaxation tests were also applied to the agarose-chondrocyte discs to determine their peak stress and equilibrium modulus as an indication of functionality of the formed matrix. One μM Ral treatment resulted in decrease of apoptotic activity, ALP and collagen X gene expressions. This dose of Ral enhanced collagen synthesis and improved mechanical properties of the discs. Hence, our results suggested that 1 μM Ral has the potential to reverse OA-like alterations. However, with dose increment, these positive effects on OA-like chondrocytes were reversed, except ALP gene expression and sGAG deposition in the discs. Here, the reversal effect in matrix degeneration and chondroprotective effect of Ral on OA-like chondrocytes were documented in a 3-D model for the first time which were supported with gene expression results in our 2-D model.

2. Materials and methods

The time plan and steps followed in this study are given in figure 1. Five experimental groups were used:

1. Control chondrocytes (C): Neither Aza-C-exposed nor Ral-treated chondrocytes
2. Osteoarthritis-like chondrocytes (OC): Aza-C-exposed and Ral-untreated chondrocytes
3. OA-like chondrocytes treated with 1 μM Ral (OC+1 μM Ral): Aza-C-exposed and 1 μM Ral-treated chondrocytes
4. OA-like chondrocytes treated with 5 μM Ral (OC+5 μM Ral): Aza-C-exposed and 5 μM Ral-treated chondrocytes
5. OA-like chondrocytes treated with 10 μM Ral (OC +1 μM Ral): Aza-C-exposed and 10 μM Ral-treated chondrocytes

2.1 *Culture of articular chondrocytes*

Approval from Ethical Board of Hacettepe University School of Medicine was obtained for the use of animals in cell isolation. Articular cartilage was harvested from knees of Sprague-Dawley rats and the chondrocytes were isolated with enzymatic digestion from the removed cartilage following the modified procedure of Hidvegi *et al.* (2006). Briefly, cartilage pieces were treated with 0.1% trypsin/g cartilage (Sigma, USA) for 45 min at 37°C, then with 100 U/mL collagenase type II (PAA, Austria) for 10.5 h at 37°C. The isolated chondrocytes were cultured in DMEM/HAM'S F-12 (PAA, Austria) supplemented with 10% FCS (PAA, Austria), 0.1 mM non-essential amino acid (PAA, Austria), 0.4 mM proline (Sigma, USA) and 0.1% penicillin/streptomycin (PAA, Austria) at 37°C with 5% CO_2. In the study, the chondrocytes at 3rd–4th passage were used.

2.2 *In vitro OA model*

Agarose-chondrocyte discs were prepared by modifying the procedures of Mauck *et al.* (2000) and Toyoda *et al.* (2003). Briefly, agarose type VII (Sigma, USA) (4%, w/v) in phosphate-buffered saline (PBS) (0.01 M, pH 7.2) was mixed with an equal volume of chondrocyte suspension (4×10^6 cells/mL) in chondrogenic differentiation medium [DMEM/HAM'S F-12 supplemented with 2% FCS, 1%

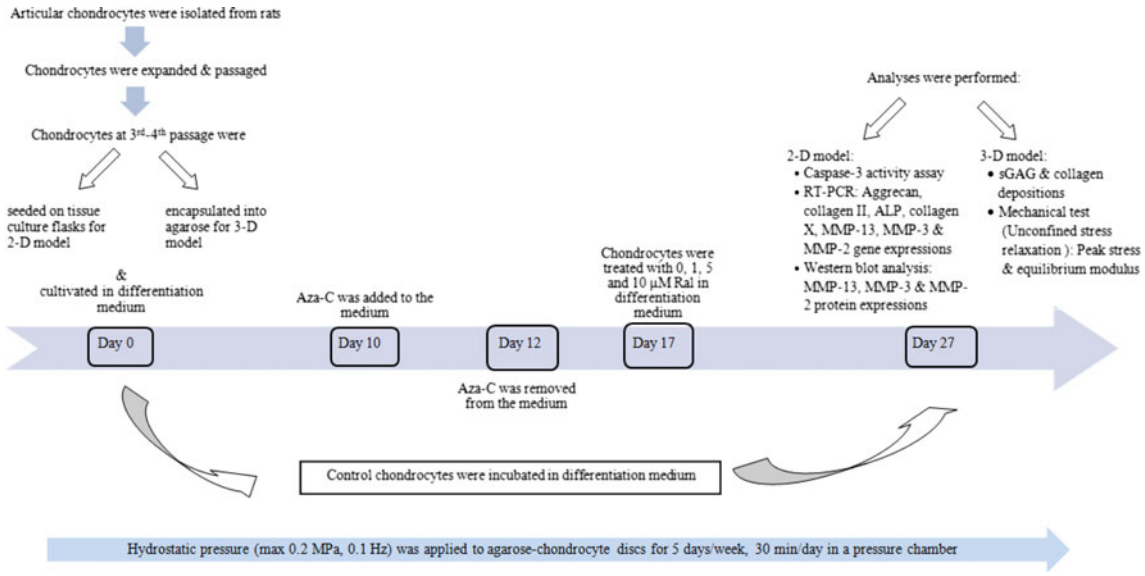

Figure 1. Experimental design of the study.

ITS (Sigma, USA), 1% sodium pyruvate (PAA, Austria), 100 nM dexamethasone (Sigma, USA), 80 μM ascorbic acid (Aldrich, Germany) and 0.1% penicillin/streptomycin]. The mixture was cast between parallel glass plates separated by ~3 mm thick spacers. Subsequent to gelation at room temperature, discs were obtained (~5 mm in diameter and 3 mm in height) using a skin punch. The discs were cultured in the differentiation medium in 6-well plates (15 discs/well). Fifteen μg/mL of Aza-C (Sigma, USA) was added to the medium on the 10th day and maintained for 48 h (Ho *et al.* 2006). The discs were then cultured in the same medium without Aza-C for an additional 5 days. All discs were subjected to hydrostatic pressure (0.2 MPa, 0.1 Hz) (Hansen *et al.* 2001), 5 days/week (Mauck *et al.* 2000), 30 min/day in a custom-made pressure chamber (Biolab Ltd, Turkey) throughout the entire culture period. For 2-D *in vitro* OA model study, chondrocytes were seeded on tissue culture flasks and the above-described steps for induction of OA-like alterations were followed except the application of hydrostatic pressure.

2.3 *Raloxifene treatment*

Following the establishment of the *in vitro* OA models, chondrocytes cultured as a monolayer and in agarose discs (1 disc/1 well of 48-well plates) were incubated with various concentrations of Ral (1, 5 and 10 μM) (Sigma, USA) for 10 days. The medium with Ral was refreshed every 3rd day. After 10 days of Ral treatment, chondrocytes were scraped off from the tissue culture flasks for 2-D *in vitro* model analyses and agarose discs were harvested for 3-D *in vitro* model analyses described below. The control and OA-like chondrocytes were used for comparison. All discs were subjected to hydrostatic pressure as described above.

2.4 *Analyses for 2-D in vitro OA model study*

2.4.1 *Caspase-3 activity assay:* In order to assess apoptotic activity of the chondrocytes, caspase-3 activity assay was determined with a Biovision caspase-3 kit. Cells were collected and resuspended in 50 μL chilled cell lysis buffer, and incubated on ice for 10 min. Then, they were centrifuged at 10000*g* for 1 min. The supernatant (cytosolic extract) was transferred to a fresh tube. The protein concentrations were determined by a modified Bradford Assay. One-hundred μg cell lysate from each sample was mixed with 50 μL chilled cell lysis buffer and 50 μL of 2X reaction buffer was added to each sample. Then, 5 μL of 4 mM DEVD-ρNA substrate was added and the mixture was incubated for 2 h at 37°C. The absorbance of samples was measured at 405 nm in a spectrophotometer (Analytic Jena Spekol, Germany).

2.4.2 *Reverse transcription polymerase chain reaction:* RT-PCR was conducted to study effects of Ral on aggrecan, collagen II, collagen X, ALP, MMP-13, MMP-3 and MMP-2 gene expressions. Total RNA was extracted from chondrocytes with RNeasy Mini Kit (QIAGEN). The first strand cDNA was converted from 1 μg RNA by adding RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) and oligo(dT) primer. PCR was performed with a thermocycler (2720, Applied Biosystems, USA) using Taq DNA polymerase (Fermentas). PCR steps included initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s and extension

at 72°C for 40 s. The final extension was performed at 72°C for 7 min. PCR products were resolved by electrophoresis on a 2% agarose gel and the bands were quantified by ImageJ image analysis software (National Institutes of Health, USA). Mitochondrial intermediate peptidase (Mipep) was used as a housekeeping gene and all gene expressions were normalized with Mipep. The sequences of the PCR primers are given in table 1.

2.4.3 *Western blot analysis:* Cell lysates were isolated using M-PER assay buffer (Pierce, Rockford, IL, USA) containing protease inhibitors (Roche, Mannheim, Germany). The protein content was measured using the modified Bradford Assay using a Coomassie Plus protein assay reagent (Pierce, Rockford, IL, USA). Whole-cell extracts (40 μg) and Prestained Pageruler protein ladder (Fermentas) were separated in a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) at 4°C for 2 h. The membranes were blocked in 5% skim milk and were probed with the appropriate antibodies overnight: MMP-3 (1:100), MMP-2 (1:100), MMP-13 (1:100) (Santa Cruz, CA, USA) followed by a horseradish peroxidase–conjugated donkey anti-goat (1:2000), or goat anti-mouse (1:2500) secondary antibody. The bands were visualized by using an enhanced chemiluminescence kit (ECL Plus, Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Equal protein loading was confirmed by stripping the same membrane probing for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000).

2.5 *Analyses for 3-D in vitro OA model study*

2.5.1 *sGAG deposition:* sGAG (sulphated glycosaminoglycan) content of the discs was determined to investigate effect of Ral on aggrecan deposition by OA-like chondrocytes. The agarose discs were lyophilized and then digested with papain (Sigma, USA) as described by Hoemann *et al.* (2002). Subsequent to papain digestion, the sGAG deposited in the discs was determined with 1,9 dimethyl methylene blue (DMMB) (Aldrich, Germany) assay (Farndale *et al.* 1986). Chondroitin sulphate from bovine trachea (Sigma, USA) was used as a standard. The sGAG amount deposited in the discs was normalized with wet weight of the discs and expressed as a percentage of wet weight.

2.5.2 *Collagen deposition:* Collagen content of the discs was determined using Sirius red S (Fluka, Switzerland) after papain digestion of agarose discs. The method of Na *et al.* (2006) was followed with some modifications as described in our previous study (Kavas *et al.* 2011). Bovine collagen from tracheal cartilage (Sigma, USA) was used as a standard for construction of the calibration curve. The collagen content of the discs was normalized with wet weight of the discs to express the collagen content as a percentage of wet weight.

2.5.3 *Mechanical test:* Mechanical properties of the agarose-chondrocyte discs were evaluated to determine the functionality of the matrix deposited by Ral-treated chondrocytes. Static compression tests were applied to the discs using the method of Mauck *et al.* (2000) with some changes as

Table 1. The sequences of the PCR primers

Primer	Amplicon size	Sequence
Aggrecan	728	Forward: 5′ AAACTTCTTCGGAGTGGGTGGTGA
		Reverse: 5′ TCCTGGAAGGTGAACTGCTCCATT
ALP	783	Forward: 5′ AACACCAACGTGGCCAAGAACATC
		Reverse: 5′ AGGGTCAGTCAGGTTGTTCCGATT
Collagen II	870	Forward: 5′ ACTGATGGTATTCCCGGAGCCAAA
		Reverse: 5′ ATACCCTGGTCACCTTGCTTTCCA
Collagen X	398	Forward: 5′ TTTCTAAAGTGCACTCCGGGACCA
		Reverse: 5′ ACCATGGAGTGATGCACCATCAGA
Mipep	588	Forward: 5′ AGAGCACATTCAGCACCACTTTGC
		Reverse: 5′ TAGCCCAGCAACCCTTCAGATTCA
MMP-2	439	Forward: 5′ TCCGGAGATCTGCAAACAGGACAT
		Reverse: 5′ ATTCCAGGAGTCTGCGATGAGCTT
MMP-3	305	Forward: 5′ TGGAATGGTCTTGGCTCATGCCTA
		Reverse: 5′ ATTTGGTGGGTACCACGAGGACAT
MMP-13	783	Forward: 5′ AGGATCACCTGATTCTTGGGTGCT
		Reverse: 5′ AGGAGCATGAAAGGGTGGTCTCAA

mentioned in our previous study (Kavas *et al.* 2011). Briefly, unconfined compression stress relaxation tests were performed at room temperature between two rigid-impermeable platens with a computer-controlled testing device (Lloyd LS 500, UK) equipped with Nexygen MT Software Version 4.5 (Ametek Inc., UK). Before the test, the free swelling thickness and diameter of each disc were measured by a digital caliper. The discs in PBS were subjected to stress relaxation tests with a constant speed of 3 mm/min until reaching 10% of the free swelling thickness and then allowed to relax to equilibrium (600 s). Peak stress of each disc was obtained from the maximum stress value at 10% strain. Equilibrium modulus (an evaluation of static equilibrium stiffness) was calculated from the equilibrium stress divided by applied strain.

2.6 *Data analysis*

All results were represented as mean±standard error of the means (SEM). Data were analysed with independent-samples *t*-test (SPSS-9 Software, SPSS Inc., USA). Statistical significance was assigned at $P \leq 0.05$.

3. Results

Rat chondrocytes are known to express estrogen receptor alpha and beta (ERα and β) (van der Eerden *et al.* 2002); therefore Ral would be expected to bind to these receptors on the chondrocytes leading to the functional effects described below.

3.1 *Analyses for 2-D in vitro OA model study*

3.1.1 *Apoptosis assay:* Caspase-3 assay demonstrated that the apoptotic activity of the OA-like chondrocytes was significantly higher than that of the control chondrocytes (figure 2). Treatment with 1 μM Ral significantly decreased caspase-3 activity of these cells to a level that was not statistically different from the control chondrocytes. However, the same effect on apoptosis was not observed when the Ral dose was increased to 5 or 10 μM.

3.1.2 *Reverse transcription polymerase chain reaction:* RT-PCR results showed that aggrecan mRNA expression level was significantly higher in the control chondrocytes compared to the OA-like chondrocytes (figure 3A). Although 1 μM Ral treatment showed a significantly higher mRNA expression of aggrecan with respect to OA-like chondrocytes, the aggrecan mRNA levels in all Ral treatment groups were significantly lower than that of the control chondrocytes. A negative correlation was observed between the Ral dosage and aggrecan mRNA expression.

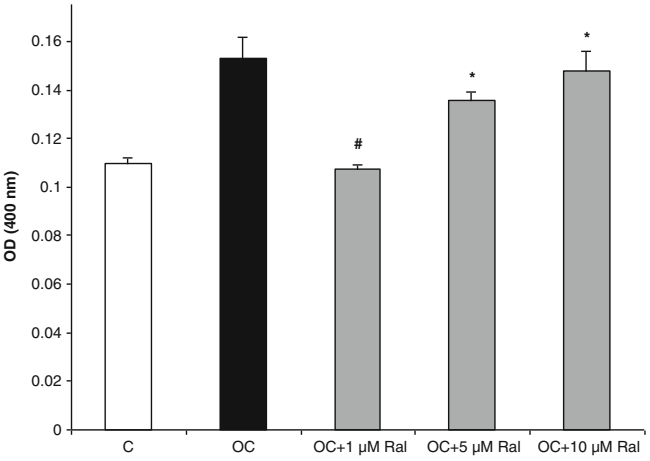

Figure 2. Effect of Ral treatment on apoptosis of OA-like chondrocytes. Data are presented as mean±SEM ($n=2$). Independent-samples *t*-test: $^{*}P \leq 0.05$ versus the control chondrocytes, $^{\#}P \leq 0.05$ versus the OA-like chondrocytes. Statistically significant difference ($P \leq 0.05$) was observed between the control and the OA-like chondrocytes.

Control chondrocytes had the highest collagen II mRNA level, which was significantly higher than that of the OA-like chondrocytes (figure 3B). Following the control chondrocytes, 1 μM Ral treatment group showed the second highest expression of collagen II mRNA, which was significantly higher than those of 5 μM and 10 μM Ral-treated groups. Treatment with two high doses of Ral resulted in significantly reduced levels of collagen II mRNA expression relative to all other groups.

Maximum ALP mRNA expression was observed in OA-like chondrocytes with significant differences from all the other groups (figure 3C). Control chondrocytes and 1 μM Ral-treated chondrocytes had comparable ALP mRNA expressions and both were significantly higher than those of 5 and 10 μM Ral-treated chondrocytes. It was observed that 5 and 10 μM Ral treatment groups had the lowest ALP mRNA levels among the groups.

Similar to the expression of ALP, OA-like chondrocytes had the highest collagen X mRNA expression level and it was significantly different from all other groups (figure 3D). Ral treatment of OA-like chondrocytes resulted in a remarkably decreased collagen X mRNA expression, which was even significantly lower than that of the control chondrocytes. The lowest collagen X mRNA level was found in 1 μM Ral treatment group and it was significantly lower than that in 10 μM Ral group.

Consistent with ALP and collagen X gene expression results, the OA-like chondrocytes had the maximum level of MMP-13 expression, which was significantly higher than those observed in the control chondrocytes, 1 and 5 μM Ral-treated chondrocytes (figure 4A). Among all, 1 μM Ral

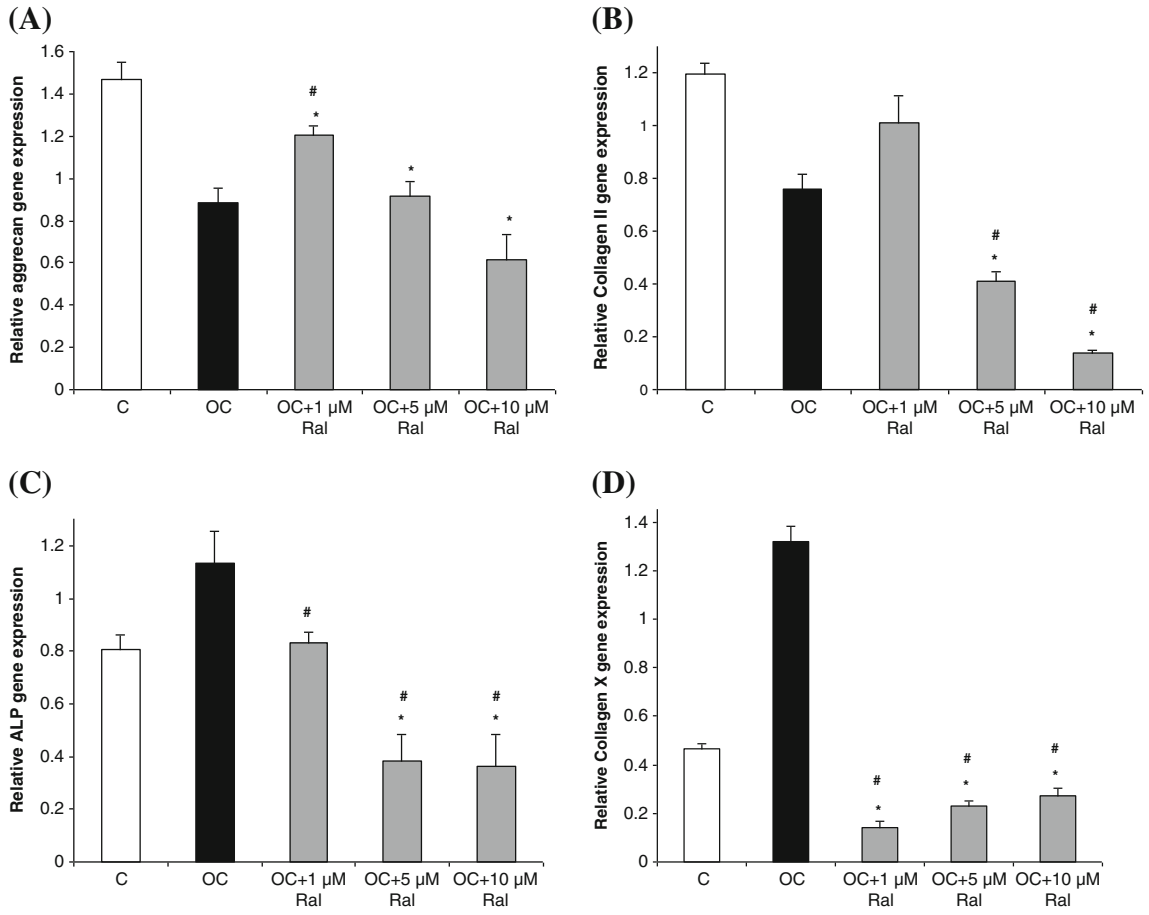

Figure 3. Effect of Ral treatment on (**A**) aggrecan, (**B**) collagen II, (**C**) ALP and (**D**) collagen X gene expressions in OA-like chondrocytes. Mipep was used to normalize the gene expression levels. Data are presented as mean±SEM: (n=6, aggrecan), (n=2, collagen II), (n=6, ALP) and (n=3, collagen X). 'n' number indicates to two independent biological replicates, with three technical replicates. Independent-samples t-test: *$P \leq 0.05$ versus the control chondrocytes, $^{\#}P \leq 0.05$ versus the OA-like chondrocytes. Statistically significant difference ($P \leq 0.05$) was observed between the OA-like and the control chondrocytes for each gene expression.

group was observed to result in minimum MMP-13 mRNA expression, which was significantly different from other two Ral doses.

MMP-3 mRNA expression profile of groups was similar to that of MMP-13. The only difference was that MMP-3 mRNA level was lower in the control chondrocytes relative to all Ral treatment groups (figure 4B). Additionally, control chondrocytes were significantly different from 5 and 10 μM Ral-treated chondrocytes and MMP-3 mRNA expression for 1 μM Ral treatment group was significantly lower than other two Ral doses.

Although the differences in MMP-2 mRNA expression among groups did not reach statistical significance, it was found that the OA-like chondrocytes had higher MMP-2 mRNA level compared to the control chondrocytes (figure 4C). Moreover, 1 μM Ral treatment resulted in a slight decrease in MMP-2 mRNA expression relative to OA-like chondrocytes.

3.1.3 *Western blot analysis:* According to Western blot analysis, MMP-13 protein expression was higher in OA-like chondrocytes compared to the control chondrocytes, 1 and 10 μM Ral treatment groups (figure 4D). However, 5 μM Ral treatment group showed MMP-13 protein expression at a comparable level with OA-like chondrocytes. MMP-3 protein was highly expressed in OA-like chondrocytes with respect to all other groups. Following the OA-like chondrocytes, control chondrocytes demonstrated a decreased level of MMP-3 protein expression. Moreover, all Ral treatment groups had markedly decreased MMP-3 protein expressions. MMP-2 protein level was very low and did not change among the groups.

3.2 *Analyses in 3-D in vitro OA model*

3.2.1 *sGAG content:* The sGAG percentage of agarose discs of control chondrocytes was significantly higher than that of

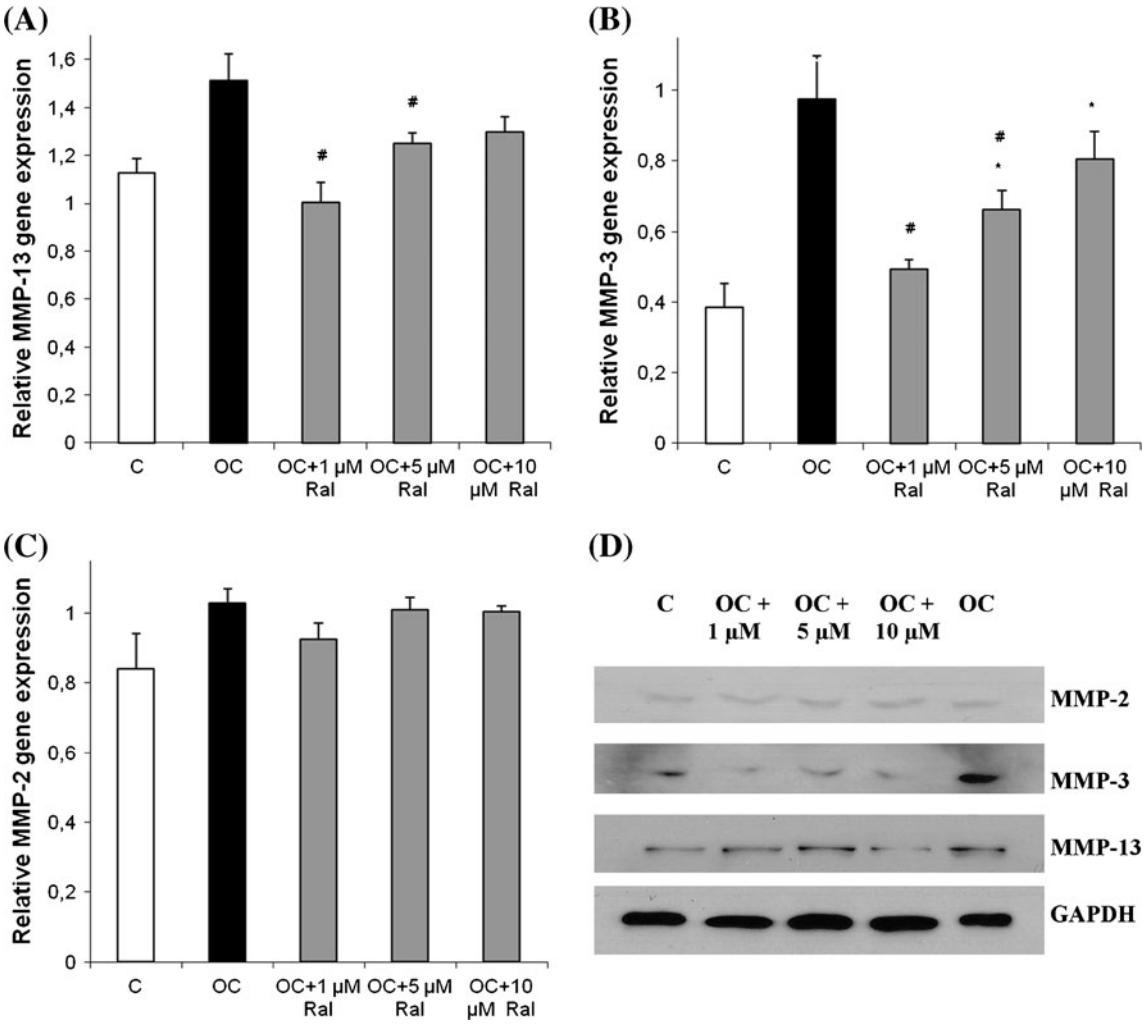

Figure 4. Effect of Ral treatment on (**A**) MMP-13, (**B**) MMP-3 and (**C**) MMP-2 gene expressions in OA-like chondrocytes. Mipep was used to normalize the gene expression levels. Data are presented as mean±SEM: (n=6, MMP-13), (n=4, MMP-3) and (n=6, MMP-2). '*n*' number indicates to two independent biological replicates, with three technical replicates. Independent-samples *t*-test: $^{*}P \leq 0.05$ versus the control chondrocytes, $^{\#}P \leq 0.05$ versus the OA-like chondrocytes. Statistically significant difference ($P \leq 0.05$) was observed between the OA-like and the control chondrocytes for MMP-13 and MMP-3 gene expressions. (**D**) Effect of Ral treatment on protein expressions of MMP-2, MMP-3 and MMP-13 in OA-like chondrocytes. GAPDH was used to normalize the amount of proteins.

the OA-like chondrocytes (0.057±0.007% versus 0.026±0.005%) (figure 5A). Ral treatment groups did not demonstrate any significant increase in the sGAG deposition compared to both control and OA-like chondrocytes. Only a slight increase was observed for 5 μM Ral treatment group relative to OA-like chondrocytes. Treatment with 10 μM Ral demonstrated a substantial decrease in the sGAG content relative to all the other groups with a significant difference from the control chondrocytes and 1 μM Ral treatment group.

3.2.2 *Collagen content:* It was observed that the collagen content of agarose discs of the control chondrocytes was significantly higher than that of OA-like chondrocytes (0.238±0.004% versus 0.221±0.005%) (figure 5B). Among the groups, the highest collagen deposition was observed in 1 μM Ral treatment group (0.275±0.010%), which showed a significant increase compared to both the control and OA-like chondrocytes and 10 μM Ral treatment group. A decrease in collagen content of agarose discs was observed as the Ral dose was increased to 10 μM with a significantly lower collagen amount relative to the control and OA-like chondrocytes.

3.2.3 *Mechanical properties:* The peak stress of agarose discs of OA-like chondrocytes was significantly lower than that of the control chondrocytes (3.464±0.072 versus 5.078±0.443)

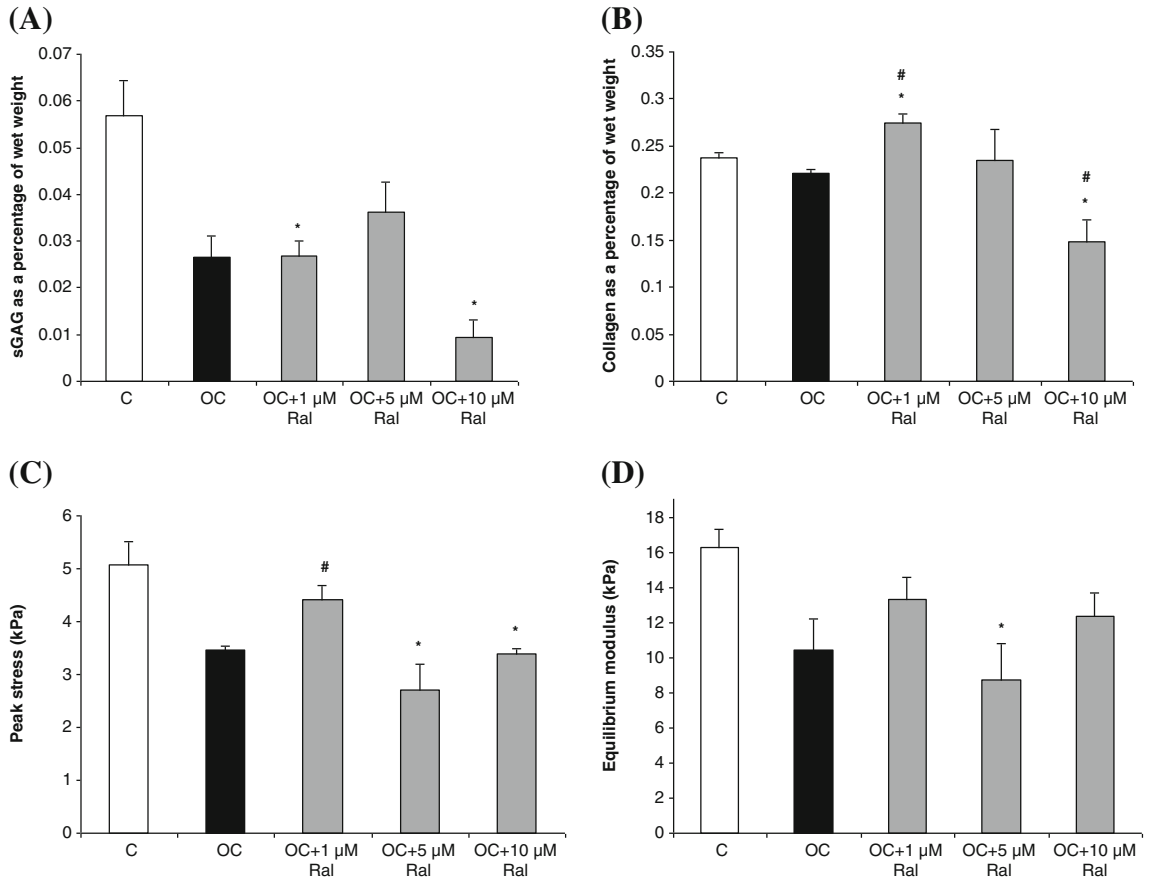

Figure 5. Effect of Ral treatment on (**A**) sGAG and (**B**) collagen deposition in the discs. Data are presented as mean±SEM: (n=3, sGAG) and (n=3, collagen). Independent-samples t-test: *$P \leq 0.05$ versus the control chondrocytes, $^{\#}P \leq 0.05$ versus the OA-like chondrocytes. Statistically significant difference ($P \leq 0.05$) was observed between the OA-like and the control chondrocytes. Amounts of both sGAG and collagen were normalized with wet weight of the agarose discs. Effect of Ral treatment on (**C**) peak stress and (**D**) equilibrium modulus of agarose-chondrocyte discs. Data are presented as mean±SEM (n=3). Independent-samples t-test: *$P \leq 0.05$ versus the control chondrocytes, $^{\#}P \leq 0.05$ versus the OA-like chondrocytes. Statistically significant difference ($P \leq 0.05$) was observed between the OA-like and the control chondrocytes.

(figure 5C). Treatment with 1 μM Ral resulted in a significant increase in peak stress with respect to OA-like chondrocytes, 5 μM Ral and 10 μM Ral treatment groups. One μM Ral treatment group showed a lower peak stress value compared to the control chondrocytes although this difference did not reach to statistical significance level. The agarose discs with OA-like chondrocytes had a significantly lower equilibrium modulus than that of the control group (10.398±1.830 versus 16.301±0.978) (figure 5D). Among Ral treatment groups, agarose-chondrocyte discs treated with 1 μM Ral had the highest equilibrium modulus followed by 10 μM Ral treatment group. Although not statistically significant, both of these groups demonstrated higher equilibrium moduli compared to the OA-like chondrocytes.

All Ral treatment effects (except MMP-13, MMP-3 and MMP-2 protein expressions) are compared with control and OA-like chondrocytes in table 2.

4. Discussion

Osteoarthritis (OA) is a chronic, progressively degenerating disease of the joints that includes loss of cartilage, bone thickening and synovial inflammation (Roman-Blas *et al.* 2009). Postmenopausal OA, resulting from hormone deficiency, is very common; therefore, treatment with hormone replacement therapy or the use of selective estrogen receptor modulators (SERMs) such as Raloxifene (Ral) has been explored in recent years. Direct estrogen application in animal models of OA has led to inconclusive results, whereas the use of SERMs appeared to be more beneficial, which has led to the recommendation of Raloxifene in postmenopausal OA patients (Snikers *et al.* 2008). However, the mechanisms by which Ral ameliorates the symptoms of OA are not clear. In this study, we have isolated chondrocytes from rats and induced OA-like changes with Aza-C and hypothesized the

Table 2. Summary of Ral treatment effects on osteoarthritis-like chondrocytes in 2-D and 3-D *in vitro* models

	C	OC	OC + 1 μM Ral		OC + 5 μM Ral		OC + 10 μM Ral	
			2-D Analyses					
Caspase-3 activity	▼*	Δ*	▼*	▽	▼	Δ*	▼	Δ*
Aggrecan expression	▲*	▽*	▲*	▽*	▲	▽*	▼	▽*
Collagen II expression	▲*	▽*	▲	▽	▼*	▽*	▼*	▽*
ALP expression	▼*	Δ*	▼*	Δ	▼*	▽*	▼*	▽*
Collagen X expression	▼*	Δ*	▼*	▽*	▼*	▽*	▼*	▽*
MMP-13 expression	▼*	Δ*	▼*	▽	▼*	Δ	▼	Δ
MMP-3 expression	▼*	Δ*	▼*	Δ	▼*	Δ*	▼	Δ*
MMP-2 expression	▼	Δ	▼	Δ	▼	Δ	▼	Δ
			3-D Analyses					
sGAG deposition	▲*	▽*	▲	▽*	▲	▽	▼	▽*
Collagen deposition	▲*	▽*	▲*	Δ*	▲	▽	▼*	▽*
Peak stress (kPa)	▲*	▽*	▲*	▽	▼	▽*	▼	▽*
Equilibrium modulus (kPa)	▲*	▽*	▲	▽	▼	▽*	▲	▽

Closed and open arrows are used for comparison of the groups with osteoarthritis-like chondrocytes (OC) and control chondrocytes (C), respectively. * indicates significant difference. Upward and downward arrows are used to show an increase and decrease in the parameter relative to C or OC, respectively.

amelioration of these changes with different doses of Ral. 1, 5 and 10 μM Ral were chosen as the appropriate doses based on a recent report by Tinti *et al.* (2011), who showed that a lower dose of 0.1 μM and 1 μM Ral had very similar effects in an IL-1β-induced OA model.

During the progression of OA, chondrocytes undergo three main changes: terminal differentiation, mineralization and finally apoptosis (Blanco *et al.* 1998; Kirsch *et al.* 2000). One set of mediators implicated in the apoptotic pathway belongs to the asparate-specific cysteinyl proteases or caspases. Caspase-3 (CPP32) is a key mediator of apoptosis in mammalian cells. In order to evaluate the induction of apoptosis in the chondrocytes, their caspase-3 activities were measured. It was found that caspase-3 activity of OA-like chondrocytes was significantly higher compared to that of the control chondrocytes (figure 2). This finding was in agreement with the study of Ho *et al.* (2006), who observed that the apoptotic rate of articular chondrocytes increased significantly after Aza-C exposure with respect to Aza-C-unexposed chondrocytes. Additionally, it was reported that a higher percentage of articular chondrocytes in OA patients underwent apoptosis relative to normal articular chondrocytes (Blanco *et al.* 1998; Hashimoto *et al.* 1998). In our study, 1 μM Ral treatment resulted in a significant decrease in caspase-3 activity of chondrocytes compared to OA-like chondrocytes. This result was in parallel with the study of Hattori *et al.* (2012), who reported that Ral significantly inhibited TNF-α-induced apoptosis in normal human knee chondrocytes (NHAC-Kn) and HCS-2/8 cells (chondrocytic cell line). Similarly, Tinti *et al.* (2011) reported that Ral (0.1 μM and 1 μM doses) restored the viability of IL-1β-stimulated human OA chondrocytes.

In the current study, aggrecan and collagen II gene expressions were significantly lower in OA-like chondrocytes compared to the control chondrocytes in accordance with changes occurring in osteoarthritic chondrocytes (figure 3A and B). This was another indication of OA-like alterations in the chondrocytes caused by Aza-C. Ho *et al.* (2006) also reported that collagen II mRNA expression decreased subsequent to Aza-C treatment. Additionally, Eid *et al.* (2006) observed that collagen II mRNA expression decreased more as the severity of OA increased. On the other hand, treatment with 1 μM Ral resulted in an increase in both aggrecan and collagen II gene expressions with respect to OA-like chondrocytes and other Ral treatment groups. Moreover, ALP and collagen X gene expressions were significantly higher in OA-like chondrocytes compared to control chondrocytes (figure 3C and D). Similarly, Ho *et al.* (2006) reported that mRNA expressions of collagen type X and ALP increased after Aza-C treatment. It was also reported that chondrocytes in articular cartilage, upon calcification, display features of hypertrophic growth plate chondrocytes and synthesize collagen X and ALP, both of which are the protein markers for hypertrophy (Rees and Ali 1988; Gannon *et al.* 1991). Kirsch *et al.* (2000) reported that ALP and collagen X were expressed by chondrocytes in the upper zone of early- and late-stage human osteoarthritic cartilage. In the current study, it was also found that ALP and collagen X mRNA levels in all Ral treatment groups were significantly lower compared to the OA-like chondrocytes.

Several studies have shown that MMP-13 plays a major role in the cleavage of collagen II and this situation increases in OA (Billinghurst *et al.* 1997). In our study, it was found that the OA-like chondrocytes had the highest MMP-13 gene expression among all groups, which was also significantly higher than that of the control chondrocytes (figure 4A). Treatment with 1 μM Ral resulted in the lowest MMP-13 gene expression, which was significantly lower than that of the OA-like chondrocytes. Lu *et al.* (2006) reported that Ral inhibited the MMP-13 promoter in rabbit synoviocytes by about 35–40% in the presence of estrogen receptor α isoform. Considering protein expression data, it was found that MMP-13 protein expression was the highest in OA-like chondrocytes and 5 μM Ral-treated cells (figure 4D). Moreover, 1 μM Ral treatment resulted in a lower protein level of MMP-13 relative to the OA-like chondrocytes although the expression level in this treatment group was not the lowest one. Differences in the patterns between gene and protein expression levels for the groups may originate from the fact that gene expression is also regulated at the post-transcriptional and post-translational levels. Thus, gene and protein expressions are sequential phenomena depending on time (De Croos *et al.* 2006). MMP-2 and MMP-3 are also the enzymes that are involved in the degradation of cartilage. According to Wu *et al.* (1991), fibrillar collagen II can be cleaved by MMP-3. Several studies have reported that MMP-3 can degrade glycosaminoglycan and type II collagen (Aigner *et al.* 2001; Malemud *et al.* 2003; Cawston and Young 2010). Saito *et al.* (1998) indicated that MMP-3 was important for the full activation of proMMP-1. It was also reported that MMP-2 had a direct effect on collagen. Besides that, it activates interstitial collagenase that cleaves collagen types I and II (Sang *et al.* 1996). In the current study, it was found that both MMP-3 gene expression (figure 4B) and protein level (figure 4D) in the OA-like chondrocytes were higher than those in the control chondrocytes. Lower MMP-3 at both mRNA and protein levels were detected in 1 μM Ral treatment group compared to the OA-like chondrocytes and 5 μM Ral group. This result was in agreement with the findings of Bellosta *et al.* (2007), who demonstrated that the expression of MMP-3 in the intimal cells was decreased when Ral was used in rabbit carotid lesions. On the other hand, in a recent study (Tinti *et al.* 2011), it was also shown that low amount of MMP-3 detected in the culture medium of human articular OA chondrocytes did not change upon incubation with Ral (0.1 and 1 μM) alone. However, after induction of MMP-3 with IL-1β, Ral treatment resulted in a statistically significant but very similar reduction of MMP3 at both concentrations of Ral. Unlike other MMPs, there was no statistical difference in MMP-2 gene expression among the groups in the current study (figure 4C). Only the control chondrocytes and 1 μM Ral treatment group had slightly lower level of MMP-2 gene expression compared to OA-like chondrocytes. MMP-2 protein levels were very low and similar among all groups (figure 4D). In the literature, Ral was shown to have different influences on MMP-2 gene expressions and protein levels in different cell types and tissues (Wołczynski *et al.* 2001; Christodoulakos *et al.* 2004; Bellosta *et al.* 2007; Zbucka *et al.* 2007). In this study, the effect of Ral on MMP-13 and MMP-2 in OA-like chondrocytes was demonstrated for the first time.

Matrix elements were also analysed in the 3-D *in vitro* OA model. It was observed that sGAG deposited in the discs by control chondrocytes was significantly higher than that by OA-like chondrocytes (figure 5A). This result was in agreement with the gene expression results. However, for the sGAG measurements, only a slight increase was observed for 5 μM Ral treatment group relative to the OA-like chondrocytes and sGAG content of 1 μM Ral treatment group was almost the same with that of the OA-like chondrocytes. sGAG deposited in discs (figure 5A) for 1 and 5 μM Ral treatment groups did not match with the results of PCR analysis conducted in 2-D model (figure 3A). Gene expression may not necessarily correlate with enzymatic activity of a protein as enzymes are frequently expressed as zymogens. Ten μM Ral treatment resulted in a remarkable decrease in the sGAG deposition even lower than that of OA-like chondrocytes (figure 5A). This negative effect for 10 μM Ral was also observed in aggrecan mRNA levels (figure 3A). In the study of Tinti *et al.* (2011), it was shown that Ral treatment (at 0.1 μM and 1 μM) significantly, but similarly, increased the proteoglycan level in IL-1β exposed human OA chondrocytes. In our study, it was also observed that the amount of collagen in the OA-like chondrocytes was significantly lower compared to that in the control chondrocytes (figure 5B). A similar significant difference was also observed for collagen II gene expression levels (figure 3B). Moreover, the amount of collagen for 1 μM Ral treatment group was significantly higher than that for the OA-like chondrocytes (figure 5B). This result was also consistent with gene expression findings since 1 μM Ral treatment resulted in a higher collagen II gene expression (figure 3B) compared to the OA-like chondrocytes and lower MMP-13 and MMP-3 gene expressions (figure 4A and B). It was reported that main collagen type in articular cartilage is collagen II constituting over half of the cartilage dry weight (Deshmukh and Nimni 1973). Accordingly, it was thought that, although the amount of various types of collagen may change in disease conditions, the trend observed for collagen II gene expression levels could be related to the total collagen amount.

The equilibrium modulus and peak stress of the agarose discs were analysed to evaluate biomechanical properties of the matrix. Compared to the agarose discs with control chondrocytes, the peak stress and the equilibrium modulus of those with OA-like chondrocytes were significantly

lower, indicating a decrease in these intrinsic mechanical properties upon OA-like changes. Such a decline might be related with the cumulative effect of differences in cellularity, sGAG and collagen depositions for the control and OA-like chondrocytes. The peak stress and the equilibrium modulus of 1 μM Ral treatment group were the highest values among Ral treatment groups and they were the ones closest to those of the control chondrocytes. Since 1 μM Ral treatment did not have a notable positive effect on sGAG synthesis, this outcome can be explained by the highest collagen content of this group. Williamson *et al.* (2001) also reported that matrix stiffness of cartilage explants was increased incrementally with collagen amount and, to a lesser extent, by proteoglycan amount. Similarly, it was also indicated that mechanical properties of agarose constructs were influenced much more by collagen content than sGAG content (Mauck *et al.* 2002). In our study, it was also observed that 5 μM Ral treatment group resulted in lower peak stress and equilibrium modulus compared to the OA-like chondrocytes, although its sGAG amount was higher and collagen amount was nearly the same compared to OA-like chondrocytes. Nearly similar peak stress value and slight improvement in the equilibrium modulus over those obtained for OA-like chondrocytes were observed for 10 μM Ral treatment group, despite its lower matrix deposition. According to Poole (2003), spatial organization of the extracellular matrix components synthesized by the chondrocytes had an influence on the functional behavior of articular cartilage. Thus, these results for the OA-like chondrocytes, 5 and 10 μM Ral treatment groups could be attributed to variations in the organization of the matrix elements. In our previous study (Kavas *et al.* 2011), the effect of Pluronic F-68 on matrix synthesis of OA-like chondrocytes was investigated in 3-D *in vitro* OA model, and it was shown that not only amount of matrix elements deposited by the chondrocytes but also spatial arrangement of matrix components had an important role in mechanical properties of 3-D constructs. Furthermore, Bastiaansen-Jenniskens *et al.* (2008) demonstrated that number of collagen cross-links in addition to the collagen content significantly affected mechanical features of extracellular matrix of bovine articular chondrocytes cultured in alginate beads.

5. Conclusion

As a conclusion, in this study, 2-D and 3-D *in vitro* OA models demonstrated the stimulatory effect of 1 μM Ral treatment on enhancement of collagen synthesis and improvement of mechanical properties. Treatment with 1 μM Ral also decreased the apoptotic activity of the OA-like chondrocytes to the same level with that of the control chondrocytes. This dose of Ral is likely to induce deposition of collagen both by up-regulating collagen II gene expression and down-regulating the gene and protein expressions of MMP-13 and MMP-3. Furthermore, gene expressions of ALP and collagen X were also reduced by 1 μM Ral treatment, showing recovery from OA-like alterations. Thus, 1 μM dose of Ral had a positive effect on functional matrix formation by OA-like chondrocytes, while increasing doses of this drug reversed these effects except ALP gene expression and sGAG deposition. Therefore, Ral at an optimum dose may have the potential to reverse OA-like alterations and have chondroprotective effect. However, the current study is an *in vitro* evaluation of the effects of Ral treatment on OA-like chondrocytes using 2- and 3-D models. Cell culture studies impose limitations on drawing conclusions about clinical results. The authors, therefore, suggest further *in vivo* studies for dose-dependent effects of Ral treatment on OA.

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