ARP Rheumatology 2022;1:30-41

ORIGINAL ARTICLES

Potential effect of Enterolactone and Raloxifene in reversing osteoarthritis markers in cultured Human articular chondrocytes

Hamdi T[1], Gouissem I[2]

ABSTRACT

Background: Osteoarthritis (OA) is associated with cartilage destruction. Maintenance of differentiated chondrocyte biomarkers is important in prevention of degeneration. Selective estrogen receptor modulators (SERMs) such as raloxifene (Ral) and phytoestrogens like enterolactone (Ent) which have structural and functional homologies with estrogen can mime their action. This study was undertaken to evaluate in vitro the possible effects of Ral and Ent on the expression of differentiated, dedifferentiated and hypertrophic biomarkers in human articular chondrocytes (HAC).

Methods: Chondrocytes of osteoarthritic patients, harvested from one passage cell culture, were treated with Ral (0.1 and 1 µM) and Ent (1 and 5 µM) for 10 and 12 days in two- (2D) and three-dimensional (3D) models. Genes expression of type I collagen (COL1A1), type II collagen (COL2A1), type X collagen (COL10A1), aggrecan (ACAN) and small novel rich in cartilage (SNORC), were detected by real-time PCR and by Western-Blotting.

Results: Our study revealed that Ral increased COL2A1, ACAN and SNORC and decreased concomitantly COL1A1 and COL10A1 genes expression when compared to untreated OA chondrocytes. Ent increased COL2A1 and decreased genes expression of the other biomarkers cited above like COL1A1. Our results demonstrated that low doses of 17β-oestradiol (E2), Ral and Ent had positive effects on the expression of differentiated chondrocyte markers such as COL2A1, ACAN and SNORC whereas high dose of these compounds inhibited their effects. Our results showed that 1µM of Ral induced an up-regulation of COL2A1, ACAN and SNORC expression and a down-regulation of COL1A1 and COL10A1 expression in HAC incubated in 3D for 12 days.

Conclusions: This study showed that SERMs like Ral and a phyto-estrogen like Ent induced the expression of differentiated chondrocyte markers of hyaline cartilage: COL2A1, ACAN and SNORC and decreased COL1A1 and COL10A1 genes expression on HAC of osteoarthritis patients.

Keywords: Raloxifene; Enterolactone; Osteoarthritis; Collagens; Cartilage; Chondrocytes.

INTRODUCTION

Osteoarthritis (OA) is a chronic disease due to cartilage degradation. The risk of developing this disease increases with age, so this pathology remains the leading cause of disability for the elderly. Indeed, the cartilage cellular component is disrupted in articular cartilage leading to its erosion. Moreover, other components of the joint are involved in OA such as subchondral bone alterations and synovial membrane inflammation[1]. Over many years, the mechanical overload and biochemical mediators are involved in cartilage destruction. Mechanical stress sets off degenerative cartilage factors cascade. Thus, chondrocytes are exposed to autocrine, paracrine and other catabolic factors which involved the synthesis of metalloproteinases (MMPs), aggrecanases, cyclooxygenases, cytokines, nitric oxide (NO), and prostaglandins[2-4]. Moreover, hormonal changes occurring around menopause induce the occurrence of OA[5]. The depletion of sex hormones has been considered as a risk factor for OA but the effects of estrogen replacement in OA patients are controversial. Some studies have reported a chondroprotective effect of estrogen, while others have shown no effect of these substances on cartilage[6]. To verify these controversies, we test the effect of two alternative sex hormone analogs raloxifene (Ral) and enterolactone (Ent) on the redifferentiation of human arthritic chondrocytes (HAC).

It has been reported that Ral, a selective Estrogen Receptor Modulators (SERMs) mixed estrogen agonist/

[1] Biochemistry Department LR99ES11 / University of Carthage, Faculty of Sciences of Bizerte, Zarzouna, Bizerte-Tunisia University of Tunis El Manar, Research Laboratory LR99ES11, Biochemistry Department, Rabta University Hospital, Tunis-Tunisia; [2]University of Carthage, Faculty of Sciences of Bizerte, Zarzouna, Bizerte-Tunisia

Submitted: 02/05/2021
Accepted: 03/10/2021

Correspondence to: Thouraya Hamdi
E-mail: thouraya.hamdi14@gmail.com

antagonist[7-9], prevents breast cancer and osteoporosis in women[10] and rats[11]. Additionally, Ral is approved worldwide for the prevention and treatment of post-menopausal osteoporosis[12,13], and reduction of the risk of invasive breast cancer[14,15]. Ral acts on lipids, on the coagulation system, and uterine tissues[16]. In contrast, another study reported that Ral has neutral effects on endometrium[17]. Otherwise, Ral had protective effect on human OA chondrocytes from interleukin-1β which involved the matrix metalloproteinase (MMP-3) and NO release[18].

Furthermore, Ent a phytoestrogen substance produced naturally in plants and has similar structure to estrogen, binds to the estrogen receptors (ERα and ERβ) and acts as agonist in some tissues and as antagonist estrogen in others[19]. Binding of phytoestrogens to ERs induces a conformational change which promotes their interaction with promoter regions on DNA[20,21]. This conformational change precisely at the position of helix 12, depends on the effect of the phytoestrogen so activates or represses the transcription of estrogen target genes[22]. The relative affinities of phytoestrogen for the ER favor them to act as agonists and/ or antagonists in each ER subtype[23,24] depend on concentrations and tissue sites[25].

The aim of the present study was to evaluate in vitro the possible effects of Ral and Ent on the expression of differentiated biomarkers type II collagen (COLII), aggrecan (ACAN) and SNORC, dedifferentiated type I collagen (COLI) and hypertrophic type X collagen (COLX) in HAC using both monolayer 2D and biomaterial 3D models.

MATERIAL AND METHODS

Human articular chondrocytes (HAC) were treated in vitro with different concentrations of 17β-E2 (0.1 and 1 nM), Ral (0.1 and 1 µM) and Ent (1 and 5 µM) for 10 days in monolayer (2D) or 12 days in type I collagen sponges (3D) models. Phenotype gene expressions of differentiated chondrocytes such as ACAN, COLII and SNORC, of dedifferentiated chondrocyte like COLI, or of hypertrophic chondrocytes such as COLX, were analyzed by real-time PCR and Western Blotting.

Chondrocytes primary culture

HAC were obtained following the laying of total knee arthroplasty from 15 patients donors (six women and nine men) aged 63-85 years (mean 74 ± 11 years) from orthopedic surgery department of Caen Saint Martin Polyclinic (Normandy, France). The mean body mass index of patients was 29.9 ± 2.5 kg/m2. The study was conducted in accordance with the ethical guidelines of the Helsinki Declaration. The design of the study was approved by the Ethics Committee of Caen (Comité de Protection des Personnes Nord Ouest III, Caen, France). All patients gave written informed consent for cartilage samples used in this study. HAC extraction is based on a well-defined protocol. HAC from macroscopically healthy areas of osteoarthritic joints were cut into small chips, rinsed in Phosphate Buffered Saline (PBS) and then subjected to a wash cycle decantation. The fragments were then subjected to the following enzymatic treatments: Protease type XIV (6.2 U / mL) for 45 minutes at 37°C and Clostridium histolyticum collagenase (290 U / mL) overnight at 37°C. These enzymes were diluted in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose, buffered to pH 7.1/7.4 and supplemented with 10% of decomplemented fetal calf serum (FCS). To this mixture was added a mixture of antibiotics and anti- fungal compound of penicillin (100 IU / mL), erythromycin (100 mg / mL) and fungizone (0.25 microgram / mL). Chondrocytes were then dissociated and the cell suspension was filtered and centrifuged for 10 minutes at 700 g. Cells were seeded in culture dishes at a density of 40 000 cells/cm^2 in DMEM containing 10% FCS at 37°C and 5% CO_2. During treatment of the cells with Ral, Ent or 17β-E2 (used as positive control), This reduction is due to the glycolytic production of NAD(P)H in viable cells. The number of viable cells is determined according to the cleavage of the tetrazolium salts WST-1 added to the culture medium into soluble formazan by cellular dehydrogenases (Berridge). So, the amount of the culture medium was DMEM 1% L-glutamine, 1% sodium pyruvate and supplemented with 2% steroid-free FCS. Cells were seeded either in monolayer at 40 000 cells/cm^2 or in type I collagen sponge 3D at 400 000 cells/cm^2.

Prior to study the expression of articular biomarkers on cultured HAC, cell viability of chondrocytes is evaluated using WST-1 assay[26] with kit from Roche Diagnostics. Following WST-1 test, cell proliferation was performed by crystal violet test allows cell to be counted by staining adherent cell[27]. In our study, cell viability rate of chondrocytes was 80 %.

RNA extraction, reverse transcription and real-time Polymerase Chain Reaction

Total RNA was isolated with TRIzol reagent according to the manufacturer's instructions (Invitrogen). One microgram (µg) of total RNA was reverse-transcribed with DNase I (Invitrogen) into complementary DNA (cDNA) at 37°C for 1 hour using 50 pmoles of oligo dT, 5 mM of each dNTPs, reverse transcriptase buffer 5X, 40 units/µL of RNase inhibitor and 200 units/µL of "Moloney Murine Leukemia Virus" enzyme (Invitrogen) in a final volume of 25 µL. The cDNA samples were diluted 1/100 before amplifications in real-time PCR analyses, with appropriate primers, SYBR Green PCR Master Mix

and a ABI Prism 7000 SDS apparatus (all from Applied Biosystems). Ribosomal protein L13a (RPL13a) was used as an endogenous reference gene. The relative gene expression was calculated with the 2-ÄÄCt method or the standard curve method depending on the amplification deficiency of each RPL13a and target gene, and expressed as mean of triplicate samples. Primers were: SNORC (S: 5'-GTGCCCACGCTGTGGAACGA-3', AS: 5'-CTCCTGGTCCAGCCGCTCCT-3'), COL2A1 (S: 5'-GGCAATAGCAGGTTCACGTACA-3', AS: 5'-CGATAACAGTCTTGCCCCACTT-3'), COL10A1 (S: 5'-CAGCCGGTCCAGGGATTC-3', AS: 5'-AAACCAGGAGAGAGAGGACCATATG-3'), COL1A1 (S: 5'-CAGATCACGTCATCGCACAAC-3', AS: 5'-ACCAATCACCTGCGTACAGAA-3'), ACAN (S: 5'-TCGAGGACAGCGAGGCC-3', AS: 5'-TCGAGGGTGTAGCGTGTAGAGA-3'), RPL13 (S: 5'-GAGGTATGCTGCCCCACAAA-3', AS: 5' -GTGGGATGCCGTCAAACAC-3').

Western-blotting

After cells treatment, they were washed once with ice-cold PBS crushed and lysed in RIPA buffer to prepare cellular extracts for Western-blotting. Whole-cell extracts (20 µg) were separated in a 10% polyacrylamide gel and transferred onto polyvinylidenedifluoride (PVDF) membrane (Millipore, Molsheim, France) at 4°C for 75 minutes. The membranes were blocked for 1 hour in Tris buffer saline (20 mM Tris, 137 mM NaCl, 0.1% Tween 20) containing 10% skim milk and were probed with the appropriate antibodies (Santa Cruz Biotechnology, Inc.) overnight at 4°C. The membranes were then washed and incubated for 1 hour at room temperature with anti-goat secondary peroxidase-conjugated antibody (Santa-Cruz Biotechnology, Inc.). The bands corresponding to the desired proteins were visualized by enhanced chemiluminescence Luminol Reagent (Tebu-Bio) and exposed to X-ray sensitive film. The type I and II collagen signals were quantified by densitometry with the Image Quant software (Amersham Biosciences, Orsay, France) in reference to the GAPDH control signal.

Statistics

Results were representative of one experiment, performed in triplicates unless otherwise precisions. For real-time PCR analysis, data were representative of four experiments performed in triplicate each one. Difference between control and treated group was assessed by the Student's t test. Structure of the data was first tested in order to assess normality (Kolmogorov-Smirnov test) and equality of variance (Levene test). One-way analysis of variance (One-Way ANOVA) were used to test for overall differences between controls and treatments and LDS or Dunn multiple comparisons tests were used in pairwise comparisons of treatments and control. Statistically significant differences were set at *p<0.05, **p<0.01 and ***p<0.001 **versus control and †p<0.05 ; ‡p<0.001 versus 1µM Ent or 0.1µM Ral treated groups.**

RESULTS

Expression of articular chondrocyte biomarkers in the presence of Ent cultured in 2D and / or 3D models.

Analyses of Western-Blotting of 2D and 3D models after treatment with Ent

We determined the effect of Ent (1 and 5 µM) on protein expression of type I collagen (COL1A1) and type II collagen (COL2A1) by Western blotting analysis of HAC cultured in monolayer (2D) for 10 days and in 3D model for 12 days (Figure 1A). Ent at 1 µM concentrations did not modify protein level of COL2A1 but 5 µM increased COL2A1 protein level by 1.63 -fold. On the other hand, Ent 1 and 5 µM reduced those of COL1A1 by 44% and 21% respectively in monolayer culture (Figure 1B). The HAC culture of another patient for 12 days in 3D model (Figure 1B) showed that Ent at 1 and 5 µM stimulated the production of COL2A1 protein levels by 1.60 -fold and 1.37 -fold respectively, and COL1A1 by 1.55 -fold and 1.44 -fold respectively (Figure 1B).

In our study, the Ent has the same effects on protein expression of both collagens (COL2A1 and COL1A1) regardless of the dose used on HAC incubated on 3D model. Whereas in monolayer culture (2D), the Ent (1 µM) did not change the COL2A1 protein expression but decreased COL1A1 protein level.

Analyses of real-time Polymerase Chain Reaction of 2D and 3D models

When HAC of three patients are incubated in hypoxia for 10 days in monolayer 2D model, the analysis of real-time PCR indicated that Ent at 1 and 5 µM increased significantly the expression of COL2A1 by 3.21-fold (p<0.001) and 1.71-fold (p<0.05) respectively (Figure 2A). On the other hand, these doses induced a significant reduction in mRNA levels of the dedifferentiation marker, the COL1A1, by 48% and 79% respectively (p<0.001) (Figure 2B). In addition, we evaluated the COL2A1/COL1A1 mRNA ratio, differentiation index which provides an indication of the differentiation status of chondrocytes and their functional index[28-31]. The differentiation index calculated from COL2A1/ COL1A1 was higher : 6.22 -fold and 7.95 -fold in monolayer cultured chondrocytes stimulated with 1 and 5 µM of Ent respectively in 2D model (Figure 2C). Thereafter, we have measured the impact of a biomaterial (sponge of type I collagen) on the mRNA expression of COL2A1 and COL1A1. We used the 3D model as previously des-

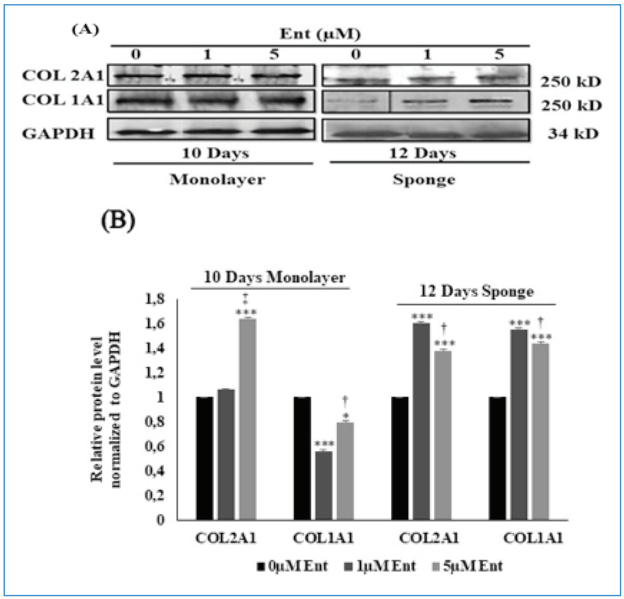

Figure 1. (A): Effect of enterolactone (ENT) on protein expression of type II collagen (COL2A1) and type I collagen (COL1A1) in human articular chondrocytes cultured after one passage in hypoxia monolayer (2D) or collagen sponge (3D). (B): Quantification of the relative expression of COL2A1 and COL1A1 after treatment with Ent (1 and 5 µM) by densitometric analysis of the blots using the Image J software and normalized to GAPDH protein control signal. The significance of differences was assessed using the Student's t-test *p<0.05 ; ***p<0.001 vs control ; †p<0.05 ; ‡p<0.001 vs 1µM Ent.

Human articular chondrocytes (HAC) obtained following digestion of the fragments of the human articular cartilage by specific enzymes, were cultured and amplified in the presence of 21% oxygen in a medium containing phenol red DMEM + 10% FCS. After one passage, HAC from two patients were cultured in monolayer (at 40 000 cells per cm2) for 24 hours in normoxia in phenol red-free DMEM + 10% steroid-free and decomplemented FCS. For 3D culture, HAC from these patients were first grown in sponge of type I collagen (at 400 000 cells per sponge) in the presence of 21% oxygen for 16 hours in a medium containing phenol red-free DMEM + 10% steroid-free and decomplemented FCS. For both models (2D and 3D), the next day (day 0 of treatment), HAC were treated with phenol red-free DMEM, supplemented with 2% steroid-free FCS, treated or not with Ent (1 and 5 µM) for 10 and 12 days in a hypoxic environment (3% oxygen). Control represents cells without Ent treatment (0 µM). Then, the total protein extracts (20 µg) from these HAC were subjected to SDS-PAGE for analysis by "Western-blotting" with anti-type II collagen (COL2 A1), anti-type I collagen (COL1 A1) and anti-glyceraldehyde 3-phospho dehydrogenase (GAPDH) reference gene antibodies. Western-blots were repeated two times with similar results, and a representative western blot is displayed.

cribed[32] Our data revealed that the expression of COL2A1 mRNA was enhanced by 3.5 -fold ($p < 0.001$) (Figure 2A), while the expression of COL1A1 mRNA was significantly inhibited by 80% ($p<0.001$) (Figure 2B). Moreover the differentiation index COL2A1 / COL1A1 was higher (17.5 -fold) in 3 D model (Figure 2C). This ratio was in the range of 3.76 -fold and 24.52 -fold in the presence of 0.1 and 1 nM 17β-E2 respectively used as positive control. These results suggested the redifferentiation of chondrocytes by the Ent.

In addition, comparison of COL2A1 and COL1A1 mRNA expression induced by Ent in the two models (2D and 3D) revealed that in sponge 3D, the relative expression of CO2A1 was slightly higher than that obtained in 2D model. While the inhibition of COL1A1 mRNA expression in 3D model was comparable to that found in the 2D model.

Expression of articular chondrocyte biomarkers in the presence of Ral cultured in 2D and / or 3D models

Analyses of Western-Blotting of 2D and 3D models after treatment with Ral

First, we tested the effect of Ral on protein expression of COL1A1 and COL2A1 on HAC of a patient cultured in 2D and of another patient in 3D for 10 and 12 days respectively by Western Blotting (Figure 3A). Whatever the dose or model used, the protein expression of COL2A1 is promoted by Ral especially in 3D system, compared to the untreated HAC (Figure 3B). Ral at 0.1 and 1 µM increased COL2A1 by 1.57 -fold and 1.87 -fold respectively in 3D culture and by 1.44 -fold and 1.29 -fold respectively in monolayer model (Figure 3B). However, these two doses 0.1 and 1 µM of Ral slightly modified the biomarker of dedifferentiation COL1A1 by 1.10 -fold and 1.17 -fold respectively in 3D model. While in monolayer (2D) model, the protein expression of COL1A1 remained unchanged (1.09 -fold) with 0.1 µM Ral but increased (1.52 -fold) with Ral at 1 µM (Figure 3B).

In addition, comparison of the protein expression of COL2A1 and COL1A1 in these models revealed that the expression of COL2A1 was higher in 3D than in 2D model but COL1A1 protein expression was less in sponge 3D than in monolayer model.

Analyses of real-time Polymerase Chain Reaction of 2D and 3D models.

When HAC from 4 patients are incubated in hypoxia for 10 days in monolayer 2D system, the analysis of real-time PCR showed that Ral at 0.1 and 1 µM, increased significantly the expression of COL2A1 by 1.95 -fold ($p<0.05$) and 6.93 -fold ($p<0.001$) respectively (Figure 4A). Looking at the effect of the positive control, 17β-E2, there was a significant increase in monolayer 2D model

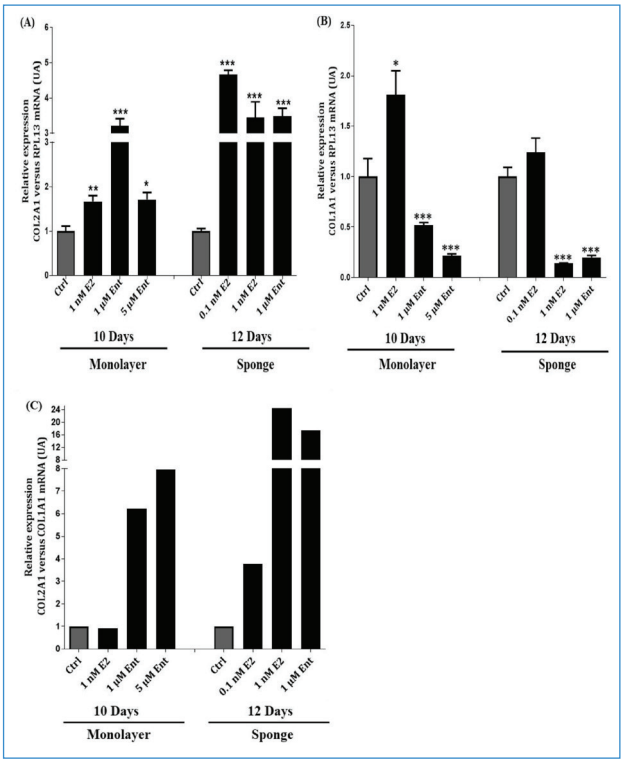

Figure 2. Effect of enterolactone (Ent) on mRNA expression of COL2A1 (A) and COL1A1 (B) in HAC from different patients and differentiation index (COL2A1/COL1A1 ratio) (C) of HAC under the effect of Ent in monolayer (2D) and sponge (3D).

Human articular chondrocytes (HAC) obtained following digestion of the fragments of the human articular cartilage by specific enzymes, were cultured and amplified in the presence of 21% oxygen (normoxia) in a medium containing phenol red DMEM + 10% FC. After one passage, HAC from three patients were cultured in monolayer (at 40 000 cells per cm2) for 24 hours in normoxia in phenol red-free DMEM + 10% steroid-free and decomplemented FCS. HAC from these patient were initially grown in a biomaterial (at 400 000 cells per sponge) in the presence of 21% oxygen for 16 hours in a medium containing phenol red-free DMEM + 10% steroid-free and decomplemented FCS. For both culture models (2D and 3D), the next day (day 0 of treatment), HAC are treated with phenol red-free DMEM, supplemented with 2% steroid-free FCS, treated or not with Ent (1 and 5 µM) for 10 and 12 days and 17β-E2 (0.1 and 1 nM) in a hypoxic environment (3% oxygen). Control (Ctrl) represents cells without Ent and 17β-E2 treatment. Then the RNAs from these HAC were extracted for analysis by RT-PCR with primers specific genes collagen type II (COL2A1) and primers specific genes collagen type I (COL1A1). The domestic reference gene is the gene RPL13 ("Ribosomal Protein L13"). The results represent the mean of three measurements (triplicate) ± SD. The significance of differences was assessed using the Student t test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Differentiation index are calculated by the ratio of the normalized relative expression of COL2A1 mRNA relative to that of COL1A1. U.A: arbitrary unit.

with 0.1 nM dose ($p<0.05$). For ACAN mRNA expression, we observed in monolayer that the two concentrations of 0.1 and 1 µM of Ral increased significantly the expression by 2.14 -fold and 3.74 -fold ($p<0.05$ and $p<0.001$) respectively (Figure 4B). Concerning the positive control, we observed a significant expression in monolayer model with the two doses of 0.1 and 1 nM of 17β-E2 ($p<0.01$). On the other hand, in monolayer we observed that Ral at 0.1 µM increased the expression of mRNA SNORC but not significantly, while Ral (1 µM) decreased this expression not significantly (Figure 4C). As regards the chondrocyte marker of dedifferentiation, the expression of COL1A1 in monolayer is decreased but not significantly with 0.1 µM of Ral whereas 1 µM of Ral increased significantly this expression by 1.42 -fold ($p<0.01$) (Figure 4D). The effect of Ral on the expression of COL10A1 showed a significant increase by 1.2 -fold ($p<0.05$) in monolayer with 0.1 µM and a significant decrease with 1 µM of Ral by 93% ($p<0.01$) (Figure 4E).

Thereafter, we used the 3D model to determine the mRNA expression of the biomarkers (COL2A1, ACAN, SNORC, COL1A1 and COL10A1). We observed that the Ral (0.1 µM and 1 µM) increased significant the COL2A1 mRNA expression by 1.42 -fold ($p<0.01$) and 1.35 -fold ($p<0.01$) respectively (Figure 4A). Moreover, the Ral (1µM) increased significantly the expression of 17β-E2 mRNA ($p<0.001$), the positive control (Figure 4A). On the other hand, the Ral (0.1 and 1 µM) enhanced significantly the ACAN mRNA expression by 1.34 -fold and 1.53 -fold respectively ($p<0.05$) (Figure 4B) and mRNA SNORC expression by 1.43 and 1.99 -fold ($p<0.05$ and $p<0.001$) respectively (Figure 4C). In contrast, Ral (0.1 and 1 µM) inhibited the expression of COL1A1 mRNA by 23% ($p<0.05$) and 42% ($p<0.01$) respectively (Figure 4D) and only the dose of 1 µM Ral decreased significantly the expression of COL10A1 by 85% ($p<0.001$) (Figure 4E).

To evaluate the differentiation index of chondrocytes under the effect of Ral, we determined the ratios of COL2A1 / COL1A1 mRNA, COL2A1 / COL10A1, ACAN / COL1A1, ACAN / COL10A1, SNORC / COL1A1 and SNORC / COL10A1. In 3D model, Ral (0.1 and 1 µM) increased the ratios of COL2A1 / COL1A1 by 1.84 -fold and 2.32 -fold respectively; and COL2A1 / COL10A1 by 1.30 fold and 9.0 -fold; and ACAN / COL1A1 by 1.74 -fold and 2.63 -fold; ACAN / COL10A1 by 1.22 -fold and 10.2 -fold; SNORC / COL1A1 by 1.85 -fold and 3.43 -fold; and finally SNORC / COL10A1 by 1.31 -fold and 13.26 -fold. In 2D monolayer model, the Ral (0.1 and 1 µM) increased ratios of COL2A1 / COL1A1 by 2.09 -fold and 4.80 -fold; COL2A1 / COL10A1 by 1.68 -fold and 7.57 -fold; ACAN / COL1A1 by 2.31 -fold and 2.63 -fold; ACAN / COL10A1 by 1.84 -fold and 53.42 -fold; and finally, SNORC / COL10A1 by 1.12 -fold and

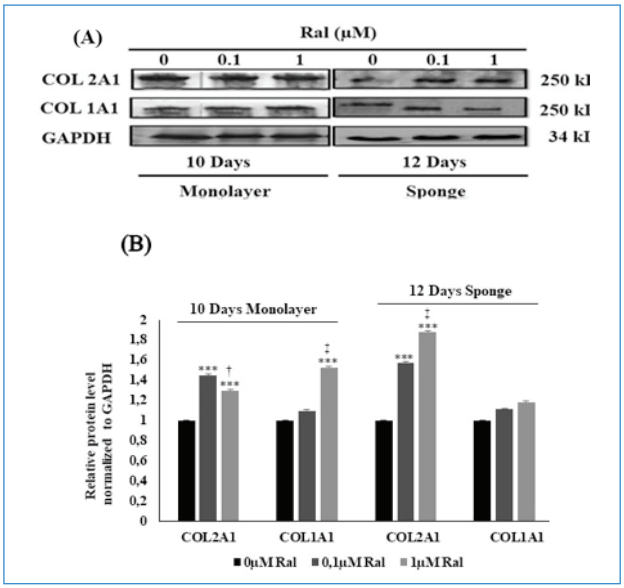

Figure 3. (A): Effect of raloxifene (Ral) on protein expression of type II collagen (COL2A1) and type I collagen (COL1A1) in human articular chondrocytes cultured after one passage hypoxia monolayer (2D) or collagen sponge (3D). (B): Quantification of the relative expression of COL2A1 and COL1A1 after treatment with Ral (0.1 and 1 µM) by densitometric analysis of the blots using the Image J software and normalized to GAPDH protein control signal. The significance of differences was assessed using the Student's t-test ***p<0.001 vs control ;†p<0.05 ; ‡p<0.001 vs 0.1µM Ral.

Human articular chondrocytes (HAC) obtained following digestion of the fragments of the human articular cartilage by specific enzymes, were cultured and amplified in the presence of 21% oxygen in a medium containing phenol red DMEM + 10% FCS. After one passage, HAC from two patients were cultured in monolayer (at 40 000 cells per cm2) for 24 hours in normoxia in DMEM phenol red-free + 10% steroid-free and decomplemented FCS. HAC from these patients were first grown in a biomaterial (at 400 000 cells per sponge) in the presence of 21% oxygen for 16 hours in a medium containing phenol red-free DMEM + 10% steroid-free and decomplemented FCS. For both models of cultures (2D and 3D), the next day (day 0 of treatment), HAC are treated with phenol red-free DMEM, supplemented with 2% steroid-free FCS, treated or not with Ral (0.1 and 1 µM) for 10 and 12 days in a hypoxic environment (3% oxygen). Control represents cells without Ral treatment (0 µM). Then, the total protein extracts (20 µg) from these HAC were subjected to SDS-PAGE for analysis by "Western-blotting" with type anti type II collagen (COL2 A1) anti type I collagen (COL1 A1) and anti-glyceraldehyde 3-phospho dehydrogenase (GAPDH) reference gene antibodies. Western-blots were repeated two times with similar results, and a representative western blot is displayed.

11.28 -fold respectively. The SNORC / COL1A1 ratio was increased by 1.4 -fold but decreased by 55% with 0.1 and 1 µM of Ral respectively.

Mean studied chondrocytes biomarkers of HAC obtained from four patients cultured in 3D with Ent and Ral

Considering all the results of HAC of different patients obtained individually, and in order to confirm the chondrogenic effects of the studied molecules, we maintained the cell culture conditions that gave the best differentiation index and repeated the experiments of HAC from four patients cultured in hypoxia environment and 3D model. Thereby, we used Ent at 1 µM and Ral at 1µM and 1 nM of 17β-E2 as positive control. The results of these experiments are presented in figure 5. The analysis of RT -PCR indicated that Ral at 1 µM increased slightly but not significantly the mRNA expression of COL2A1 (Figure 5A), ACAN (Figure 5B) and SNORC (Figure 5C), whereas the mRNA expression of COL1A1 was reduced significantly by 43% ($p<0.01$) (Figure 5D) but the mRNA expression of COL10A1 was almost unchanged (Figure 6E). In contrast, the Ent at 1 µM enhanced significantly the mRNA expression of COL2A1 by 1.3 -fold ($p<0.05$) (Figure 5A), but inhibited the mRNA expression of COL1A1 by 46% ($p<0.05$) (Figure 5D) and COL10A1 by 45% ($p<0.05$) (Figure 5E).

The chondrogenic differentiation index revealed that the concentration of 1 µM of Ral increased significantly the ratios of COL2A1 / COL1A1 by 1.88 -fold ($p<0.01$) (Figure 6A) and SNORC / COL1A1 by 2.32 -fold ($p<0.05$) (Figure 6E) and enhanced but not significantly the ratios of COL2A1 / COL10A1 by 2.87 -fold (Figure 6B), ACAN / COL1A1 by 2.32 -fold (Figure 6C), ACAN / COL10A1 by 5.60 -fold (Figure 6D) and SNORC / COL10A1 by 4.98 -fold (Figure 6F). On the other hand, Ent at 1 µM increased significantly ($p<0.05$) the ratios of COL2A1 / COL1A1 by 4 -fold (Figure 6A), COL2A1 / COL10A1 by 3 -fold (Figure 6B) and ACAN / COL1A1 by 1.92 -fold (Figure 6C). When multiple comparisons between differentiation index were made COL2A1 / COL1A1 and SNORC / COL10A1 were found significantly different ($p <0.039$).

DISCUSSION

The imbalance between the anabolic and catabolic activities of chondrocytes is responsible for the pathogenesis of osteoarthritis. Indeed, the arthritic process is a modulation of the chondrocyte phenotype inducing the decreased production of type II collagen and aggrecan and the increased expression of type I and X collagen[33]. Several factors are involved in the process of cartilage degradation. Among them interleukins (ILs)[18] are implicated in the cartilage resorption by stimulation of

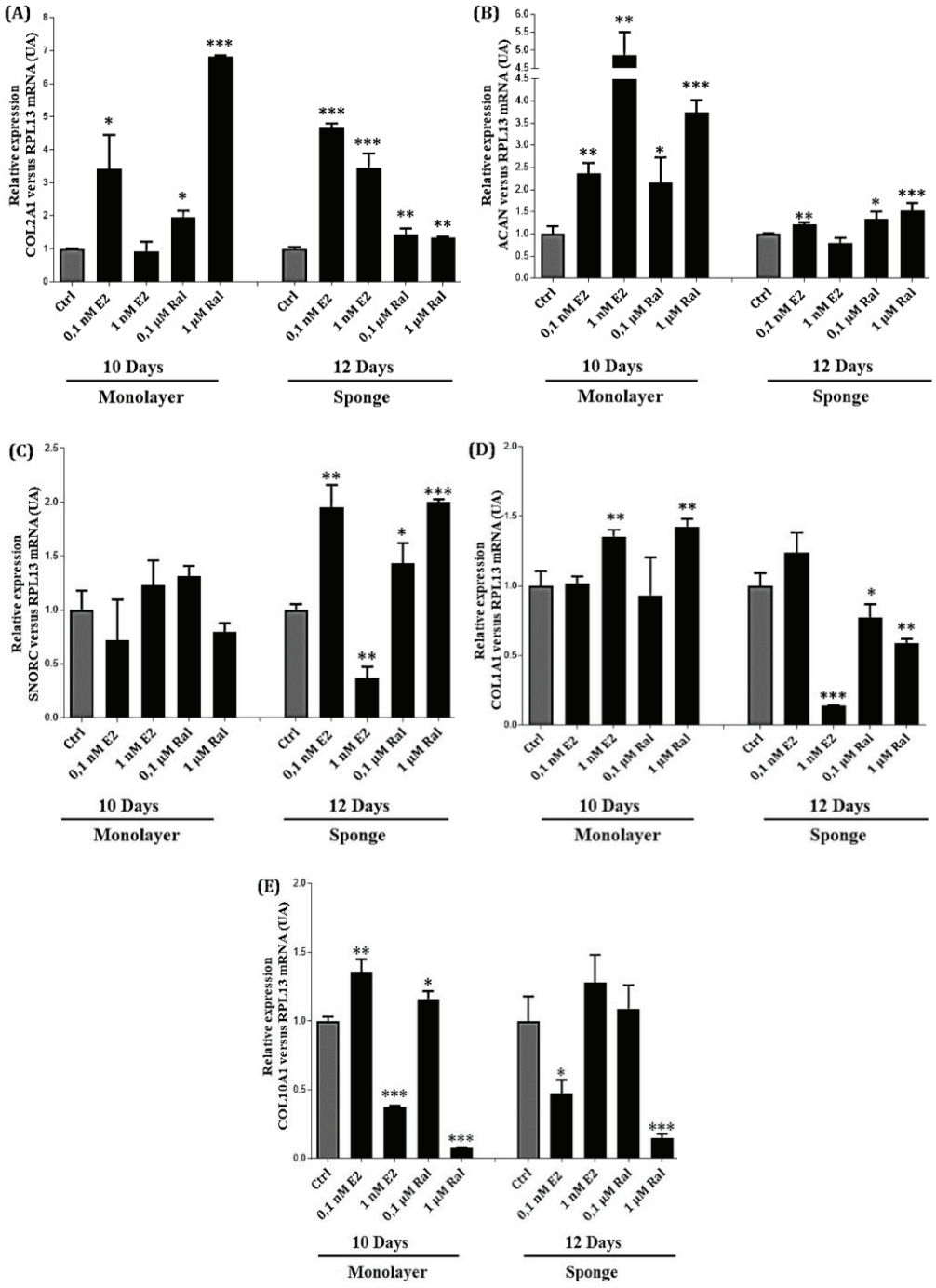

Figure 4. Effects of raloxifene (Ral) on mRNA expression of COL2A1 (A), of ACAN (B) SNORC (C), COL1A1 (D) and of COL10A1 (E) in HAC from different patients incubated in hypoxia monolayer (2D) or sponge (3D) for 10 or 12 days.

Human articular chondrocytes (HAC) obtained following digestion of the fragments of the human articular cartilage by specific enzymes, were cultured and amplified in the presence of 21% oxygen (normoxia) in a medium containing phenol red DMEM + 10% FCS. After one passage, HAC from four patients were cultured in monolayer (at 40 000 cells per cm2) for 24 hours in normoxia in DMEM phenol red-free + 10% steroid-free and decomplemented FCS. HAC from these patients were first grown in a biomaterial (at 400 000 cells per sponge) in the presence of 21% oxygen for 16 hours in a medium containing phenol red-free DMEM + 10% steroid-free and decomplemented FCS. For both models, the next day (day 0 of treatment), HAC are treated with phenol red-free DMEM, supplemented with 2% steroid-free FCS, treated or not with Ral (0.1 and 1 µM) and 17β-E2 (0.1 and 1 nM) for 10 and 12 days in hypoxic environment (3% oxygen). Then the RNAs from these HAC were extracted for analysis by RT-PCR with primers specific gene type II collagen (COL2A1) from "Small Novel Rich in Cartilage" (SNORC), aggrecans (ACAN), collagen type I (COL1A1), and X-type (COL10A1). The domestic reference gene is the gene RPL13 ("Ribosomal Protein L13"). The results represent the mean of three measurements (triplicate) ± SD. The significance of differences was assessed using a Student t test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Ctrl: control, Ral: raloxifene, E2: 17β-E2, U.A: arbitrary unit.

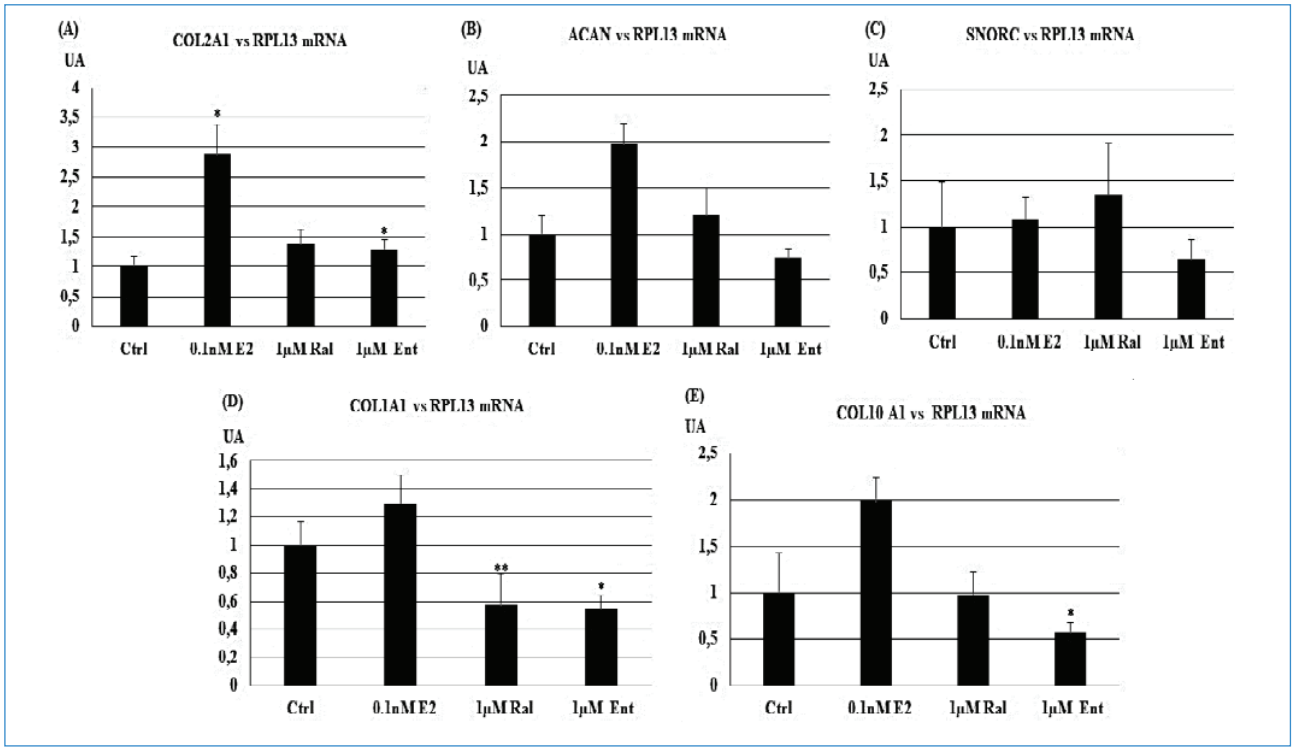

Figure 5. Effect of 17β-E2 (E2), Raloxifene (Ral) and Enterolactone (Ent) on mRNA expression of COL2A1 (A), ACAN (B), SNORC (C), COL1A1 (D) and COL10A1 (E) in HAC from four patients (n = 4) incubated in hypoxia and 3D environment.

Human articular chondrocytes (HAC) obtained following digestion of the fragments of the human articular cartilage by specific enzymes, were cultured and amplified in the presence of 21% oxygen in a medium containing phenol red DMEM + 10% FCS. After one passage, HAC from four patients were initially grown in a biomaterial (at 400 000 cells per sponge) in the presence of 21% oxygen for 16 hours in a medium containing phenol red-free DMEM + 10% steroid-free and decomplemented FCS. The next day (day 0 of treatment), HAC are treated with phenol red-free DMEM, supplemented with 2% steroid-free FCS, treated or not with 17β-E2 (0.1 nM), Ral (1 µM) and Ent (1 µM) for 12 days in hypoxic environment (3% oxygen). Then the RNAs from these HAC were extracted for analysis by RT-PCR with specific primers of the genes for type II collagen (COL2A1), aggrecans (ACAN), "Small Novel Rich in Cartilage" (SNORC), type I collagen (COL1A1) and type X collagen (COL10A1). The domestic reference gene is the gene RPL13 ("Ribosomal Protein L13"). The results represent the mean of three measurements (triplicate) ± SD. The significance of differences was assessed using a student t test (* $p < 0.05$). Ctrl: control, E2: 17β-E2, Ral: raloxifene, Ent: enterolactone, U.A: arbitrary unit.

the MMPs[34] and catabolic factors of chondrocytes, involved in the degradation of extracellular matrix. Moreover, depletion of estrogens in postmenopausal women is a risk factor of OA[35] because estrogens play an important role in the regulation of collagen metabolism, both in the bone, intervertebral discs and in articular cartilage[36,37]. Our findings revealed that Ent and Ral, a phytoestrogen and a selective estrogen receptor modulator respectively increased type II collagen and aggrecan and reduced type I and X collagens in cultured human chondrocytes using both monolayer 2D and biomaterial 3D systems.

Our results with Ent 1 µM showed that this phytoestrogen modulated the biomarkers. Indeed, the mRNA and protein expression of type II collagen, the specific differentiated chondrocytes marker are increased by the stimulation of Ent in cultured HAC. The trend was similar when considering the COL1A1 which decreased. In addition, the differentiation index[28-31] based on the ratio of mRNA levels of collagen COL2A1 / COL1A1 indicated change of HAC favoriting type II collagen expression, a differentiated chondrocytes marker. Taken together these results suggested that the Ent could protect cartilage from the loss of its integrity. Our results were in accordance with a previous study[38] testing the effect of another phytoestrogen, the genistein, and has reported that this molecule had not significant effect on human cartilage glycoprotein 39 (YKL-40), a marker of cartilage catabolism. Thus, these authors[38] have obtained a positive effect of this phytoestrogen on chondrocytes by inhibiting the production of pro-inflammatory molecules COX-2 and nitric oxide (NO)[39] which promote

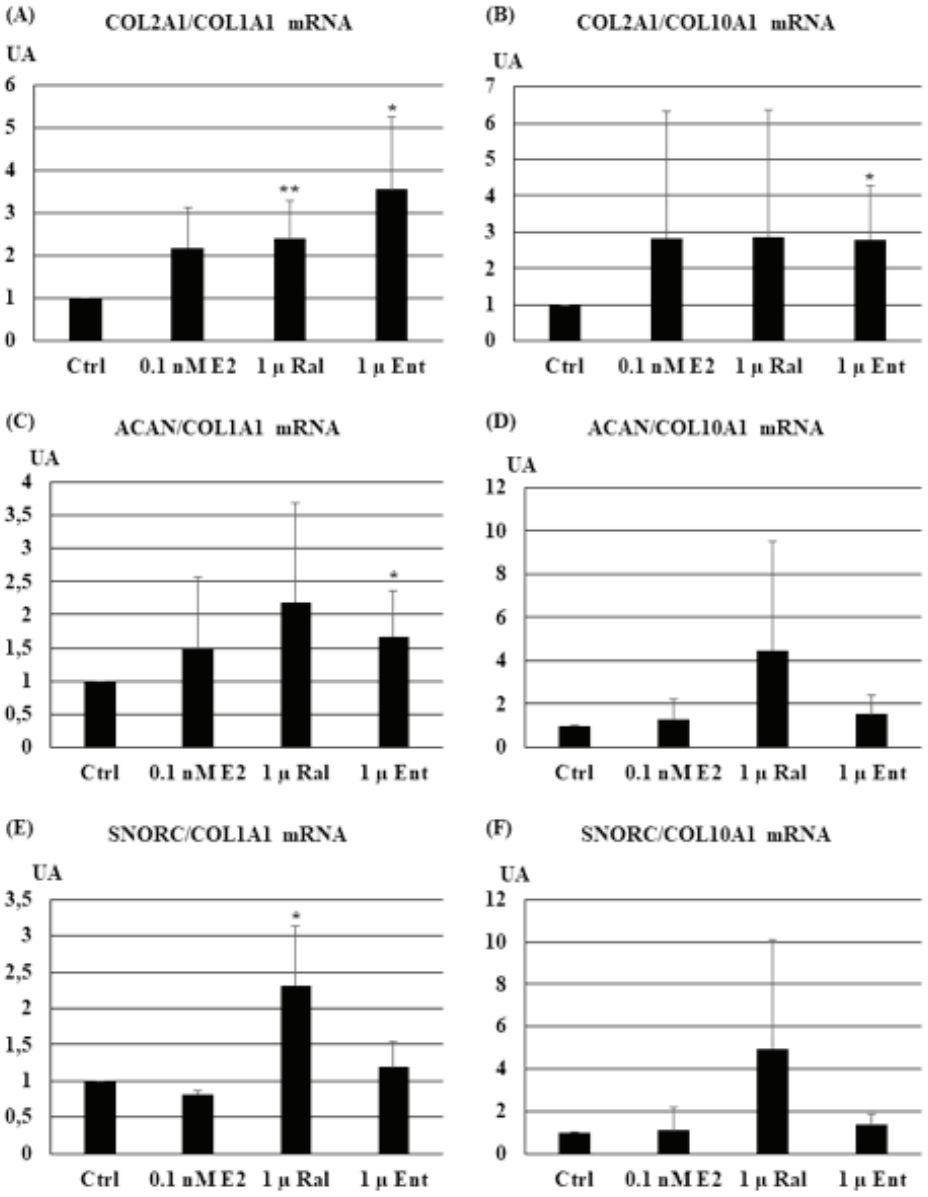

Figure 6. Differentiation index of COL2A1 / COL1A1 (A), COL2A1 / COL10A1 (B), ACAN / COL1A1 (C), ACAN / COL10A1 (D), SNORC / COL1A1 (E) and SNORC / COL10A1 (F) of HAC from four patients (n = 4) incubated in sponge 3D in the presence of 17β-E2 (E2), Raloxifene (Ral) and Enterolactone (Ent).

Human articular chondrocytes (HAC) obtained following digestion of the fragments of the human articular cartilage by specific enzymes, were cultured and amplified in the presence of 21% oxygen in a medium containing phenol red DMEM + 10% FCS. After one passage, HAC from four patients were initially grown in a biomaterial (at 400 000 cells per sponge) in the presence of 21% oxygen for 16 hours in a medium containing phenol red-free DMEM + 10% steroid-free and decomplemented FCS. The next day (day 0 of treatment), HAC are treated with phenol red-free DMEM, supplemented with 2% steroid-free FCS, treated or not with 17β-E2 (0.1 nM), Ral (1 µM) and Ent (1 µM) for 12 days in hypoxic environment (3% oxygen). Then the differentiation index is calculated respectively by the ratio of the relative mRNA expression of COL2A1, ACAN and SNORC normalized relative to those of COL1A1 and COL10A1. Statistical analysis was performed using the One-Way ANOVA test. Comparison of the differences between biomarkers ratios was performed using the One-Way ANOVA test. Ctrl: control, E2: 17β-E2, Ral: raloxifene, Ent: enterolactone, U.A: arbitrary unit.

matrix degradation in human chondrocytes. Moreover, some studies have shown that the asian vegetarian diet allowed women less osteoporosis[40]. Indeed, vegetarians and people who consume a high and variable quantity of products rich in lignans like flaxseed and sesame seeds can reach micro-molar concentrations of Ent in their serum[41] that protect them from osteoarthritis[42]. Thus, phytoestrogens effects depend particularly on the used dose.

Concerning the effect of Ral on chondrocytes cultured in 2D and 3D models, we observed a significant increased mRNA and protein expression of type II collagen and a decreased COL1A1 mRNA expression in the HAC. We have used Ral at 0.1 and 1 µM concentrations as it is reported in a previous study[18] which suggested positive effects of Ral on HAC by induction of proteoglycans secretion and inhibition of IL-1β which induced MMP-3 and NO release. Our data showed also in 3D model that Ral significantly increased the major markers of hyaline cartilage COL2A1, ACAN and SNORC. Furthermore, the differentiation index of COL2A1/ COL1A1 and COL2A1 / COL10A1 revealed a positive effect of Ral on type II collagen expression. Similarly, the differentiation index indicated that the Ral (1 µM) increased the ratios of ACAN / COL1A1, ACAN / COL10A1, SNORC / COL1A1 and SNORC / COL10A1. Indeed, in articular cartilage, aggrecan are responsible for providing compressive and elastic strength to articular cartilage and regulating fluid in the cartilage matrix. Consistent with our findings, a previous study[43] on rat chondrocytes cultures in 2D and 3D models reported a significant increase in the expression of COL2A1 and ACAN under the effect of Ral 1 µM. Indeed, hormone replacement therapy could prevent and treat OA by these molecules having the same effect of estrogen on cartilage[44]. The present study suggested that Ral and Ent may have protective role on HAC.

Finally, we must acknowledge that the present study has a number of limitations. The results remain to be confirmed by using a large sample size. Furthermore, the western blotting analyses revealed differences that were not highlighted by the RT-PCR experiments. Indeed, comparison of the protein levels of COL2A1 and COL1A1 in 2 D and 3 D cultures treated by Ent or Ral showed differentially expression and provided divergent results. It might be explained by the fact that in vitro, the treatment of cartilage defects have some limitations. Moreover, divergent biomarkers results between 2D and 3D cultures were obtained suggesting that chondrocytes interactions in 3D culture may have an impact on specific protein expression of cells. It seems that cells in 3D culture have a higher degree of structural complexity and a more real chondrocytes microenvironment than in 2D.

CONCLUSION

The present study showed that SERMs like Ral and a phyto-estrogen like Ent can act as natural hormones, the estrogens, on articular chondrocytes of osteoarthritis patients by reshaping differentiation indexes and stimulating expression of COL2A1, ACAN and SNORC, the differentiated biomarkers of hyaline cartilage. These results could open investigations fields concerning the use of these molecules in cellular engineering of the cartilage but also in the context of the hormonal replacement therapy for pre- or post-menopausal women to reduce side effects of natural estrogen.

ACKNOWLEDGEMENTS

We would like to thank Dr Sylvain Leclercq from orthopedic surgery department of Caen Saint Martin Polyclinic (Normandy, France) and the whole team of the laboratory of Microenvironnement Cellulaire and Pathologies (MILPAT) EA 4652/ BIOTARGEN EA 7450 for their help.

DECLARATION OF INTEREST

The authors declare that there are no conflicts of interest.

FUNDING

We are grateful to the laboratory of Microenvironnement Cellular and Pathologies (MILPAT) EA 4652/ BIOTARGEN EA 7450, UFR Santé, Université de Caen Normandie 14032 Caen France and the French Ministry of Research and Technology.

REFERENCES

1. Goldring MB, Goldring SR. Osteoarthritis. J Cell Physiol 2007;213:626–634. https://doi.org/10.1002/jcp.21258.
2. Moos V, Fickert S, Muller B, Weber U, Sieper J. Immunohistological analysis of cytokine expression in human osteoarthritic and healthy cartilage, J Rheumatol 1999;126:870–879. https://pubmed.ncbi.nlm.nih.gov/10229409/.
3. Attur MG, Dave M, Cipolletta C, Kang P, Goldring MB, Patel IR, et al. Reversal of autocrine and paracrine effects of interleukin 1 (IL-1) inhumanarthritis by type II IL-1 decoy receptor. Potential for pharmacological intervention. J Biol Chem 2000;275:40307–40315. https://doi.org/10.1074 / jbc.M002721200.
4. Hardy MM, Seibert K, Manning PT, Currie MG, Woerner BM, Edwards D, et al. Cyclooxygenase 2–dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants. Arthritis Rheum 2002 ;46 :1789–1803. https://doi.org/10.1002 / art.10356.
5. Felson DT, Zhang Y. An update on the epidemiology of knee and hip osteoarthritis with a view to prevention. Arthritis Rheum 1998;41:1343–1355. https://doi.org/10.1002 / 1529-0131 (199808) 41: 8 <1343 :: AID-ART3> 3.0.CO; 2-9.
6. Felson DT, Nevitt CM. The effects of estrogen on osteoarthritis. Curr Opin Rheumatol 1998;10:269–272. https://doi.org/10.1097 / 00002281-199805000-00019.
7. Muchmore, D. Raloxifene: a selective estrogen receptor modulator (SERM) with multiple target system effects. Oncologist 2000;5:388-392. https://doi.org/10.1634 / théoncologue.5-5-388.
8. Taras TL, Wurz GT, DeGregorio MW. In vitro and in vivo biologic effects of FC-1271a in breast cancer. J Steroid Biochem Mol Biol 2001;7:271-279. https://doi.org/10.1016 / s0960-0760 (01) 00066-8.

9. Cho CH, Nuttall ME. Therapeutic potential of oestrogen receptor ligands in development for osteoporosis. Expert Opin Emerg Drugs 2001;6:137-154. https://doi.org/10.1517 / 14728214.6.1.137.
10. Maximov PY, Lee TM., Jordan VC. The Discovery and Development of Selective Estrogen Receptor Modulators (SERMs) for Clinical Practice. Curr Clin Pharmacol 2013;8:135-155. https:// doi.org/10.2174 / 1574884711308020006.
11. M, Kim J, Short LL, Slemenda CW, Bryant HU. Longitudinal and cross-sectional analysis of raloxifene effects on tibiae from ovariectomized aged rats. J Pharmacol Exp Ther 1995;272:1252–1259.
12. Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 1999;282:637-645. https://doi.org/10.1001 / jama.282.7.637.
13. Delmas PD, Ensrud KE, Adachi JD, Harper KD, Sarkar S, Gennari C, et al. Efficacy of raloxifene on vertebral fracture risk reduction in postmenopausal women with osteoporosis: four-year results from a randomized clinical trial. J Clin Endocrinol Metab 2002;87:3609-3617. https://doi.org/10.1210 / jcem.87.8.8750.
14. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA 1999;281:2189-2197. https://doi.org/10.1001 / jama.281.23.2189.
15. Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA., Purdie DW, at al. Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Multiple outcomes of raloxifene evaluation. Breast Cancer Res Treat 2001;65:125-134. https://doi.org/10.1023 / a: 1006478317173.
16. Barrett-Connor E. Raloxifene: risks and benefits. Ann N Y Acad Sci 2001;949:295–303. https://doi.org/10.1111/j.1749-6632.2001.tb04036.x.
17. DeMichele A, Troxel AB, Berlin JA, Weber AL, Bunin GR, Schinnar ET, et al. Impact of raloxifene or tamoxifen use on endometrial cancer risk: a population-based case–control study. J Clin Oncol 2008;26:4151–4159. https://doi.org/10.1200 / JCO.2007.14.0921.
18. Tinti L, Niccolini S, Lamboglia A, Pascarelli NA, Cervone R, ¬ Fioravanti A. Raloxifene protects cultured human chondrocytes from IL-1β induced damage: A biochemical and morphological study. Eur J Pharmacol 2011;670:67–73. https://doi.org/10.1016 / s0960-0760 (01) 00066-8.
19. Albertazzi P, Purdie D. The nature and utility of the phytoestrogens: a review of the evidence Maturitas 2002;42:173–175. https://doi.org/10.1016 / s0378-5122 (02) 00024-5.
20. McDonnell DP, Connor CE, Wijayaratne A, Chang CY, Norris JD. Definition of the molecular and cellular mechanisms underlying the tissue-selective agonist/antagonist activities of selective estrogen receptor modulators. Recent Prog Horm Res 2002;57:295–316. https://doi.org/10.1210 / rp.57.1.295.
21. Smith CL, O'Malley BW. Coregulator function : a key to understanding tissue specificity of selective receptor modulators. Endocr Rev 2004;25:45–71. https://doi.org/10.1210 / er.2003-0023.
22. Osborne CK., Zhao H, Fuqua SAW. Selective estrogen receptor modulators: structure, function, and clinical use. J Clin Oncol 2000;18:3172-3186. https://doi.org/10.1200 / JCO.2000.18.17.3172.
23. Miksicek RJ. Estrogenic flavonoids: structural requirements for biological activity. Proc Soc Exp Biol Med 1995;208:44–50. https://doi.org/10.3181 / 00379727-208-43830.
24. Paige LA, Christensen DJ, Grøn, H, Norris JD, Gottlin EB, Padilla KM., et al. Estrogen receptor (ER) modulators each induce distinct conformational changes in ER alpha and ER beta. Proc Natl Acad Sci U S A 1999;96:3999–4004. https://doi.org/10.1073/ pnas.96.7.3999.
25. Cassidy A. Potential tissue selectivity of dietary phytoestrogens and estrogens. Curr Opin Lipidol 1999;10:47–52. https://doi.org/10.1097 / 00041433-199902000-00009.
26. Berridge MV, Tan AS, Herst PM. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol Annu 2005; 11:127–152. doi: 10.1016/S1387-2656(05)11004-7.
27. Saotome K, Morita H, Umeda M. Cytotoxicity test with simplified crystal violet staining method using microtitre plates and its application to injection drugs. Toxicol In Vitro 1989; 3:317–321.doi: 10.1016/0887-2333(89)90039-8.
28. Barlič A, Drobnič M, Maličev E, Kregar-Velikonja N. Quantitative analysis of gene expression in human articular chondrocytes assigned for autologous implantation. J. orthopaedic research 2008 ; 26 : 847-853. https://doi.org/10.1002/jor.20559
29. Diaz-Romero J, Nesic D, Grogan SP, Heini P, Mainil-Varlet P. Immunophenotypic changes of human articular chondrocytes during monolayer culture reflect bona fide dedifferentiation rather than amplification of progenitor cells. J Cell Physiol 2008 ;214 :75-83. doi: 10.1002/jcp.21161.
30. Toh WS, Guo XM, Choo AB, Lu K, Lee EH, Cao T. Differentiation and enrichment of expandable chondrogenic cells from human embryonic stem cells in vitro. J Cell Mol Med 2009 ;13 :3570-3590. doi: 10.1111/j.1582-4934.2009.00762.x.
31. Agar G, Blumenstein S, Bar-Ziv Y, Kardosh R, Schrift-Tzadok M, Gal-Levy R, et al. The Chondrogenic Potential of Mesenchymal Cells and Chondrocytes from Osteoarthritic Subjects: A Comparative Analysis. Cartilage 2011 ;21 :40-49. doi: 10.1177/1947603510380899.
32. Campoccia D, Doherty P, Radice M, Brun P, Abatangelo G, Williams DF. Semisynthetic resorbable materials from hyaluronan esterification. Biomaterials 1998;19:2101-2127. https://doi.org/10.1016 / s0142-9612 (98) 00042-8.
33. Goldring MB, Birkhead J, Sandell LJ, Kimura T, Krane SM. Interleukin 1 Suppresses Expression of Cartilage-specific Types 11 and IX Collagens and Increases Types I and III Collagens in Human Chondrocytes. J Clin Invest 1988; 82: 2026-2037. doi: 10.1172/JCI113823.
34. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, et al. Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. J. Clin. Invest 1997;99:1534–1545. https://doi.org/10.1172 / JCI119316.
35. Christgau S, Tanko LB, Cloos PA, Mouritzen U, Christiansen C, Delaissé JM, et al. Suppression of elevated cartilage turnover in postmenopausal women and in ovariectomized rats by oestrogen and a selective oestrogen-receptor modulator (SERM). Menopause 2004;11:508–518. https://doi.org/10.1097 / 01.wcb.0000121484.18437.98.
36. Movérare-Skrtica S, Börjessona AE, Farmana HH, Sjögrena K, Windahla SH, Lagerquista MK., et al. The estrogen receptor antagonist ICI 182,780 can act both as an agonist and an inverse agonist when estrogen receptor α AF-2 is modified. Proc Natl Acad Sci U S A 2014;111:1180–1185. https://doi.org/10.1073 / pnas.1322910111.
37. Gambacciani M, Pepe A, Cappagli B, Palmieri E, Genazzani AR. The relative contributions of menopause and aging

to post-menopausal reduction in intervertebral disc height. Climacteric 2007;10:298-305. https://doi.org/10.1080 / 13697130701457729.
38. Hooshmand S, Soung DY, Lucasc EA, Madihallyd SV, Levensona CW, Arjmandi BH. Genistein reduces the production of proinflammatory molecules in human chondrocytes. J Nutr Biochem 2007;18:609–614. https://doi.org/10.1016/j.jnutbio.2006.11.006.
39. Amin AR, Dave M, Attur M, Abramson SB. COX-2, NO, and cartilage damage and repair. Curr Rheumatol Rep 2000;2:447-453. https://doi.org/10.1007 / s11926-000-0019-5.
40. Ho-Pham LT, Vu BQ, Lai TQ, Nguyen ND, Nguyen TV. Vegetarianism, bone loss, fracture and vitamin D: a longitudinal study in Asian vegans andnon-vegans. Eur J Clin Nutr 2012;66(1):75-82. https://doi.org/10.1038 / ejcn.2011.131.
41. Adlercreutz H, Bannwart C, Wähälä K, Makela T, Brunow G, Hase T, et al. Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. J Steroid Biochem Mol Biol 1993;44:147-153. https://doi.org/10.1016/0960-0760(93)90022-O.
42. Phitak T, Pothacharoen P, Settakorn J, Poompimol W, Caterson B, Kongtawelert. P. Chondroprotective and anti-inflammatory effects of sesamin. Phytochemistry 2012;80:77-88. https://doi.org/10.1016 / j. phytochem.2012.05.016.
43. Kavas A, Cagatay ST, Banerjee S, Keskin D, Tezcaner A. Potential of Raloxifene in reversing osteoarthritis-like alterations in rat chondrocytes: An in vitro model study. J Biosci 2013;38:1–13. https://doi.org/10.1007 / s12038-012-9282-7.
44. Bolognese MA. SERMs and SERMs with estrogen for postmenopausal osteoporosis. Rev Endocr Metab Disord 2010;11:253–259. https://doi.org/10.1007 / s11154-010-9137-1.