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The Use of Bone Morphogenetic Protein-7 and Resveratrol in Collagen Type II of Articular Cartilage

Molaba Gloria Mmadira and Motaung Shirley Keolebogile

Tshwane University of Technology, Faculty of Science, Department of Biomedical Sciences, Private Bag X 680, Pretoria, South Africa, 0001

Abstract: This study aimed to investigate the effects of resveratrol and bone morphogenetic protein 7 on type II collagen from superficial and middle zone of porcine articular chondrocytes. Articular cartilage was isolated from dissected porcine knee joint n = 12. Isolated cells were plated as monolayers at a density of 1×10^5 cells/well in 12-well culture plates and incubated at 37°C in a humid atmosphere of 5% carbon dioxide and 95% air. Cell cultures were treated for four days with various concentrations of bone morphogenetic protein-7 and resveratrol. Cells were then collected and analysed for collagen type II expression by real time polymerase chain reaction and protein level quantification by enzyme-linked immunosorbent assay. Cartilage tissue sections were localised for collagen type II by immunohistochemistry. Moreover, resveratrol and bone morphogenetic protein-7 effects on cartilage matrix contents were analysed by histology. Resveratrol and bone morphogenetic protein-7 stimulates expression of collagen type II mRNA and protein level accumulation in the surface zone and middle zone at 50 µM + 300 ng/ml (RSV + BMP-7). Immunohistochemistry results confirmed the presence of collagen type II on articular cartilage. Histological tissue sections confirmed that chondrocytes were obtained from different zones of articular cartilage. The study suggests that a combination of bone morphogenetic protein-7 and resveratrol up-regulate the expression and synthesis of collagen type II.

Key words: Articular cartilage, osteoarthritis, collagen type II, resveratrol, bone morphogenetic protein-7.

1. Introduction

Articular cartilage is critical to the normal function of human and animal joints, providing lubrication and load-bearing to allow locomotion and movement. It is a uniquely avascular, aneural, and alymphatic tissue comprised of an ECM (extensive extracellular matrix) with very few cells [1]. Cartilage has a defined stratified structure composed of superficial, middle, deep, and calcified zones [2, 3], each with distinct cell densities and phenotypes, molecular architecture, and mechanical properties [4, 5]. It is composed of ECM macromolecules such as type II collagen, aggrecan, hyaluronan, chondroitin sulfate, and decorin. Collagen type II fibrils provide the tensile strength and maintain the integrity of mammalian articular cartilage by forming a network that resists the swelling pressure resulting from the hydration of the polyanionic proteoglycan aggregates in the extracellular matrix. Damage to this fibrillar meshwork, made up of primarily type II collagen may be a critical event in the pathology of many arthritides, due in part to the very slow rate of collagen turnover within the cartilage [6-9].

On a microscopic scale, the collagen and proteoglycan content in the tissue varies with depth from the articulating surface. Collagen is the major organic constituent of cartilage and accounts for 15-22 % of the wet tissue weight [10-12]. Collagen content is highest in the surface zone and decreases by approximately 15 % in the middle and deep zones of the tissue [1, 4, 6-8]. Proteoglycan accounts for 4-7 % of the wet tissue weight [1, 13-15]. In contrast to the distribution of collagen content, proteoglycan content is lowest in the surface zone and increases by approximately 15 % in the middle and deep zones [6-8]. Water distribution throughout the tissue is similar to

Corresponding author: Dr SCKM Motaung, D. Tech. biomedical technology, research field: tissue engineering.

that of collagen, accounting for more than 80 % of the wet tissue weight at the surface and 65 % in the deep zone [8, 16].

The surface zone of the articular cartilage has flattened discoid cells that are aligned tangentially to the surface and secrete a proteoglycan called SZP (surface zone protein) [17, 18]. The surface zone of articular cartilage is responsible for appositional growth, and appears to contain progenitor cells [19]. The middle zone consists of spherical cells arranged in perpendicular columns and is characterised by type II collagen, aggrecan, and CILP (cartilage intermediate layer protein) [20]. The deep zone includes the calcified area of cartilage the tidemark distinguishes between the noncalcified and calcified areas [6, 7, 13]. The characteristic zonal architecture is intimately linked to the biological function [9, 15, 21].

The ECM (extracellular matrix) of cartilage contains numerous non-collagenous proteins, collagens and proteoglycans [22, 23]. The main type of proteoglycan found in cartilage is aggrecan, which provides the compressive strength of cartilage [24]. Others include decorin, biglycan and fibromodulin. One of the more prominent non-collagenous proteins is COMP (cartilage oligomeric matrix protein) [25-27].

Cartilage repair is dependent on the production of matrix by chondrocytes, which are stimulated by anabolic morphogenetic proteins, such as BMPs (bone morphogenetic proteins), TGF-β (transforming growth factor), and IGF- (1insulin-like growth factor-1), and the actions of catabolic cytokines such as IL-1β (Interleukin-1 beta), and TNF-α (tumour necrosis factor alpha). Changes in the cartilage homeostasis are thought to precede the shift to OA (osteoarthritis). It is well established that morphogens and growth factors play an important role in cartilage homeostasis [28-34]. Bone morphogenetic proteins are pleiotropic regulators of the cartilage and bone differentiation cascade including chemotaxis of progenitor cells, mitosis of mesenchymal stem cells, and differentiation of cartilage and bone. It has been shown that BMPs induce new bone and cartilage formation *in vitro* and *in vivo* [28]. BMP-7 (bone morphogenetic protein-7), also called human OP-1 (osteogenic protein-1), plays an important role in human and bovine cartilage homeostasis and repair [30, 35-37]. Many studies have shown that BMP-7 and TGF-β1 (transforming growth factor-beta 1) can synergistically promote increased survival and matrix synthesis by normal and osteoarthritic human articular chondrocytes [31, 38-41]. Other growth factors, such as basic FGF-2 (fibroblast growth factor-2), IGF-1 (insulin-like growth factor-1) [42, 43] and PDGF (platelet-derived growth factor), have all been shown to be anabolic for cartilage and chondrocytes [41, 44].

RSV (resveratrol) is isolated from the roots of white hellebore. Resveratrol (3,5,4-trihydroxystilbene) is a polyphenolic phytoalexin compound found in various plants, such as grape vines, berries, peanuts, seeds and roots; the highest concentration is in the skin of red grapes [45-48]. This component of red wine has potent anti- inflammatory properties and may reduce the side effects of non-steroidal anti-inflammatory drugs that are currently used and may thus offer new opportunities for the treatment of OA. The anti-inflammatory effects of resveratrol have been shown in several animal model studies [49]. Therefore, resveratrol might be the relevant compound for potential use in osteoarthritis therapy. There is therefore a dire need to detect cartilage loss before it is severe. Natural products such as RSV do not have the disadvantages mentioned above like the non-steroidal anti-inflammatory drugs. This offers novel and alternative treatment opportunities for OA. The existence of traditional and complementary medicine is known to be a fertile ground source of western medicine. Both of these molecules RSV and BMP-7 are involved in bone and cartilage regeneration [50-52]. However, the regulation of collagen type II by RSV and BMP-7 in the different zones of porcine articular cartilage has not been fully investigated.

2. Materials and Methods

2.1 Tissue Acquisition for Cell Culture

Total of 12 different porcine stifle (knee joint) from 3 month-old pigs, representing a sample size of 12 experiments were obtained from local abattoir and dissected under aseptic conditions to expose the femoral condyles as described by [53]. The superficial zone cartilage (100 μm) of the femoral condyles was harvested using a dermatome (Phoenix Surgical, Cape Town, SA). Osteochondral plugs were excavated from the femoral condyles using 3 mm diameter steel dermal puncher (Thermo Scientific, Waltham, MA) and the middle zone was removed from each plug using a custom cutting jig.

2.2 Isolation and Monolayer Culture

The superficial zone was digested with 0.2% collagenase- P (Roche Pharmaceuticals, Nutley, NJ, USA) for 45 minutes and the middle zone for 1hour. Chondrocytes were plated as monolayers at a density of 1 x 10^5 cells/well in 12-well culture plates and incubated at 37 ^{0}C in a humid atmosphere of 5% carbon dioxide and 95% air. Chondrocytes were cultured overnight in DMEM/F-12 medium (Life Technologies, Carlsbad, CA, USA) containing 1% fetal bovine serum (FBS; Thermo Scientific). The next day the medium was changed to serum-free DMEM/F-12 medium with insulin transferrin selenium (ITS) + Premix (Life Technologies). Cells were then treated with different concentrations of RSV (20 μM, 50 μM and 100 μM) [54] and BMP-7 (100 ng/ml and 300 ng/ml) [53] and incubated for 4 days. Untreated control was assigned for all the experiments.

2.3 RT-PCR (Real Time Polymerase Chain Reaction) Analysis

Total RNA was extracted from cultured monolayer cells using RNeasy mini kit (Qiagen, Valencia, CA, USA) with on-membrane DNase 1 (Qiagen) digestion to avoid genomic DNA contamination. Extracted RNA quality and quantity were checked by Qubit 2.0 fluorometer instrument (Life Technologies) accordingly. Total RNA was reverse-transcribed into single- stranded cDNA, using Veriti® Thermal Cycler (Life Technologies). Real time quantitative PCR was performed in triplicate on cDNA with StepOne plus Real-Time PCR sequence detector system and Tag-Man gene expression reagents (Life Technologies), following the recommended protocols. Collagen type II mRNA levels were normalized to GAPDH (glyceraldehydes 3-phosphate dehydrogenase) levels and expressed relative to the control (untreated) culture levels (ΔCT methods; Life Technologies). The primers for collagen type II (Ss 03373345 g1) and GAPDH (Ss03375435 μL) were predesigned by Life Technologies.

2.4 ELISA (Enzyme-Linked Immunosorbent Assay) Analysis of Collagen Type II Protein

An ELISA kit was used for quantitative determination of collagen type II (MD Biosciences, St. Paul, MN, USA) levels in monolayer cell culture. The assay is based on colorimetric based immunoassay. The collagen type II ELISA is a heterogeneous sandwich ELISA. A monoclonal anti-collagen type II antibody is pre-coated onto a 96-well microplate and any collagen type II present in the sample will be bound to the microplate by the antibody. A secondary biotinylated antibody is then added and will bind to the immobilized antigen forming the antibody-antigen complex. Substrate is then added and converted by the enzyme to produce a quantifiable coloured product that is in direct proportion to the amount of antigen bound in the initial reaction.

2.5 Immunolocalization of Collagen Type II

Full-thickness articular cartilage sections were dissected from porcine femoral chondyles. The specimens were fixed with 10% neutral buffered formalin (Sigma-Aldrich) and embedded in paraffin. Tissue sections (5 μm thick) were prepared, sectioned

vertically from the surface to the bottom and collagen type II was localised using novalink min polymer detection system kit (Leica Microsystems, Newcastle, UK). The sections were deparaffinised and endogenous peroxidase was blocked with 1 % hydrogen peroxidase. The sections were then incubated over night at 4^0C with a 1:1000 dilution of rabbit polyclonal collagen type II antibody (Leica Microsystems). The sections were incubated with biotinylated link antibody. No primary antibody was applied in the control experiment. Visualisation was achieved using DAB chromagen resulting in a brown precipitate evaluated microscopically. Control experiments were incubated with PBS only. Images were obtained on Leica DMIL LED biological microscope with appropriate photographic software (Leica Microsystems) at 10X magnification.

2.6 Histological Analysis

To confirm that cartilage slices had been acquired from the specific zones of articular cartilage, consecutive formalin-fixed and paraffin-embedded tissue sections were stained with 1% toluidine blue, safranin O, Masson's trichrome and haematoxylin-eosin staining for histologic evaluation using standard procedure. Each experiment was assigned a set of control and treated sections with RSV (20 μM, 50 μM and 100 μM) and BMP-7 (100 ng/ml and 300 ng/ml). Images were obtained on a Leica DMIL LED biological microscope with appropriate photographic software (Leica Microsystems) at 10 X magnification

2.7 Statistical Analysis

The sample size for all experiments was twelve (n = 12), which represent twelve different animals. All the quantitative data were presented as means ± standard deviations. ANOVA (A one-way analysis of variance) with Tukey's HSD (Honestly Significant Differences) to account for multiple comparisons was used to determine the effects of RSV and BMP-7 on collagen type II expression in different zone of articular cartilage. A paired t-test was performed to determine the difference between the control and treated cells. P-values less than 0.05 were considered significant for all comparisons.

3. Results

3.1 Chondrocytes Morphology Analysis

Articular chondrocytes from the superficial and middle zones were cultured as monolayers. Before treatment, the chondrocytes morphology is similar in both surface and middle zone cultures (Figs. 1A-1D and 1E-1H). Both cultures were then treated with various concentrations of RSV (20 μM, 50 μM and 100 μM) and BMP-7 (100 ng/ml and 300 ng/ml) and incubated. After day one of treatment, there is a slight change in cell morphology with chondrocytes differentiating and moving away from a round shape nature (Figs. 2B-2D and 3B-3D). Control cell morphology after day one treatment remains the same as cells before treatment (Figs. 2 A and 3.A). The effect of RSV and BMP-7 was successfully observed through transition of chondrocytes from round to an elongated polygonal morphology on day four of incubation (Figs. 2E-2H and 3E-3H). It was observed that there was more cell development on a concentration of RSV (50 μM) + BMP-7 (300ng/ml) in both surface and middle zone cultures (Figs. 2C and 3C) compared to control and other treatment concentrations on day four.

3.2 Influence of RSV and BMP-7 on Collagen Type II mRNA and Protein Level

Articular chondrocytes obtained from the surface and middle zone were cultured for four days as monolayers and treated with different concentrations of RSV+BMP-7 (20 μM + 100 ng/ml, 50 μM + 300 ng/ml and 100 μM + 100 ng/ml) and TGF-β1 (3 ng/ml). Collagen type II mRNA expression was assessed by means of RT-PCR as shown in Figs. 4A and 4C) and collagen type II protein level was

quantified by means of ELISA as shown in Figs. 4B and 4D). The insignificant in collagen type II mRNA expression and protein levels by surface zone chondrocytes was observed on TGF-β1 as compared to RSV + BMP-7 (50 μM + 300 ng/ml), Figs. 4A and 4B). Treatment with RSV and BMP-7 at 20 μM + 100

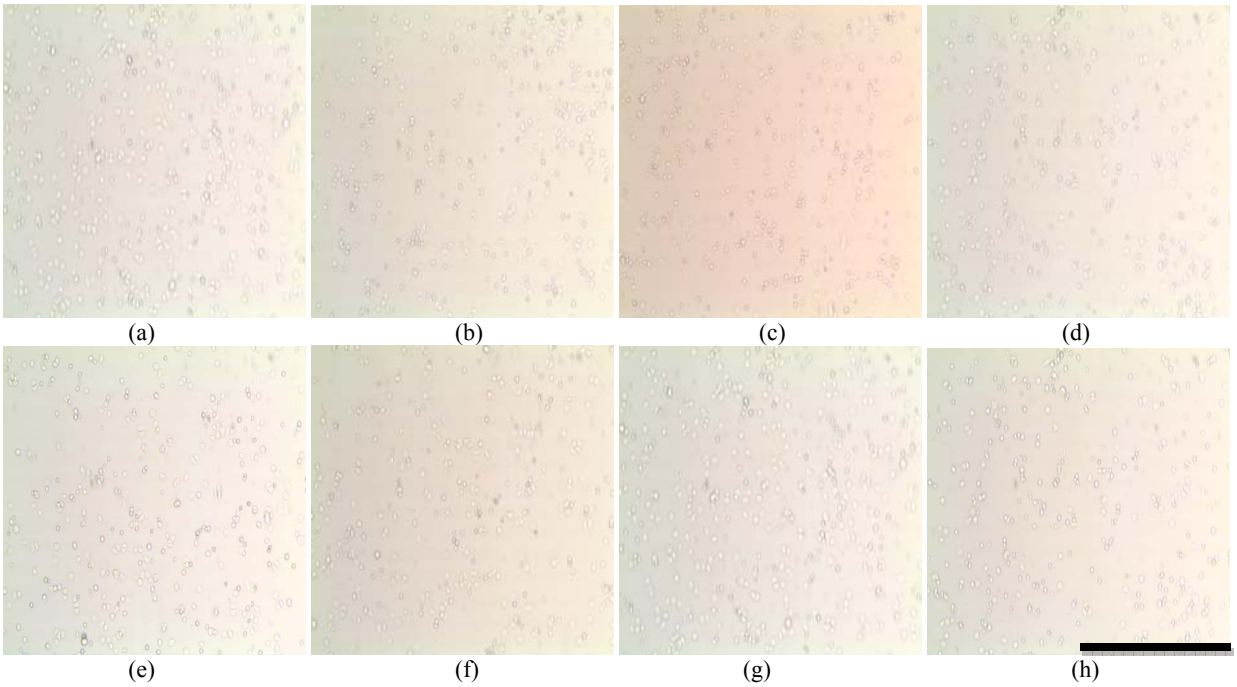


Fig. 1 A-D Represents superficial zone chondrocytes before treatment and E-H middle zone chondrocytes before treatment.

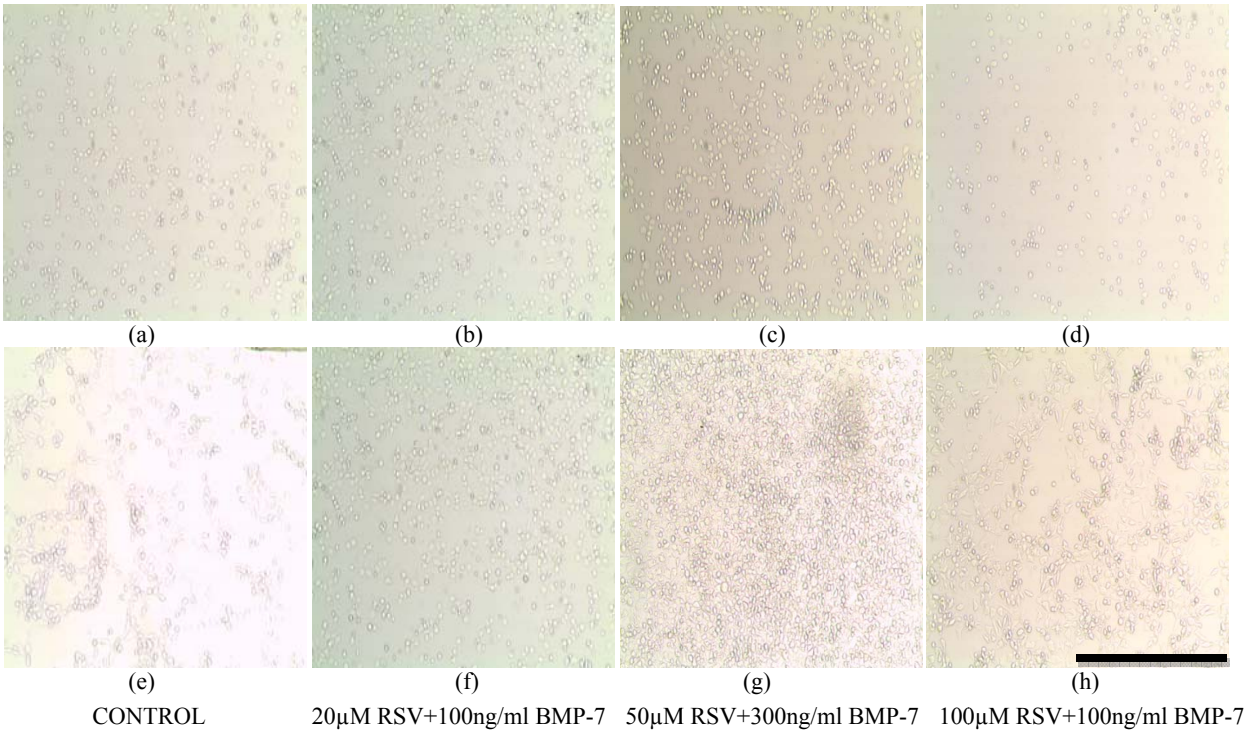


Fig. 2 A-D Represents superficial zone chondrocytes after treatment by different concentrations of RSV: 20 μM, 50 μM and 100 μM and BMP-7: 100 ng/ml and 300 ng/ml on Day 1 and E-H represents superficial zone chondrocytes after treatment by different concentrations of RSV: 20 μM, 50 μM and 100 μM and BMP-7: 100 ng/ml and 300 ng/ml on Day 4.

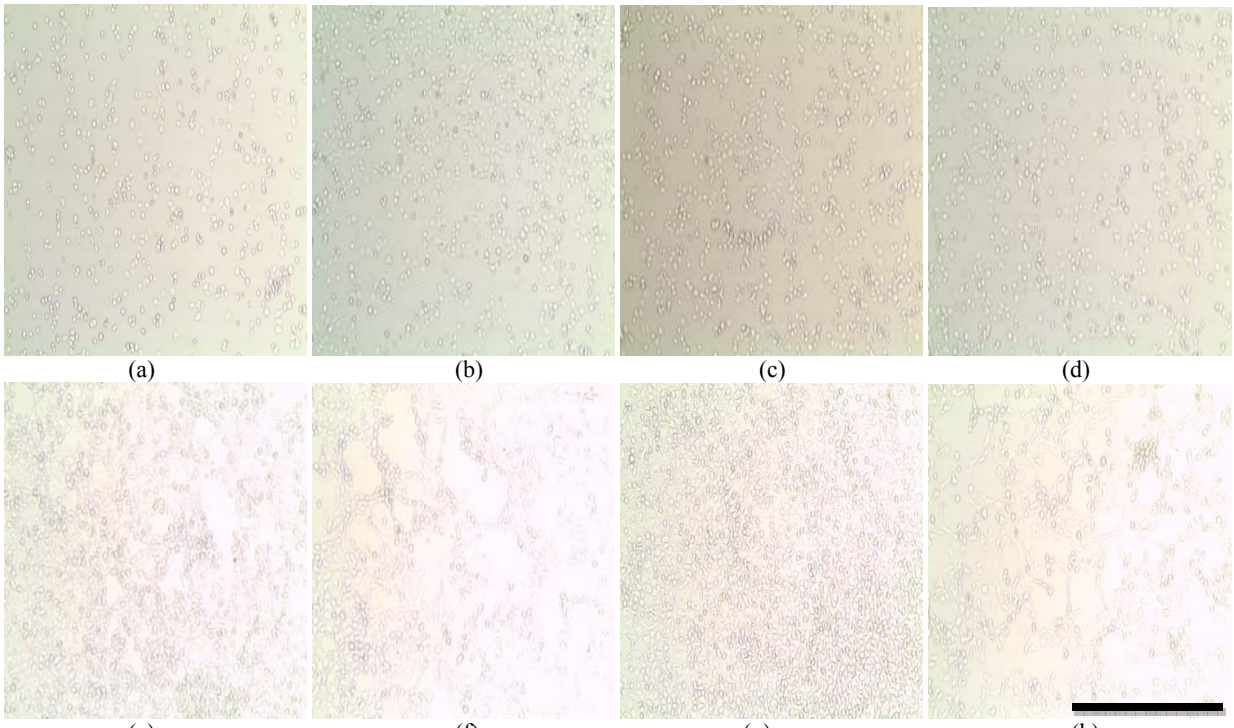


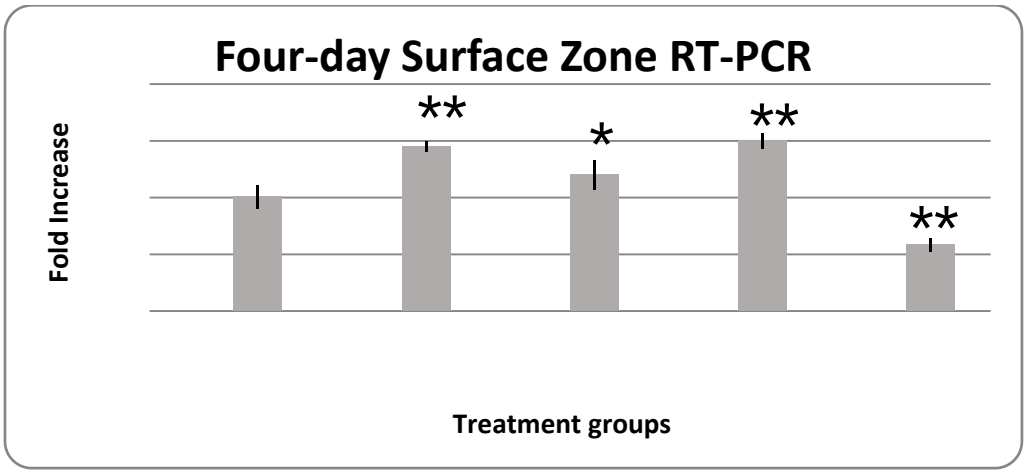
Fig. 3 A-D Represents middle zone chondrocytes after treatment by different concentrations of RSV: 20 μM, 50 μM and 100 μM and BMP-7: 100 ng/ml and 300 ng/ml on Day 1 and E-H represents middle zone chondrocytes after treatment by different concentrations of RSV: 20 μM, 50 μM and 100 μM and BMP-7: 100 ng/ml and 300 ng/ml on Day 4.

ng/ml for 4 days significantly enhanced the expression of Collagen type II mRNA at 1.27 fold increase and collagen type II protein level at the concentration of 1.30 ng/ml in the surface zone as in Figs. 4A and 4B compared to the middle zone where the expression of collagen type II mRNA expression was 1.64 fold increase and protein concentration was 1.32 ng/ml as in Figs. 4C and D.

Treatment with RSV and BMP-7 at 50 μM + 300 ng/ml for four days significantly enhanced the expression of Collagen type II mRNA at 1.50 fold increase (Fig. 4A) and collagen type II protein level at the concentration of 1.49 ng/ml (Fig. 4B) in the superficial zone compared to the middle zone where the expression of collagen type II mRNA expression was 2.53 fold increase (Fig. 4C) and protein concentration was 1.56 ng/ml (Fig. 4D). However, treatment with RSV and BMP-7 at 100 μM + 100 ng/ml for four days significantly decreased collagen type II expression relative to the control had a 0.58 fold increase and 1.19 ng/ml concentration for superficial zone (Figs. 4A and 4B) and 1.09 fold increase and 1.15 ng/ml for middle zone (Figs. 4C and 4D). A higher increase in the expression of collagen type II mRNA and protein level was observed at RSV (50 μM) + BMP-7 (300 ng/ml concentration as shown in Fig. 4B compared to the control and other concentrations (Figs. 4B and 4D)

3.3 Immunohistochemistry Analysis

Negative control (without primary anti-body) and positive control (with primary anti-body) untreated cartilage tissue sections prepared were observed under a light microscope for collagen type II staining (Fig. 5). Positive staining was identified by a brownish colour with round chondrocyte morphology (Figs. 5B and 5D). Negative (without anti-body incubation) and positive (incubated with anti-body) treated with RSV (50 μM) and BMP-7 (300 ng/ml) prepared cartilage tissue sections were also observed


Four-day Surface Zone RT-PCR
Fold Increase
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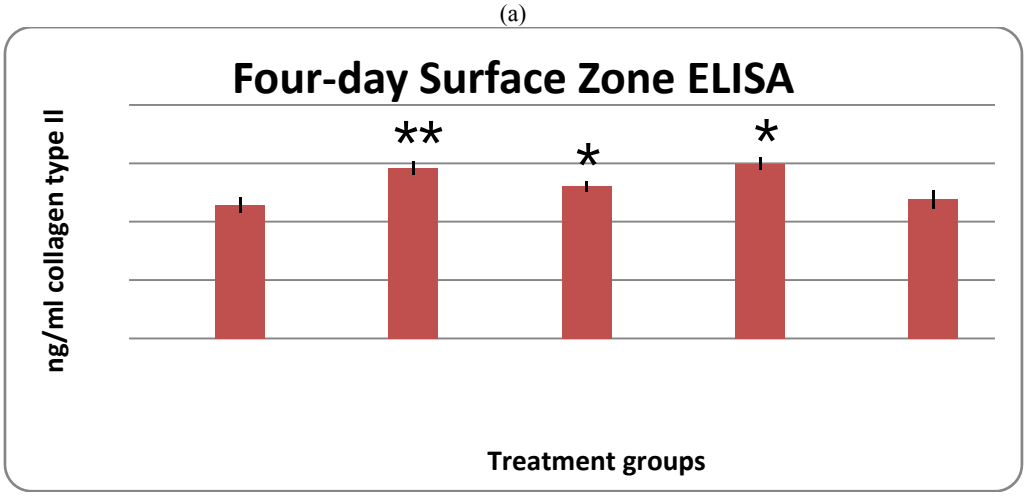
Treatment groups

(a)


Four-day Surface Zone ELISA
ng/ml collagen type II
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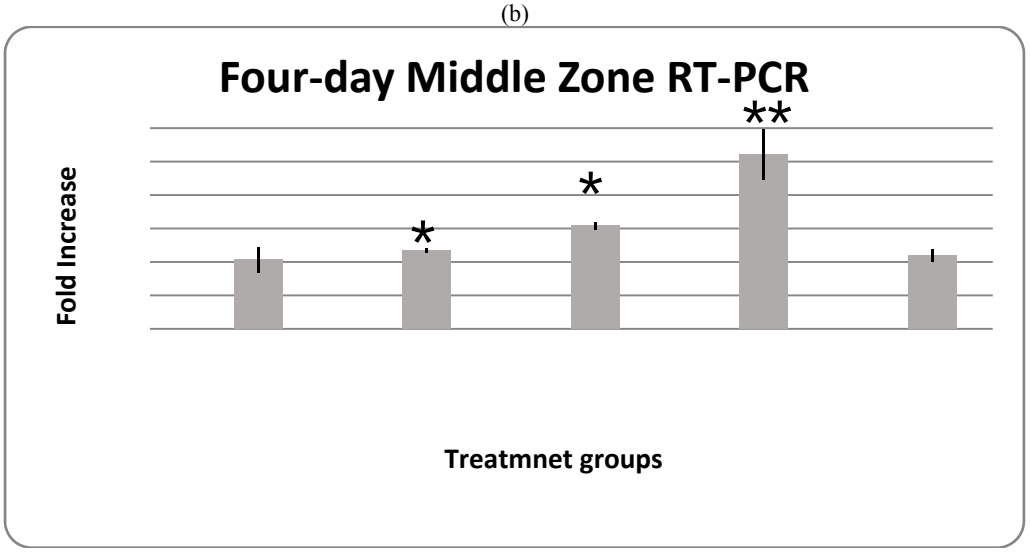
Treatment groups

(b)


Four-day Middle Zone RT-PCR
Fold Increase
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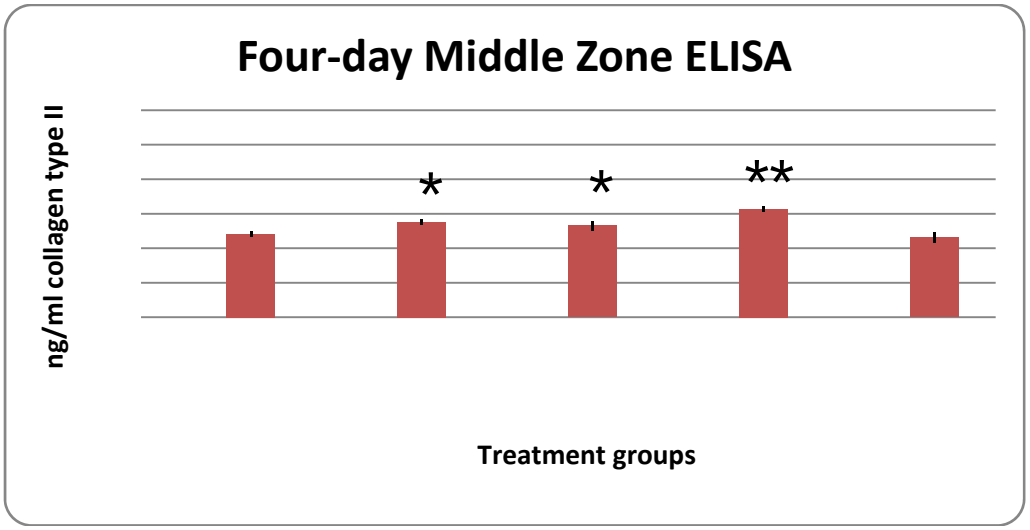
(c)



(d)

Fig. 4 (A) Indicates the effects of RSV and BMP-7 on collagen type II mRNA expression on the surface zone. (B) Indicates the effects of RSV and BMP-7 on collagen type II protein level on the surface zone. (C) Indicates the effects of RSV and BMP-7 on collagen type II mRNA in the middle zone and (D) Indicates the effects of RSV and BMP-7 on collagen type II protein level in the middle zone. Articular cartilage surface and middle zone chondrocytes were treated with different concentrations of RSV and BMP-7 (20 µM+100 ng/ml, 50 µM+300 ng/ml and 100 µM+100 ng/ml). Untreated cells and TGFβ1 (3 ng/ml) served as positive control. A significance change of * represents a p value = 0.05 and ** p value = 0.01.

under the light microscope for collagen type II staining (Figs. 5C and 5D). Sections were no primary antibody was added, showed no staining demonstrating the reliability and validity of the method (Figs. 5A and 5C). Larger chondrocytes emerging from the deeper zone of the RSV 50 µM and BMP-7 300 ng/ml treated cartilage section (Figs. 5C and 5D) compared to the control sections (Figs. 5A and 5B) were observed. The staining was strong in the superficial zone, mild in the middle zone and decreasing further in the deep zone.

3.4 Histology Analysis

Histological evaluation confirmed that cartilage tissue sections had been obtained from the superficial and middle zones of articular cartilage. The upper layer had small cells with flattened or ellipsoidal cellular morphology, whereas the middle and lower layers had large cells with oval or round cellular morphology. The upper zone had the highest cellularity, followed by the middle zone and lower zone. Cells of the superficial zone were smaller than cells of the middle and deep zones (Fig. 6). Tissue sections' staining was positive for articular cartilage matrix components. The intensity of toluidine blue was highest in the lower zone of the cartilage (Figs. 6A and 6E). Safranin O staining, demonstrating proteoglycan content was highest in the lower zone, followed by the middle zone and upper zone (Figs. 6B and 6F). The intensity of Masson's trichrome staining was most noteworthy in the middle zone, followed by the upper zone and lower zone, which directly relates to collagen cartilage matrix distribution (Figs. 6D and 6H). The purpose of the Masson's trichrome stain is the demonstration of collagen and muscle in normal tissue and also to differentiate collagen and muscle in tumors. The middle zone had the highest collagen type II content, which was in line with the highest collagen content observed in Masson's trichrome staining. Lastly, haematoxylin-eosin stained the cartilage matrix positive pink and the nucleus an orange to green colour (Figs. 6C and 6G). Control tissue sections (Figs. 6A-6D) showed smaller round, flattened chondrocytes when compared to larger chondrocytes emerging from the deeper zone of RSV: 50 µM and BMP-7: 300 ng/ml treated sections (Figs. 6E-6H).

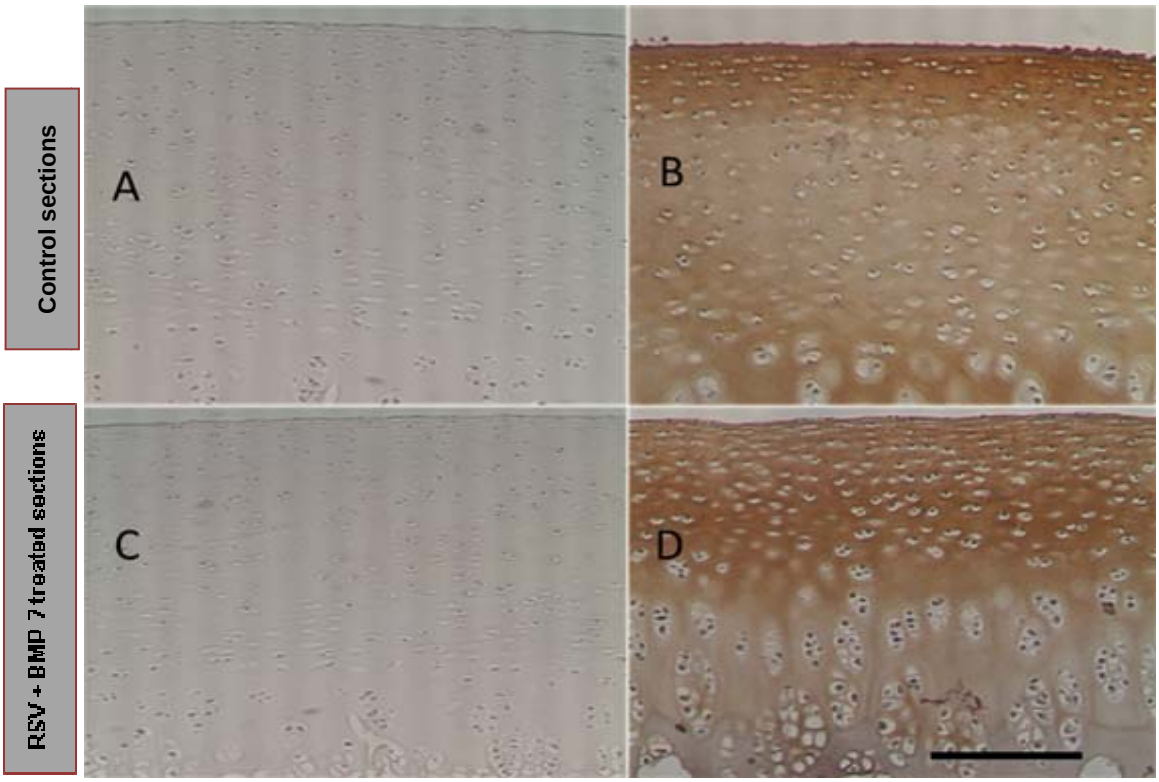


Fig. 5 Untreated collagen type II negative control without primary antibody (A) and positive control with primary antibody (B) tissue. Treated (RSV: 50 µM and BMP-7: 300 ng/ml) collagen type II negative control without primary antibody (C) and positive control with primary antibody (D) tissue sections, observed at 10X magnification. Scale bar = 200 µM.

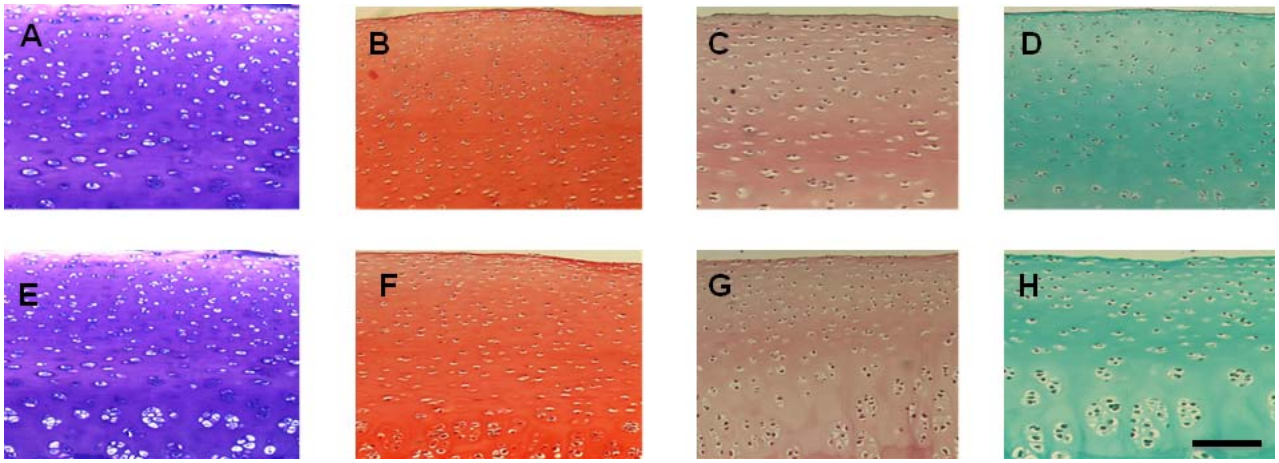


Fig. 6 Representation of cartilage tissue staining. Control tissue sections stained with Toluidine blue (A), Safranin O (B), Haematoxylin & Eosin (C) and Masson's trichrome (D) and Treated tissue sections E-H (RSV: 50 µM and BMP-7: 300 ng/ml) observed at 10X Magnification. Scale bar, 200 µm.

4. Discussion

Cartilage is an avascular tissue, which is unable to self-heal or regenerate after injury [20, 53, 56-60]. Three types of cartilage are hyaline (articular), fibrous and elastic cartilage [61] Articular cartilage consists of four different zones: superficial, middle, deep and calcified zones, which differ in matrix composition, morphology, mechanical and metabolic properties [56, 61]. Study of the different cartilage zones aim to permit a better understanding of stimuli that up-regulates biosynthesis of collagen type II in the

chondrocytes subpopulation.

Resveratrol has also proved to have chondro-protective effects on articular cartilage chondrocytes with concentrations between 25 μM and 50 μM [62]. The present study demonstrates that collagen type II mRNA and protein level is expressed in surface and middle zone chondrocytes culture (Figs. 4A-4D). However, the middle zone culture expressed more collagen type II mRNA and protein level as compared to the surface zone. Possible explanation might be that collagen type II protein is highly expressed in the middle zone of articular cartilage [55,63]. Although all the concentrations showed expression, the highest expression was shown on the 50 μM + 300 ng/ml (RSV + BMP-7) for both surface and middle zone of RT-PCR and ELISA (Figs. 4A-4D). BMP-7 has the ability to reduce the progression of OA. The expression of anabolic genes, aggrecan and collagen type II is increased when cartilage is treated with BMP-7 [64]. Resveratrol has extensive biological properties including anti-inflammatory, cardiovascular, anti-carcinogen and anti-aging effects [65, 66].

Immunohistochemistry confirms the presence of collagen type II. The staining was strong in the superficial zone, mild in the middle zone and decreasing further in the deep zone (Fig. 5D). This localisation of collagen type II confirms the observations reported in other studies [67]. Histological tissue sections confirmed that cartilage slices used had been obtained from different zones of articular cartilage. We also observed increased intensity of the stains on treated tissue sections which suggests that RSV (50 μM) + BMP-7 (300 ng/ml) stimulate synthesis of the cartilage collagen matrix (Figs. 6E-6H). These results on cell distribution and matrix composition had been observed from previous studies where the compounds were used individually, RSV [51] and BMP-7 [1, 68].

5. Conclusion

The data of this study confirm that the combination of RSV and BMP-7 may stimulate the production of collagen type II in articular cartilage zones. Collagen type II was expressed in both the surface and middle zone after being treated with different concentrations of RSV (20 μM, 50 μM and 100 μM) and BMP-7 (100 ng/ml and 300 ng/ml). This study has provided new information about the synthesis and secretion of collagen type II in different zones of articular cartilage when treated with RSV and BMP-7. Furthermore, regulation of collagen type II will be of great importance for tissue engineering to review different zones of articular cartilage. In conclusion we can safely conclude that the results support our hypothesis that the *in vitro* administration of RSV and BMP-7 to cultures of articular chondrocytes up-regulate the expression and synthesis of collagen type II.

Further research is needed to focus on exploring the mechanism of action of RSV in combination to BMP-7 and other growth factors on other articular cartilage matrix proteins such as collagen type I, X, aggrecan and SOX 9 before *in vivo* studies.

6. Funding

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