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SM Journal of Biomedical Engineering

Research Article

The Effect of WIN55, 212-2 on Protein S100, Matrix Metalloproteinase-2 and Nitric Oxide Expression of Chondrocyte Monolayer

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Article Information

Received date: Oct 03, 2016
Accepted date: Jan 03, 2017
Published date: Jan 06, 2017

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Keywords Chondrocyte; WIN55, 212-2; Wound healing; Protein expression; Collagen type-I and II; Fibronectin; MMP-2 and NO expression

Abstract

Studies have been conducted to highlight the anti-inflammatory and immunosuppressive properties of synthetic cannabinoids as well as their potential for cartilage repair. Various wound healing techniques can be used to investigate the mechanisms of chondrocyte repair in monolayers or three dimensional tissues constructs. In this work the effect of WIN55, 212-2 (WIN-2) on nitric oxide (NO) and matrix metalloproteinase-2 (MMP-2) expressed by wounded chondrocyte monolayers was investigated. Moreover, expression of collagen type-I and type-II, fibronectin and S100 proteins were detected using immunofluorescence and quantitatively verified using ELISA based techniques following treatment with 1 μM and 2 μM of WIN-2. Treating chondrocytes with 1 μM of WIN-2 significantly increased expression of collagen type-II, fibronectin and S100, and significantly reduced collagen type-I expressions as compared to the control groups. On the other hand, both concentrations of WIN-2 significantly reduced the expression of the inflammation markers NO and MMP-2 in a dose dependent manner. These findings highlight the potential use of the synthetic cannabinoids for improving cartilage healing properties as well as acting as an anti-inflammatory agent which could be used to enhance tissue engineering protocols aimed at cartilage repair.

Introduction

Articular cartilage is highly capable of withstanding repeated loading, considerable stress, and friction. However, unlike other types of connective tissue, it has very low capacity to repair due to the lack of nerves, blood vessels and lymphatics [1]. It was shown in previous studies [2] that treating primary chondrocyte monolayers with 1 μM of WIN55, 212-2 increased the rate of wound closure and cell proliferation and those treating primary chondrocyte monolayers with 2 μM of the same synthetic cannabinoid decreased rate of wound closure and cell proliferation. Moreover, both treatments notably reduced cell lengths, but by different percentages. These results prompted further investigation of the effect of WIN55, 212-2 (WIN-2) on chondrocyte protein expression. Understanding the mechanisms regulating cellular behaviour, e.g. proliferation, migration, morphology, phenotype and cell adhesion is crucial for tissue engineering [3]. Cells receptor membranes bind to their ligands by expressing different types of integrins depending on the extracellular matrix [4]. Furthermore, functionality of cells relies in part on the extra cellular matrix (ECM) protein surrounding the cell [5]. For instance, collagen type-II rich ECM promotes chondrocyte proliferation and differentiation [6].

Normally, chondrocytes within articular cartilage demonstrate a star-shaped morphology while secreting various ECM proteins including type-II collagen, cartilage oligomeric matrix protein (COMP) and aggrecan [7,8]. Nevertheless, chondrocyte dedifferentiation has been noted in osteoarthritic cartilage [8,9]. There is evidence that culturing chondrocytes in monolayers encourages the cells to lose their rounded shape and dedifferentiate into fibroblast-like morphology and produce collagen type-1 [10]. Therefore, collagen type-II can be used as a chondrocytic marker and both collagens can be used to determine the differentiation state of chondrocytes. Also, one of the major problems facing current cartilage repair strategies is the formation of fibrocartilage instead of articular cartilage due to the dedifferentiation of chondrocytes into fibrocytes. Thus, the present study was designed to investigate effect of WIN-2 on chondrocyte differentiation using immunofluorescence microscopy and validated quantitatively with an Elisa based method. Further experiments were also carried out to investigate the effect of WIN-2 on S100 protein which is considered useful for the histological evaluation of human articular cartilage repair activity [11]. It is known that S100 is an in vitro antigen synthesised by primary chondrocytes [12].

It is well established that fibronectin is a high-molecular weight glycoprotein of the ECM that binds to other ECM components via integrins such as heparan sulfate proteoglycans, fibrin and collagen [13] and that chondrocytes secrete fibronectin in the soluble form, which is then assembled into an insoluble matrix in a complex cell-mediated process [14]. It plays a key role in cell migration, adhesion, differentiation and growth, which increase its importance in processes such as wound healing and embryonic development [13]. In wound healing of connective tissue, fibronectin is vital for the remodelling and resynthesis of the tissue matrix [15]. On the other hand, changes in fibronectin organization, degradation and expression have been identified in a number of pathologies, including cancer and fibrosis [16]. Proteases including MMPs that digest the plasma fibronectin are secreted at wound sites and hence cells secrete cellular fibronectin and assemble it into an insoluble matrix [17].

The dissolution and maintenance of the extracellular matrix is mediated in part by the Matrix Metalloproteinase (MMP) family of enzymes and by their inhibitors, i.e. the Tissue Inhibitors of Metalloproteinases (TIMPs) [18]. Further, cartilage ECM may introduce a physical barrier, since some cells might be able to squeeze through matrix meshwork [19], while others may need to use proteolytic enzymes to migrate through condensed cartilage matrices [20]. It will be important to address the effect that WIN-2 has on fibronectin and proteolytic enzymes such as MMP-2 (matrix metalloproteinase-2), as the rise in chondrocytic proteases caused by osteoarthritis [21] may aid cell motility.

NO is thought to interfere with chondrocyte migration and attachment to fibronectin and cause cell death [22]. However, other studies have suggested that NO by itself is not cytotoxic to cultured chondrocytes and can even have a protective role, while it only causes cell death under conditions where other Reactive Oxygen Species (ROS) are also generated [23]. These resulted in further investigation of its role in monolayer chondrocyte wound healing described in this work. Griess reagent has been used to measure cell expression levels of nitrite and nitrate, which represent the final products of nitric oxide oxidation pathways [24]. For this reason, Griess reagent was used in this work to evaluate NO produced by different cell types.

Materials and Methods

Chondrocyte culture

Primary chondrocytes were extracted directly from 3-6 days old Sprague-Dawley rats and then cultured in a low glucose (1g/L-D-Glucose) HEPES (25mM) buffered DMEM (Dulbecco's Modified Eagle Media, Sigma Aldrich) with Ham's F12 with 1:1 ratio and culture medium supplemented with L-glutamine (2.5 mM U/mL), Penicillin (100 U/mL), Streptomycin (0.1 mg/mL), Amphotericin B or a fungizone (250 μg/ml), (Sigma Aldrich-UK) and 10% FCS v/v (fetal calf serum- PromoCell - UK). Routine sub-cultures were carried out upon reaching 80% confluency in sterile environment using a laminar flow hood by washing cells three times with Hank's Balanced Salt Solution (HBSS) each time for five minutes, and by adding 1ml of 0.25% Trypsin-EDTA (Sigma Aldrich, UK) to detach cells. Once cells became rounded, detachment was stopped by the addition of 2ml medium containing 10% FCS. Cells were centrifuged at 1500 rpm, and then diluted using complete medium and counted using a haemocytometer. Cells were plated with an initial cell density of 100,000 cell/ml, culture flaks were always incubated in a 5% CO_2 air jacket controlled incubator at 37 °C. Following the third passage chondrocytes were seeded onto 1cm2 sterile glass cover slips in 35mm petri dishes at a density of 50,000 cells/ml (15.6×10^3 Cells/cm) and left for two days to reach 100% confluency before applying the three different treatments, i.e. control, and WIN55, 212-2 (WIN-2; SIGMA-ALDRICH, UK) at final concentrations of 1 μM and 2 μM. Treated chondrocytes were then incubated at 37 °C in humidified atmosphere with 5% CO_2 for 16 hours before investigating the changes in proteins and MMP-2 expressions. In order to quantitatively investigate the effect of WIN55, 212-2 on the chondrocytes protein expression, the same treatments were applied to 100% confluent chondrocytes cultured in 96-well plates. Similarly, NO expression by wounded chondrocyte monolayers for the same treatments was investigated over a period of 16 hours, where its value was recorded every 2 hours to determine the changes in expression with time. Confluent cell cultures were wounded using a sterilized 1mm surgical plastic pipettes to reduce the possibility of contamination, and the timing window for total wound closure of a 300 μm width wound in MG-63 bone cell monolayer was found to be about 30 hours. The cells were imaged every 5 hours using a phase contrast light microscope and photographed with a digital camera.

Immunofluorescence staining for collagen Type-I and Type-II, fibronectin, and S100 protein

Treated chondrocytes were fixed using 4% formaldehyde and monoclonal anti collagen type-II (mouse IgG1 isotype) (SIGMA-ALDRICH, UK) solution was used as primary antibody and added to 9 petri dishes, 3 for each treatment. Similarly, monoclonal anti collagen type-1 (mouse IgG1 isotype) (SIGMA- ALDRICH, UK), monoclonal anti fibronectin (mouse IgG1 isotype) (SIGMA-ALDRICH, UK) and monoclonal anti S-100 (β-subunit, mouse IgG1 isotype) (SIGMA- ALDRICH, UK) antibody solutions were each added to nine petri dishes. To confirm the specificity of the primary antibodies used (staining, i.e. –ve control), three petri dishes were not treated with primary antibodies. Ab-Alexor fluor 488 nM (Invitrogen, UK) solution was used as secondary anti-body and added to all the petri dishes. Protein expression was examined using an Eclipse 80i Fluorescence microscope with an objective lens of x20.

Elisa based method for detection of collagen type-I and type-II, fibronectin and protein S-100

Anti-collagen type-II antibody solution was added to three different treatment groups each consisting of 12 wells; the 1st group was untreated (control), the 2nd was treated with 1 μM WIN-2 and the 3rd was treated with 2 μM WIN-2. Similarly, Anti-collagen type-I, anti-S100 and anti-fibronectin antibody solutions were each added to the three different treatments. Anti-mouse IgG peroxidise conjugate (8 μg/ml) was used as secondary anti-body and tetramethyl benzidine (TMB; SIGMA- ALDRICH, UK) substrate was then applied and absorbance readings were measured at 630nm wavelenght and subtracted from absorbance readings measured when the wells were empty (blank). This experiment was repeated three times, giving a total of 36 readings for each different treatment group.

MMP-2 immunoassay staining

12 samples were prepared for each of the three treatments, i.e. control, 1 μM WIN-2 and 2 μM WIN-2. Following incubation with these treatments for 16 hours, conditioned media was aspirated and

centrifuged at 1500 rpm for 5 minutes. An Elisa Kit (Invitrogen, UK) was used and staining steps were in accordance with the manufactures supplied protocol. Optical density values were obtained using ELx 800 (Bioteck instruments, USA) at wavelength of 450nm.

Nitric oxide quantitative staining

After preparation of the nine different 96-well plates, supernatants were withdrawn from culture flasks and centrifuged at 1700 rpm for 5 minutes. In a 96-well plate, 100 μl of each well was mixed with 100 μl Griess reagent (SIGMA- ALDRICH, UK) and incubated at room temperature for 10 minutes. After incubation absorbance was measured using an absorbance micro-plate reader (ELx 800, Bioteck instruments, USA) at a wavelength of 570 nm. This experiment was repeated 3 times.

Statistical analysis

Quantitative staining readings were tested for normality using a Kolmogorov Smirnov test. Normally distributed data ($p>0.05$) were analysed using SPSS via ANOVA test followed by post Hoc Bonferroni tests. Kruskal-Wallis test and serial Mann Whitney tests were used for non-normally distributed data ($p<0.05$). Statistical tests were performed such that a p value of < 0.05 was considered as indicating a significant difference.

Results

Immunofluorescence and ELISA based staining for collagen type-II and collagen type-I of chondrocytes treated with 1 μM and 2 μM of WIN55, 212-2

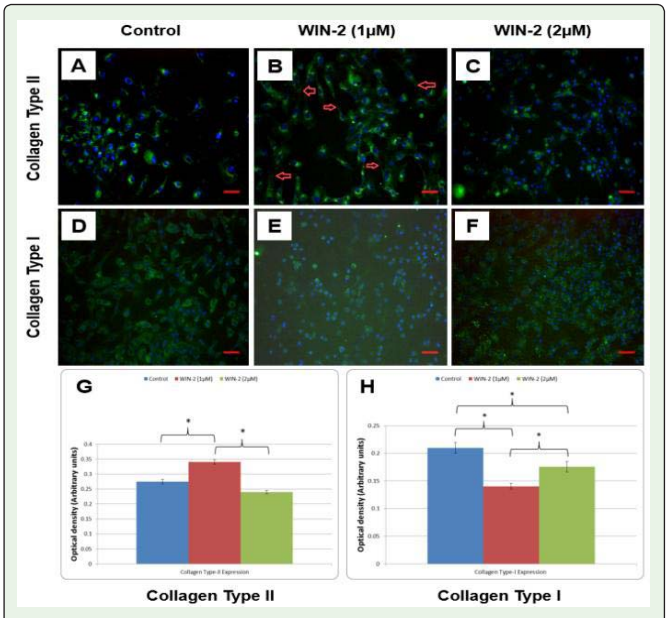

Figure 1: Immunofluorescence staining of collagen type II and type I (green). The green colour shows positive staining for collagen type II and type I in control conditions (A and D), 1 μM WIN-2 (B and E) and 2 μM WIN-2 (C and F). Individual cell nuclei are stained blue (Dapi stain). [Scale bar = 100 μm]. Arrows in Figure 1B show the increase in collagen being associated with the formation of an intercellular Type II collagen network. Optical density readings (±SD) at 630nm following immunoperoxidase staining of deposited collagen type II (G) and collagen type I (H) following 16 hours incubation with 1 μM WIN-2, 2 μM WIN-2 and control conditions (*= $p<0.05$) (n=36).

Immunofluorescence and ELISA based staining of the chondrogenic protein collagen type-II indicated that untreated cells and cells treated with 1 μM and 2 μM of WIN-2 expressed type-II collagen distributed around the cell membrane. However, there seemed to be an increase in the secretion of collagen type-II by primary chondrocytes treated with 1 μM of WIN-2 (0.341 ± 0.007SD) compared to control groups (0.275 ± 0.008SD) ($P<0.05$) (Figure 1A-C and Figure 1G), with the increase in collagen being associated with the formation of an intercellular Type II collagen network (Figure 1B). In comparison, treating chondrocytes with 2 μM of WIN-2 seemed to reduce collagen type-II secretion (0.240 ± 0.005SD) ($P<0.05$) compared to control groups (Figure 1C and 1G).

Using immunofluorescence staining of primary chondrocytes monolayer, a very weak expression of collagen type-I was visualized mainly as part of the cell membrane for the 3 different treatments. As shown in Figures 1D-F and 1H, collagen type-I was mostly expressed by untreated chondrocytes with an optical density reading of 0.210 ± 0.010SD. This value is ~33.3 % higher than the 0.140 ± 0.005SD produced by cells treated with 1μM WIN-2 ($P<0.05$). Similarly, chondrocytes treated with the higher concentration of 2μM WIN-2 expressed a lower optical density reading, but it was only ~ 16.2% lower than control conditions at a value of 0.176 ± 0.009SD ($P<0.05$) (Figure 1H). Type-I collagen was localized around the cell membrane as part of the extracellular matrix (ECM) for control groups only (Figure 1D).

Immunofluorescence and ELISA based staining for S100 of chondrocytes treated with 1 μM and 2 μM of WIN55, 212-2

Out of the 4 proteins stained, the differentiation protein S100 seemed to be highly expressed with the highest immunofluorescence

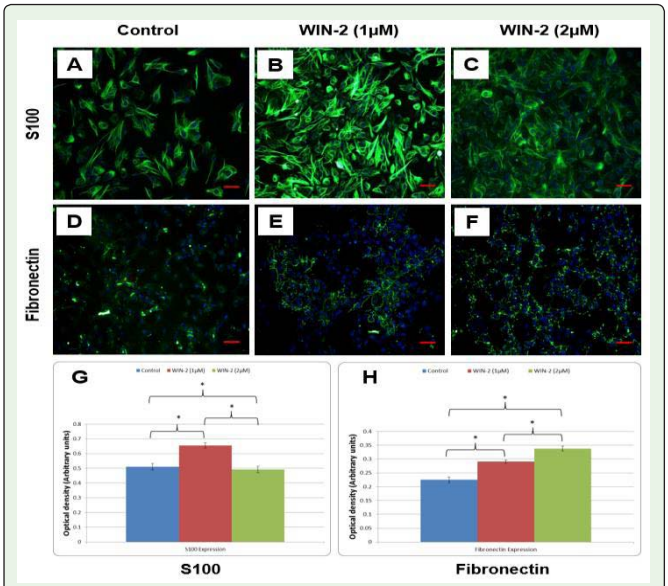

Figure 2: Immunofluorescence staining of S100 and fibronectin (green). The green colour shows positive staining for S100 and fbronectin in control conditions (A and D), 1 μM WIN-2 (B and E) and 2 μM WIN-2 (C and F). Individual cell nuclei are stained blue (Dapi stain). [Scale bar = 100μm]. Optical density readings (± SD) at 630nm following immunoperoxidase staining of deposited S100 (G) and fibronectin (H) following 16 hours incubation with 1 μM WIN-2, 2 μM WIN-2 and control conditions (*= $p<0.05$) (n=36).

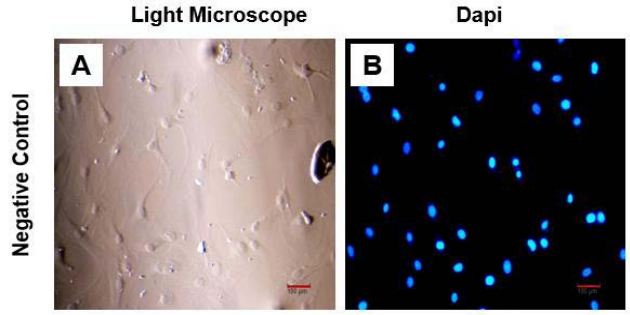

Figure 3: Negative control staining: **(a)** Light microscope image and **(b)** Immunofluorescence DAPI staining of individual cell nuclei. [Scale bar 100 μm].

staining intensity as shown in Figure 2A-C. Not only surrounding the nucleus of chondrocytes, but also predominant around the cell membrane in the ECM. Chondrocytes treated with 1 μM of WIN-2 seemed to produce the highest expression of S100 (0.655 ± 0.017SD) (Figures 2B and 2G) which is ~25% higher than control groups (0.510 ± 0.021SD). The higher concentration of WIN-2 showed an average optical density of only 0.491 ± 0.021SD ($P<0.05$) which is lower than both control and 1 μM of WIN-2.

Immunofluorescence and ELISA based staining for fibronectin of chondrocytes treated with 1 μM and 2 μM of WIN55, 212-2

Fibronectin was mainly observed as networks of bright fluorescence dots in the pericellular matrix as shown in Figure 1D-F. The fluorescence intensity and optical density produced by chondrocytes treated with 2 μM of WIN-2 were the greatest, indicating the highest expression of fibronectin for this treatment compared to the other two treatments (Figures 1D-F and 1H). The lowest expressions were for untreated cells producing an optical density reading of 0.225 ± 0.011SD. In contrast, the highest reading was produced by chondrocytes treated with 2 μM WIN-2 (0.338 ± 0.009SD), which was ~33.4% higher than untreated cells ($P<0.05$). In the same way, treating chondrocytes with 1 μM of WIN-2 increased the secretion of fibronectin by ~ 22.7% ($P<0.05$), where the optical density value was 0.291 ± 0.006SD.

Immunofluorescence staining of chondrocytes without primary antibodies

Figure 3 shows the images produced by phase contrast and fluorescence microscopy. The specificity of the primary antibodies was confirmed by the lack of specific staining because no primary antibodies were used.

Detection of MMP-2 secreted by chondrocytes treated with 1 μM and 2 μM of WIN55, 212-2 using an Elisa based method

As shown in Figure 4A, MMP-2 expression of chondrocyte monolayers was significantly lower than control groups after 16 hours of incubation with WIN-2 ($P<0.05$). The Elisa based technique, therefore, revealed that MMP-2 secreted by chondrocytes treated with 1 μM and 2 μM of WIN55, 212-2 was ~23.6% and 51.6% lower than control groups, respectively.

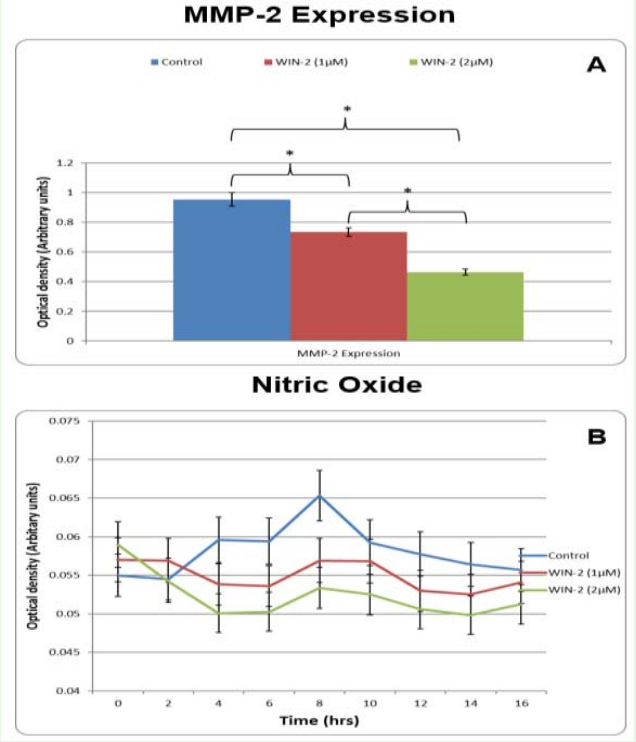

Figure 4: A shows the optical density readings (± SD) at 450nm following immunoperoxidase staining of deposited MMP-2 following 16 hours incubation with 1 μM WIN-2, 2 μM WIN-2 and control conditions (n=12). B shows the optical density readings for NO (± SD) at 570nm following nitrate Greass reagent staining for chondrocytes treated with 1 μM WIN-2, 2 μM WIN-2 and control conditions over a period of 16 hours (n=36).

Nitric oxide quantitative staining

As shown in Figure 4B, the three treatments showed similar NO expressions for the first two hours with very little difference ($P>0.05$). However, after two hours, a very large gap started to appear between the three treatments. Untreated chondrocytes expressed significantly ($P<0.05$) high levels of NO up to 8 hours, whereas WIN-2 at 2 μM induced the lowest NO expression ($P<0.05$) followed by the 1 μM WIN-2 with similar behaviour but slightly higher NO expression than 2 μM WIN-2. The remarkable differences in NO expression remained almost the same for the treated conditions over the 16 hour period whereas the control reached its peak at 8 hours and then the NO expression reduced up to 16 hours.

Discussion

In vivo, chondrocytes synthesize cartilage extracellular matrix including collagens, proteoglycans, and non-collagen proteins such as fibronectin (a glycoprotein) that bind to integrins on cell membrane. Moreover, type-II collagen is responsible for the ability of cartilage to resist tensile and shear stresses. Thus, collagen type-II is considered as a predominant protein in the formation of cartilage [25] and can be used as a chondrogenic marker [7]. On the other hand, it is a well-established fact that chondrocytes cultured in monolayers have propensity for losing their differentiated function [26]. This dedifferentiation to fibroblast like cells (fibrocytes) is marked by a reduction in the secretion of collagen type-II and increase in the

secretion of type-I collagen [27]. For these reasons, the effect that the synthetic cannabinoid WIN-2 had on chondrocytes monolayer expression of both collagen type-II and type-I was investigated. All chondrocyte monolayers, including control groups, secreted collagen type-II, indicating a chondrocytic phenotype. However, there were variations in the levels of collagen type-II secreted by the chondrocytes following the 3 different treatments.

Results reported previously [2] showed that the synthetic non-specific cannabinoid WIN-2 at 1μM significantly increased the rate of wound closure, proliferation and collagen type-II expression. Also interestingly, as seen in Figures 1D-F and 1H, collagen type-I secretion was reduced by the same treatment. Therefore, WIN-2 increased chondrocytes differentiation which might have led to an increase in cell migration and proliferation. A similar study [28] is in good agreement with current findings, i.e. in monolayers, migratory chondrocytes express more collagen type-II and less collagen type-I compared to static chondrocytes. It is also reported [29] that chondrocytes are expected to be more mobile than fibrocytes, and in monolayers, active motile chondrocytes may be more differentiated expressing higher concentrations of type-II collagen compared to less active ones.

Other factors affecting the differentiation status of chondrocytes include interleukin-1beta (IL-1β) a major catabolic pro-inflammatory cytokine involved in cartilage destruction which is known to induce chondrocyte dedifferentiation [9]. WIN-2 has been found to strongly inhibit IL-1β signaling pathway in human astrocytes in a cannabinoid receptor-independent manner [30]. In the present study there is also a possibility that WIN-2 may have induced chondrocyte differentiation by inhibiting IL-1β because blocking CB2 receptor with AM-630 (CB2 antagonist) did not significantly alter WIN-2's effect on proliferation and wound repair rate of the chondrocyte monolayers reported previously [2]. Furthermore, both CB1 and CB2 receptors are coupled to heterotrimeric Gi/o proteins and activate the mitogen-activated protein kinases (MAPK) extracellular signal regulated kinase ERK-1/2 [31], and stimulation of CB2 receptors activates ERK-1/2 pathway. ERK-1/2 pathway is required for regulating dedifferentiation and inflammation, where its inhibition was found to reverse chondrocyte induced dedifferentiation increasing collagen type-II expression [27].

Previous experiments indicated that chondrocyte monolayers treated with WIN-2 showed the roundest morphology compared to all the other treatments [2]. This can be supported by the critical role that ECM proteins like collagen type-II which play to modulate cellular phenotypes during chondrogenic differentiation [7].

Likewise, regardless of the treatment, chondrocytes expressed S100 protein confirming their chondrocytic phenotype (Figures 2A-C and 2G). However, chondrocytes treated with the lower concentration of WIN-2 displayed the highest concentration of S100, whereas treating chondrocytes with the higher concentration of WIN-2 did not alter S100's expression significantly (Figures 2A-C and 2G). Interestingly, it can be concluded from this work there is a direct correlation and an inverse correlation between S100 and collagen type-II and collagen type-I expressions, respectively. The treatment that induced the highest concentration of collagen type-II produced the highest concentration of S100 and vice versa. S100 is considered as a chondrocytes differentiation marker and in this regard, the expression of S100 has been reported [32] at different time intervals in a manually defected full thickness rabbit femoral condylar over a period of 120 days. S100 protein was present at higher levels in the early stages of the repair process. The presence of S100 protein identified cells with chondrogenic phenotype, and the lack of S100 protein in other cells embedded in conventionally stained matrix suggested that these cells were no longer of a chondroid phenotype, especially that there was very low concentration of S100 in the hypertrophic layer from a fibrocellular surface layer [32]. The fact that this experiment was carried out in vivo and gave results that agreed with current findings increases the possibilities of chondrocytes responding similarly to WIN-2 in vivo. Moreover, chondrocytes from normal cartilage specifically from perivascular areas of the neonatal articular cartilage and in the deep zone of the infant articular cartilage demonstrated a much stronger positive S100 staining compared to cells found in the intermediate zone [11]. The protein synthesized in the deep zone was greater than other regions where the chondrocytes are relatively large and have many intra-cytoplasmic filaments, Golgi bodies, abundant endoplasmic reticulum and glycogen granules. In mild Osteoarthritis (OA), S100 positive chondrocytes were only found in the intermediate zone, but also found in clusters and metaplastic cartilage arising from bone marrow in severe OA [11]. These findings also suggest that an increase in the expression of S100 indicates a rise in the metabolic activity of the cartilage matrix such as collagen and proteoglycan. Nonetheless, there is some evidence that S100 protein expression is upregulated in osteoarthritic cartilage, and may promote cartilage degeneration by activating the receptor for advanced glycation endproducts (RAGE) signalling pathway [32]. On the contrary, other studies have suggested that S100 may increase cellular proliferation [33] and that RAGE is not a universal receptor for S100 protein in other cell types which may apply to chondrocytes. In fact, S100 protein may have also contributed to the increase in the rate of wound closure associated with 1μM of WIN-2 treatment [2]. Then again, many studies have highlighted S100 protein's proinflammatory activities, and its presence at elevated levels in serum and at extravascular sites in people affected with diverse inflammatory pathologies and infections [34]. S100 also promotes monocyte and neutrophils migration and may also be involved in leukocyte migration [34]. While in vitro, raised levels of S100 proteins may have increased chondrocyte migration in the same way it did with other cell types, it may also increase the leukocytes in synovial fluid of the joints. This accumulation of leukocytes may cause loss of articular cartilage proteoglycan.

It is well recognized that cellular interactions such as cell migration, adhesion and invasion between cells and the extracellular matrix are facilitated by integrins [35], and thus, it is not surprising that not only collagen type-II and S100, but also fibronectin may promote chondrocyte migration [35]. Likewise, previous studies have shown that chondrocyte differentiation and proliferation significantly elevated fibronectin secretion compared to control groups [28]. Accordingly, chondrocytes motility was increased by an average of 3.3-fold by coating Boyden chambers with 1 μg/ml of fibronectin [28]. In other words, an increase in chondrocytes migration could be associated with an increase in the concentration of fibronectin. In this work and as reported previously [2], fibronectin expression was further increased (Figures 1D-F and 1H) whereas wound closure rate and cell proliferation were notably reduced by increasing the concentration of WIN-2 from 1 μM to 2 μM [2]. Essentially,

fibronectin is an ECM protein that is mainly responsible for cell's attachment to the ECM by binding to integrins on cell membrane [35]. Consequently, an increase in adhesion would be expected with higher concentrations of fibronectin. Some studies have also suggested that some matrix components such as the fibronectin related protein fibulin and proteoglycans decorin and versican, may provide migratory inhibitory signals [36]. In agreement with this, other studies suggest cell migration is optimised by an intermediate degree of cell attachment and that very tight adhesion to the substratum reduces locomotive ability [37]. Therefore, the highest migratory rate would be expected with the optimum and not the highest fibronectin expression. At the transcriptional level, fibronectin is one of the macromolecules that regulate matrix metalloproteinase like MMP-2 [37]. For example, activation of MMP-2 and MMP-9 was impressively down regulated for HT1080 fibrosarcoma cells cultured on fibronectin fragments compared to full length fibronectin [37]. Other cancer cells showing the same behaviour include breast cancer cells and colon cancer cells [38]. Cancer cells probably use the proteinase secreted by normal fibroblasts to facilitate tissue invasion. This does not mean that elevated levels of MMP-2 will increase normal cell migration, because cancer cells use this technique to induce self-destruction of normal tissue removing any barriers, and making it easier to invade. Then again, maybe this is what happens in arthritic joints, leading to self-destruction of cartilage. Support for this view comes from evidence indicating elevated levels of fibronectin and fibronectin fragments in arthritic synovial fluid [39]. Fibronectin fragments were found to act as proteinases themselves or encourage fibroblasts to produce metalloproteinases [39], whereas normal intact fibronectin failed to induce MMPs expression in periodontal ligament [18], which is a connective tissue. Conversely, MMP-2 and other matrix proteinases were proven to promote fibronectin degradation [40]. This may explain the noticeable reduction in MMP-2 secretion caused by WIN-2 at a concentration of 1 μM which was further decreased by higher WIN-2 concentration of 2 μM (Figure 4A). In addition, WIN-2 reduced the secretion of MMP-9 in a murine model of cigarette-smoke induced lung inflammation; MMP-2 and MMP-9 have similar substrates [41]. Therefore, numbers of studies suggest that cannabinoids may have antitumorigenic effects [42] because they reduce MMPs secretion and hence reducing healthy tissue invasion by sarcoma cells. Moreover, purified collagen type-II was cleaved by MMPs that are known to occur naturally [42], and MMP-2 has been described as a collagenase [43]. Yet, the lowest concentration of MMP-2 recorded did not correlate with the highest concentrations of the two collagens examined. Chondrocytes treated with 2 μM of WIN-2 secreted lower concentrations of collagen type-II, while reducing wound repair rates and cell proliferation, compared to control groups. This is probably due to reducing cell vitality, which will in turn reduce the protein expression. On the other hand, in vitro, osteosarcomic cells attachment to monomeric collagen type-1 increased the amount of fully active MMP-2 independently to S100 expression by elevating TIMP-1 levels, while attachment to 3D fibrilar collagen type-I had an inverse effect [44]. Nevertheless, the fact that higher levels of collagen type-I was associated with lower levels of MMP-2 increases the relevance of current findings to 3D tissue models.

Nitric oxide (NO) levels were nearly the same for the three treatments at zero hours (Figure 4B) which are released mainly by damaged chondrocytes [45]. WIN-2 at both concentrations used in this work managed to reduce NO expression up to 4 hours and then varied reduction took place until the end of the monitoring time in a dose dependent manner (Figure 4B). CB2 receptor agonists have been found [46] to stimulate anti-inflammatory healing reactions in activated microglia by inhibiting pro-inflammatory induced production of NO [46]. It has also been reported [47] that WIN-2 (similar to this work) and cannabinol induced inhibition of NO (reduced in a dose related manner) by CB1 receptor antagonist, but not CB2 receptor antagonist [47]. The findings in this work are in agreement with others and suggest that WIN-2 may inhibit NO expression by activating the function of CB1 receptor.

Conclusion

Treatment of primary chondrocytes with 1 μM of WIN-2 increased the secretion of collagen type-II and decreased that of collagen type I. It can also be concluded that there is a direct and indirect correlation between S100 and collagen type II and type I expressions, respectively. The treatment that produced the highest concentration of collagen type II produced the highest concentration of S100 and vice versa and therefore S100 can be considered as the chondrocyte differentiation marker (also reported by others). The highest expression of fibronectin was associated with 2 μM of WIN-2 and the lowest expression was for the untreated cells. MMP-2 expression of treated chondrocytes with WIN-2 was found to be significantly lower than control groups. Untreated chondrocytes expressed significantly high levels of NO and that 2 μM of WIN-2 induced the lowest NO expression. Our findings, therefore, suggest that WIN-2 is able to improve cartilage wound healing by reducing the inflammation factors such as the matrix metalloproteinase-2 (MMP2) and nitric oxide (NO). This has the potential for reducing pain and improving the quality of life for patients. The overall concluding remark is that treating chondrocytes with WIN-2 may be used to improve cartilage repair by reducing the formation of fibrocartilage and increasing the synthesization of articular cartilage via inducing chondrocytes differentiation. This is particularly important due to the loss and abnormal remodelling of the cartilage extracellular matrix including collagens, proteoglycans and non-collagen proteins causing the critical pathogenic events of osteoarthritis [48].

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