

Immunohistochemistry Evaluation of TGF- β 1, SOX-9, Type II Collagen and Aggrecan in Cartilage Lesions Treated with Conditioned Medium of Umbilical Cord Mesenchymal Stem Cells in Wistar Mice (*Rattus norvegicus*)

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ABSTRACT

Currently, umbilical cord mesenchymal stem cells have the potential to be used as treatment options for any cartilage lesion. This research aimed to evaluate the effects of conditioned medium from umbilical cord mesenchymal stem cells (UC-MSC) on damaged cartilage through the expression of proteins TGF- β 1, SOX-9, type II collagen and aggrecan, which are known to be related to chondrogenesis. UC-MSC were isolated from 19-days-pregnant Wistar mice and were cultured using the standard procedure to obtain 80% confluence. Subsequently, the culture was confirmed through a microscopic examination that was driven to be an embryoid body to obtain a pre-condition medium. This research utilized 3-month-old male Wistar mice and was categorized into 6 groups (3 control and 3 treatment groups). Each animal had surgery performed to create a femur condyle cartilage defect. The treatment groups were administered a dose of stem cells at 1 mL/kg. Next, immunohistochemical (IHC) staining was performed to examine the expression of TGF- β 1, SOX-9, type II collagen and aggrecan in the 2nd, 3rd, and 4th month of evaluation. The results were analyzed statistically using ANOVA test. For each of the treatment groups, there was increased expression ($p < 0.05$) in all proteins TGF- β 1, SOX-9, type II collagen and aggrecan when compared with control groups at the 2nd, 3rd, and 4th month of evaluation. Pre-conditioned medium from UC-MSC potentially increases the expression of TGF- β 1, SOX-9, type II collagen and aggrecan in the damaged cartilage of Wistar mice.

Keywords: *Cartilage lesions, conditioned medium, immunohistochemistry, umbilical cord mesenchymal stem cells*

INTRODUCTION

Stem cell-derived conditioned medium (CM) has the potential to be produced as a treatment option for regenerative medicine. The use of secretome containing CM has several advantages when compared to the use of stem cells. Briefly, CM can be manufactured, freeze-dried, packaged, and transported with greater ease [1]. Moreover, as it is devoid of cells there is a lower risk of host rejection. To date, there is limited data pertaining to the use of CM for cartilage repair.

Mesenchymal stem cells (MSC) are a population of cells with infinite proliferation capability and the potential to differentiate into cells of the mesoderm lineage. Research studies have reported that MSC has the capa-

bility to differentiate into osteoblasts, chondrocytes, cardiomyocytes, myocytes, adipose cells and even neural cells. MSC can be isolated from multiple areas in adult tissue such as bone marrow, adipose tissue, umbilical cords, and peripheral blood [2, 3, 4]. Umbilical Cord Derived Mesenchymal Stem Cells (UC-MSCs) have proliferation and differentiation capabilities. These stem cells have also been shown to have the ability to differentiate into unipotent cells that have a chondrocyte pattern [5].

Deterioration of cartilage leads to osteoarthritis, a disease through which articular chondrocytes become deformed, or fibrillated losing their cartilage forming function. There are several proteins that are associated

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How to cite:

Soetjahjo B, Hidayat M, Suyuti H, Fibrianto YH (2018) Immunohistochemistry Evaluation of TGF- β 1, SOX-9, Type II Collagen and Aggrecan in Cartilage Lesions Treated with Conditioned Medium of Umbilical Cord Mesenchymal Stem Cells in Wistar Mice (*Rattus norvegicus*). J. Trop. Life. Science 8 (1): 21 – 27.

with chondrogenesis. TGF- β 1 promotes chondrogenesis in cultures of early-undifferentiated MSC *in vitro* and stimulates chondrogenic differentiation *in vivo* [6]. Previous studies reported that expression and activity of TGF- β 1 signaling could potentially have detrimental effects on mature condylar cartilages [12]. TGF- β 1 has an ability to down regulate type II collagen and aggrecan in articular chondrocytes via the SOX-9 protein [7]. SOX-9 is a transcription factor belonging to the Sry-type HMG-box (SOX) protein family, which is essential for chondrogenesis and has been termed a “master regulator” of the chondrocyte phenotype. In addition, SOX-9 has been shown to activate type II collagen and aggrecan. Moreover, SOX-9 prevents chondrocyte hypertrophy and has a redifferentiation effect on osteoarthritic chondrocytes, which have been dedifferentiated. Type II Collagen, a major component of hyaline cartilage, has many advantages as a biomaterial for chondrogenesis, such as biodegradability and the capability to induce repair processes in articular cartilage [8]. Aggrecan is one of the major components of cartilage and binds to hyaluronan (HA) and links proteins to form huge aggregates. These aggregates lead to a hydrated gel-like structure of cartilage and resistibility to compression and deformation in joints [9].

Currently, there has been vast research to study factors that promote cartilage repair. However, only a few describe the secretome of UC-MSCs in chondrogenesis has been reported. Using immunohistochemical (IHC) evaluation this research would investigate the potential of CM of UC-MSCs obtained from Wistar mice with a focus on proteins TGF- β 1, SOX-9, type II collagen and aggrecan expression in a damaged cartilage wound.

MATERIALS AND METHODS

This research was performed using 24 Wistar mice 3 months old that underwent surgery within its medial condyle of the femur in right hind feet on weight bearing area. The samples were divided into 6 groups. Three control groups were 2(K2), 3(K3) and 4(K4) months, and three treatment groups were 2(E2), 3(E3) and 4(E4). The duration of the study was 4 months. This study has been approved by Ethical Clearance Commission of Gajah Mada University, Yogyakarta, in Mei 6th 2015 (Letter number: 263/KEC-LPPT/V/2015).

Isolation and cultivation of UCMSCs

The umbilical cord was obtained from 19 days pregnant Wistar mice by caesarian section according to standard procedures. Briefly, under sterile conditions,

one centimeter of umbilical cord was washed with 10% betadine and sterile NaCl. Next, that slash was placed inside a centrifuge tube containing transport media Dulbecco's Eagle Modified Medium (DMEM) with 200 μ g/L penicillin, 200 μ g/mL streptomycin, and 200 μ g/mL fungi zone). Next, the cord was cut in 20 mm³ sizes and cultured using an enzymatic technique. This cord slash was further diluted with trypsin/EDTA 0.25% and incubated for 30 minutes at 37°C. About 2 cc of complete medium (DMEM 1 \times , 10% fetal bovine serum, 50 μ g/mL penicillin streptomycin, and 2.5 μ g/mL fungi zone) was added to the tube already containing the cord that had been metabolized by the enzyme. It was then centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatant was removed and the cell suspension was mixed with complete medium and incubated at 37°C and 5% CO₂. It was replaced every three days until cell growth reached a confluence of 80%. Afterward, microscopic examination was performed using Hematoxylin and Eosin (H&E), Giemsa and Sirius Red staining to confirm the existence of the cells, and further confirm they are umbilical cord mesenchymal stem cells (UC-MSCs).

Production of conditioned medium

Cultures of UC-MSCs that have reached 80% confluence were harvested using trypsin. Following, trypsin neutralization, the cell suspension was centrifuged at 3,000 rpm for 10 minutes. The supernatant was discarded and the cell pellet was washed three times with PBS. The cell pellet was resuspended in fresh medium at 10,000 cells per mL. Next, the stem cells were pushed towards an embryoid body and seeded onto a culture plate with full medium until confluence among these bodies were formed. The production of pre-condition medium was created by washing the embryoid body culture with sterile PBS and filling the culture plate with 10 mL of complete medium without serum. After 48 hours, the pre-condition medium was stored at -20°C prior to use.

Mice treatment with conditioned medium

Prior to the treatment, the Wistar received anesthesia of ketamine : xylazine (9 : 1). Next, the Wistar were intentionally damaged using manual mechanical drilling in the medial condyle femur cartilage (750 rpm) with Kirshner wire diameter 1 mm and depth 1 mm to make a sharp slope base cartilage lesion. Afterward, they were divided into 6 groups, 3 control groups (K2, K3, K4) and 3 treatment groups (P2, P3, P4). The treatment

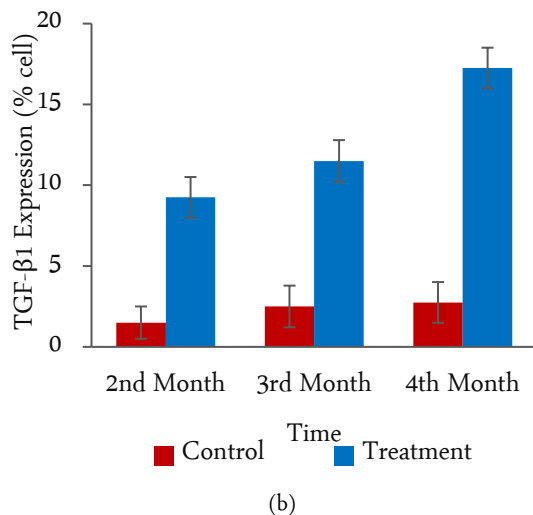
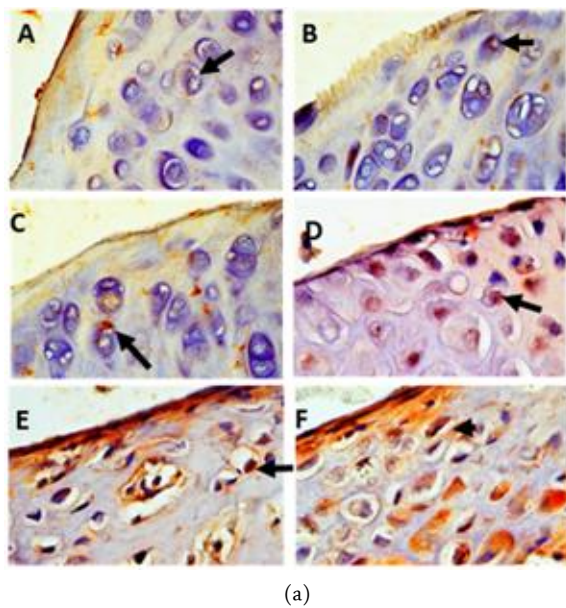


Figure 1. IHC results from the articular cartilage network joint (left panel), photomicrograph at 400 \times . (a) The chondrocytes with TGF β 1 expression are characterized by the brown color of the cell cytoplasm (arrows). A. Control 2nd month, B. Control 3rd months, C. Control 4th months, D. Treatment 2nd months, E. Treatment 3rd months and F. Treatment 4th months. (b) Graphic of the calculation analysis of all preparations of joint cartilage tissue (right panel). The X-axis is the treatment time group and Y axis represents the mean expression of TGF β 1.

groups were administered an injection of 1 cc/kg of body weight pre-condition medium 5 times over 1-week interval. The Wistar were observed at 2nd (P2 groups), 3rd (P3 groups), and 4th (P4 groups) months of evaluation. At this time, animals were humanely euthanized and the

cartilage was extracted. Articular cartilage tissue was placed inside a tube containing 10% formalin. Biopsy samples were sent to the Biochemistry-Biomolecular Laboratory of Medical Faculty - Brawijaya University, Malang.

Tissue processing (fixation and paraffin embedding) and slide preparation of 4 μ m cut thickness using a rotary microtome was performed. An immunohistochemistry technique was performed to observe the protein expression of TGF- β 1, SOX-9, Collagen type 2 and Aggrecan using specific antibodies purchased from Santa Cruz Biotech (USA) and immunohistochemistry kit using D-Bio Sys Immunostaining kit (Netherlands). The samples were reacted with primary antibody of mouse anti Col-2 (COL2A1 Antibody (M2139) cat#: sc-52658), TGF β 1 (TGF- β 1 Antibody (3C11) cat#: sc-130348), Sox-9 (Sox-9 antibody (E-9), cat#: sc-166505) and aggrecan (aggrecan antibody (4F4), cat#: sc-33695) for 24 hours at 4 $^{\circ}$ C, then cleaned with PBS pH 7.4 three times for 5 minutes. The samples were incubated using secondary antibody of biotin (rabbit anti-mouse IgG biotin labelled) for 1 h at room temperature, then washed with PBS pH 7.4 three times for 5 minutes. Strep Avidin-Horse Radin Peroxidase (SA-HRP) were added to the slides and incubated for 40 minutes then washed with PBS pH 7.4 three times for 5 minutes. Diamano Benzidine (DAB) were added to the slides and incubated for 10 minutes then washed with PBS pH 7.4 three times for 5 minutes. Counter staining the slides with Mayer Hematoxylen for 10 minutes then washed with aquadest and dried. Finally, the slides were mounted with entellan and covered with cover glass. The results were evaluated using a micrograft photo using a Nikon E-100 microscope and Sony ICLEA7 camera with 400 \times magnification. Lastly, the expression was analyzed using immunoratio software (freeware from Institute of Biomedical Technology, University of Tampere).

Statistical analysis

Control and experimental groups were analyzed using statistic parametric ANOVA and descriptively in mean \pm SD or median, frequency is presented in percentage. For normality or distribution, we use Kolmogorov-Smirnov test and two independent T-test for normal distribution data, $p < 0.05$ indicated a statistically significant difference.

RESULTS AND DISCUSSION

To investigate the potential role of conditioned medium of UC-MSc in articular cartilage repair this study

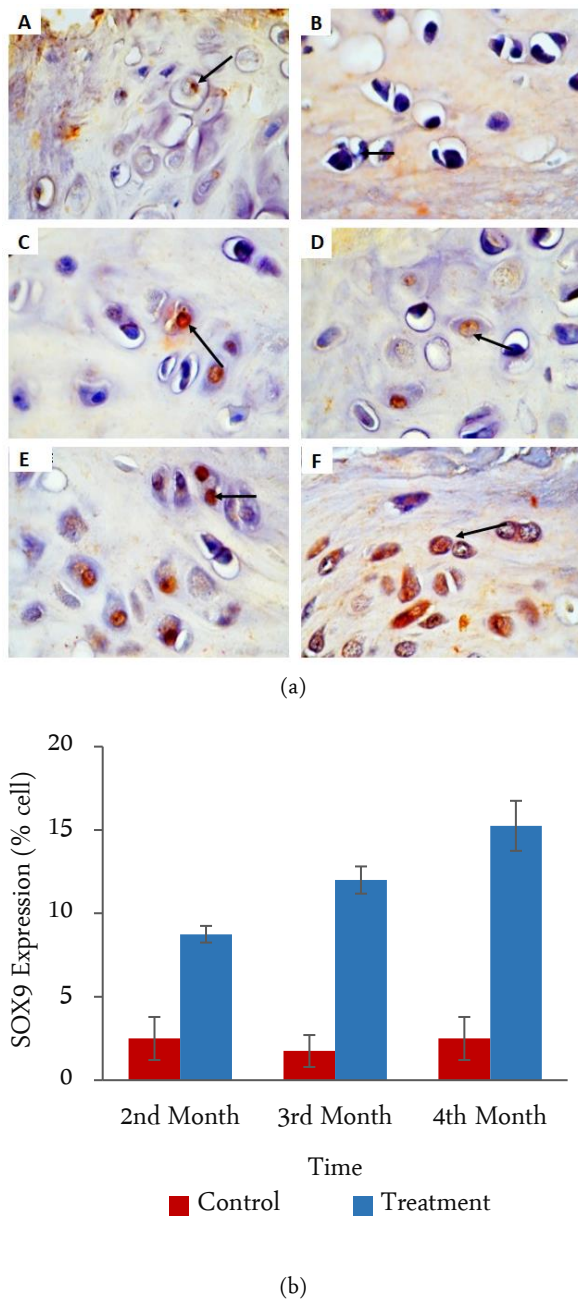


Figure 2. IHC results from the articular cartilage network joint (left panel), photomicrograph at 400x. (a) The chondrocytes with the SOX-9 expression are characterized by the brown color of the cell nucleus (arrow). A. Control 2nd month, B. Control 3rd months, C. Control 4th months, D. Treatment 2nd months, E. Treatment 3rd months and F. Treatment 4th months. (b) Graphic of the calculation analysis of all preparations of joint cartilage tissue (right panel). X axis is the treatment time group and axis Y represents the mean of SOX-9 expression.

used immunohistochemical techniques, with a peroxidase system, to show the expression of TGF-β1, SOX-9,

type II collagen and aggrecan proteins.

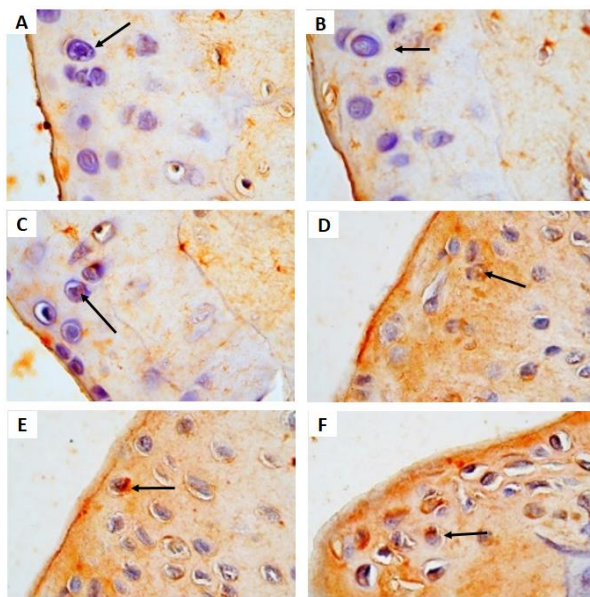
The expression of TGF-β1 was evident in all study samples. Figure 1 shows that TGF-β1 is expressed in the cytoplasm of the chondrocytes of the tegmental area, as well as the transitional layer of the joint cartilage tissue. Using statistical analysis demonstrated that TGF-β1 expression in the treatment group increased significantly when compared with the control group (bottom). Expression of TGF-β1 ($p > 0.05$) between the 2nd, 3rd, and 4th observational groups in the negative control group (untreated mice). Overall, the expression of TGF-β1 in the treatment group demonstrated a gradual increase (months 2nd, 3rd, and 4th) and was significantly different in comparison with the control group (B) ($p < 0.05$).

Similar values were observed for the expression of SOX-9 ($p > 0.05$) between the 2nd, 3rd, and 4th observational groups and the negative control group (untreated mice). Furthermore, there was a significant difference in the expression of SOX-9 ($p < 0.05$) between the 2nd, 3rd, and 4th observation groups in the treatment group (administering fasting MSC CM). SOX-9 expression demonstrated the most significant difference in the 3rd month expression (12.00 ± 0.82^b) and 4th month (15.25 ± 1.5^c) of the treatment group (Figure 2).

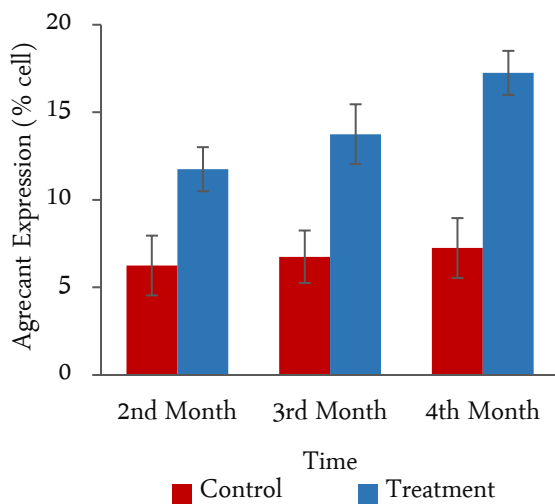
Type II collagen expression was also observed in each study sample. This result indicated that type II collagen is widely expressed in the cell cytoplasm in the transitional area of the articular tissue of cartilage of the joint. Using statistical analysis, the results showed that type II collagen expression was increased significantly compared to the control group (bottom). Overall, type II collagen expression in the treatment group demonstrated a gradual increase (2nd, 3rd, and 4th month) and was observed to be significantly different when compared to the control group ($p < 0.05$). Additionally, the expression of type II collagen under control conditions began in the 4th month ($p < 0.05$).

The aggrecan expression ($p > 0.05$) was not significantly different between the 2nd, 3rd, and 4th observational groups in the negative control group (untreated mice). Based on the mean value of aggrecan expressions, the values were similar. The significant difference in aggrecan expression ($p < 0.05$) was observed in the analysis between the 2nd, 3rd, and 4th month observational groups of the treatment group (MSC). Based on the mean values of aggrecan expression, there was a significant increase in the 4th month. While in the 2nd and 3rd months aggrecan expression remained the same (Figure 3).

UC-MSC are considered medical waste and the collection is noninvasive. Therefore, access to UC-MSC has



(a)



(b)

Figure 3. IHC results from the articular cartilage network joint (left panel), photomicrograph at 400x. (a) Chondrocytes with an expression of aggrecans are characterized by the brown color of the cell cytoplasm (arrows). A. Control 2nd month, B. Control 3rd months, C. Control 4th months, D. Treatment 2nd months, E. Treatment 3rd months and F. Treatment 4th months. (b) Graphic of the calculation analysis of all preparations of joint cartilage tissue (right panel). The X axis is the treatment time group and the Y axis represents the average of the aggrecan expression.

not been burdened with ethical problems [10]. MSC conditioned medium (CM) could be defined as secreted factors alone that are referred to as the secretome or ex-

osome without the stem cells. In addition, CM is potentially less allergenic according to the selected proteins that are transplanted to some tissues. In our study, we analyzed a group of proteins that are potentially found in the medium and have a potential to be beneficial in cartilage repair. The growth, development, maintenance, and improvement of articular cartilages are strictly regulated by multiple signaling pathways performed by several bioactive factors [11].

TGF-β1 is an especially significant factor in the chondrogenic differentiation of MSCs. It is considered to be a potent stimulator for proteoglycan and type II collagen synthesis [12]. Ye Li *et al.* reported that the expression of TGF-β1 was increased in the condylar cartilage of an injurious model of OA [7]. Injecting TGF-β1 into the periosteum of the femur stimulates chondrocyte differentiation and cartilage formation [13]. Additionally, we observed that the expression of TGF-β1 was slightly increased in all experimental groups, across all time points, when compared to the control groups. There are two important proteins, SOX-9 and RUNX2, which function as transcription regulators of the *TGF-β1* gene [14]. We speculate that the administration of UC MSC-CM activated one or both of the above regulatory proteins (SOX-9 and RUNX2), resulting in increased expression of TGF-β1. Further, this is in line with the research of Venturin *et al.* who reported that TGF-β1 is expressed in all the differentiating chondroprogenitor and chondrocytes in the proliferative zone [15].

SOX-9 is expressed in all chondroprogenitor cells, predominantly in mesenchymal condensations and cartilage. Cao *et al.* also found that the repair of cartilage defects can be enhanced by SOX-9 transduction [16]. They reported that the articular cartilage defects treated with MSC overexpressing SOX-9 showed the significantly better integration of the repair tissue at the interface with the surrounding normal articular cartilage when compared to the other groups. It is well documented that TGF-β induces expression of a transcriptional factor SOX-9 in its signaling pathway [8]. In our study, we found that SOX-9 expression was greatly increased. We suggest that a high level of SOX-9 expression will have beneficial effects on cartilage repair.

Type II collagen comprises more than 90% of the collagen found in adult articular (hyaline) cartilage. In this study, we observed that type II collagen was increased in all samples. This suggests that the CM contains potent components for activation of those proteins and displays promising potential for cartilage repair. It

is known that SOX-9 is a potent activator in the formation of collagen type II [15], which is a component of cartilage. Moreover, this is the predominant type of collagen in cartilage. The extracellular matrix is also comprised of other specific collagens such as type IX and XI [17]. Following the increase in SOX-9 mRNA expression, the production of type II collagen, aggrecan, and cartilage oligomeric matrix protein (COMP) is observed [8].

Aggrecan is one of the major structural macromolecules of cartilage. Aggrecan function is to resist any compressive forces by maintaining the osmotic pressure balance in cartilage [18]. According to our study, we found that aggrecan expression was significantly increased in all samples. We speculate that aggrecan production plays a critical role in cartilage repair techniques by maintaining the collagen network. Therefore, aggrecan participates in both the demise and survival of articular cartilage.

This suggests that there has been an increase in tissue growth according to the initial hypothesis referring to studies conducted by Mehrabani *et al.* [19] who examined cartilage defects in 12 rabbits, in which treatment groups were given mesenchymal stem transplantation. The results of the study suggest that the nature of the predominant tissue in the transplant group had a higher score than the control group.

This study has limitations to find a solution in treating cartilage defects, damaged by trauma. The outside problem is not discussed. An update of this study was the beginning of the further study for clinical experimental to find a solution to treat the problem of cartilage lesion in human as an effective, inexpensive, and convenient alternative choice.

CONCLUSION

Conditioned medium from CM-UC-MS-C contains proteins and tissue regenerative agents, which are secreted by the stem cells and allow for cellular interactions between cell-to-cell and their microenvironment for successful chondrogenesis. Results from our study indicate that when evaluated using immunohistochemistry, CM from UC-MS-C increases the expression of protein TGF- β 1, SOX-9, type II collagen and aggrecan. These increased proteins are closely related to chondrogenesis in cartilage defects in a Wistar rat.

ACKNOWLEDGMENT

The author thanks to Brawijaya University for facilitating this research.

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