

Conditioned medium of IGF-1-induced human synovial membrane mesenchymal stem cells effects on inflammatory response of osteoarthritic *in vitro* model

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ABSTRACT

Synovial membrane mesenchymal stem cells (SM-MSCs) possess excellent regenerative potential, making them a viable option for osteoarthritis (OA) therapy. Moreover, these cells can be easily obtained from the human body. Insulin-like growth factor (IGF-1) can be used to decrease the pro-inflammatory marker, these markers can trigger inflammation in OA. These challenges can be addressed by utilizing a conditioned medium (CM) derived from stem cell culture. This study aims to compare the effective way of OA therapy between CM SM-MSC and CM SM-MSC induced by IGF-1 by observe the pro-inflammatory markers such as NF-kB, RANTES, PGE₂, COL1 and the anti-inflammatory marker, Aggrecan (ACAN). Chondrocyte cells induced IL-1 β as OA model (IL1 β -CHON002) treated with IGF-1 15% and 30% and without IGF1-induced SM-MSCs-CM to know its effectiveness in decreasing the pro-inflammatory markers. ELISA and RT-qPCR methods were performed to analyze this effect. Based on the data result, SM-MSCs-CM is estimated to have the potential to treat OA as seen from the content of growth factors in CM, decreasing the markers of inflammation. The most significant reduction in PGE₂, RANTES, NF-kB, and COL1 concentration was found in the treatment of SM-MSCs-CM cultured with IGF1 concentrations of 150 ng/mL. The higher aggrecan (ACAN) concentration was found in IGF150 15%. Conclusion: CM from SM-MSCs cultured with IGF150 with concentrations of 15% and 30% resulted in the most effective concentration to decrease the concentration of pro-inflammatory markers and also to increase the anti-inflammatory markers.

Keywords: Conditioned medium; IGF-1; osteoarthritis; pro-inflammatory marker and SM-MSCs

INTRODUCTION

Osteoarthritis (OA) is a chronic joint condition that impacts multiple elements of the joint structure, encompassing the cartilage, synovial lining, ligaments, and underlying bone. It is characterized by cartilage degradation, the development of bone spurs (osteophytes), and the sclerosis of subchondral bone. These alterations result in pain, limited functionality, and a

diminished quality of life. OA is highly prevalent and debilitating in developed countries. It is estimated that approximately 9.6% of men and 18.0% of women aged 60 or above suffer from symptomatic OA. According to the World Health Organization (WHO), between 2002 and 2007 the ranking of OA as a leading cause of disability or morbidity increased significantly, moving from the 12th position to the 6th position. This upward trend can be attributed to factors such as population aging and longer life expectancy. Based on projections, it is anticipated that by 2020, OA will further climb in the rankings and become the fourth leading cause of disability (Valdes & Stocks, 2018).

Stem cells are cells that are not yet differentiated and possess the extraordinary ability to undergo self-renewal and differentiation into various specialized cell types, much like their embryonic counterparts. This unique characteristic makes them valuable for tissue regeneration and repair. Mesenchymal stem cells (MSCs) have a multipotent capacity, which can be differentiated into several mesenchymal lineages of cells, including adipocytes, chondrocytes, and osteocytes. Previous studies have revealed that MSCs possess immunomodulatory features and homing properties to the injury site is facilitated by the secretion of specific inflammatory cytokines, chemokines, and extracellular matrix (ECM) proteins. MSCs can be obtained from any part of the human body, among them is synovial membrane mesenchymal stem cells (SM-MSCs). Some studies reported that SM-MSCs are considered among the top candidates for cartilage repair. Synovial membrane have 2 layers, the intima inner layer, and the sub-intima outer layer. The intima layer is rich in fibroblast cells that function in collagen, extracellular matrix, and synovial fluid secretion. The synovial membrane that isolating MSCs was reported to have a greater potential for proliferation and chondrogenesis (Orbay et al., 2012).

Insulin-like growth factor 1 (IGF-1) is a peptide comprising 70 amino acids. It exhibits around 50% similarity in its structure to insulin. The liver is the primary source of IGF-1 production, although many cells in the body have the capability to produce this growth factor. Chondrocytes also release IGF-1, which will be increased during osteoarthritic conditions. The IGF-1 receptor is a specific tyrosine kinase receptor that mediates its effects via intracellular signaling. The circulating free level of IGF-1 is determined by several factors such as hormones, nutrition and age (Maggio et al., 2013). Growth hormone secreted from the anterior pituitary induces IGF-1 production. Calcitropic hormones such as 1,25(OH)₂ Vit D3 have also been shown to increase IGF-1 secretion (Wei et al., 2017). In osteoarthritic chondrocytes, transforming growth factor B stimulates IGF-1 secretion (Wei et al., 2017).

Conditioned medium (CM) comprises a diverse combination of growth factors and substances with tissue regenerative properties produced by the stem cells. Utilizing a stem cell-conditioned medium offers a promising substitute for stem cell transplantation, effectively tackling challenges like low grafting efficiency and the risk of tumorigenesis. However, it should be noted that the concentrations of angiogenic growth factors found in the conditioned medium may not be optimal for therapeutic applications, and certain components in the medium may not be suitable for human use (Bhang et al., 2014). CM derived from stem cells is advantageous as it is considered "cell-free," eliminating the risk of graft versus host disease (GvHD) and associated side effects. This is ensured by conducting thorough screening and testing of cord tissue donors to ensure they are free from infectious diseases. The CM obtained from stem cells is abundant in diverse growth factors and cytokines that actively contribute to cellular regeneration and angiogenesis processes (Pawitan, 2014).

RANTES, a chemotactic cytokine, is generated by cytokine-activated chondrocytes and cartilage affected by OA. It acts as a chemoattractant for monocytes. RANTES possesses the ability to stimulate chondrocyte functions linked to joint inflammation and cartilage degradation (Alaaeddine et al., 2001). Prostaglandin E₂ (PGE₂) is an anti-inflammatory protein in bone resorption and joint pain. Excessive production of PGE₂ has been associated with the pathophysiology of OA (Ying et al., 2013). During the advanced stage of osteoarthritis (OA), there is a notable elevation of type I collagen (COL1), a prominent fibrous collagen, in comparison to the early stage (Ma et al., 2013). Aggrecan (ACAN), the aggregating cartilage proteoglycan, is crucial in imparting resilient mechanical properties to the cartilage within a healthy joint. It is responsible for generating compressive ability and maintaining the overall functions of the cartilage (Flugge et al., 1999, Koga et al., 2008).

Stem cells are a promising alternative material for OA therapy, although the mechanism of action is still unclear. Recent findings revealed that MSCs possess the capability to release cytokines and growth factors with anti-inflammatory properties. Conditioned medium MSCs can reduce nitrogen oxide (NO) inflammatory mediator production in cartilage explants. Additionally, it has been observed that the conditioned medium can enhance the production of IκBα in synoviocytes and chondrocytes. IκBα plays a crucial role in activating the NF-κB pathway by phosphorylation and subsequent degradation. The NF-κB pathway is known to regulate gene expression, including key inflammatory molecules, for example interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), matrix metalloproteinase 1 (MMP1), MMP3, and MMP13. These findings underscore the significance of the NF-κB pathway in the inflammatory process and matrix degradation associated with inflammation (van Buul et al., 2012). MSCs also found as cancer inhibitors, especially for breast cancer, and confront severe low oxygen tension (Widowati et al., 2014, Widowati et al., 2015).

Therefore, this study is aimed to compare the effective way of OA therapy between CM SM-MSC and CM SM-MSC-IGF-1 by observing the pro-inflammatory markers such as NF-κB, RANTES, PGE₂, COL1, and the anti-inflammatory marker, ACAN. Generally, this study also intends to evaluate the potential of synovial membrane isolated directly from OA patients to prepare as autologous therapy.

METHODOLOGY

IGF-1 induction of synovial membrane mesenchymal stem cells (IGF1-SM-MSCs)

The fourth passage of SM-MSCs was seeded at density 5×10^5 cells in a 25 cm³ culture flask. The cells were cultured with MEM- α and supplemented by 10% FBS, 1% antibiotic-antimycotic, and 1% nano-mycopulitine and incubated at 37°C and 5% CO₂ incubator for two days until they reached 80% confluency. IGF-1 was administrated to each group treatment (0, 150 ng/mL) for a week and two weeks of incubation (Adachi et al., 2012, de Sousa et al., 2014).

Induction of CHON002 with IL-1 β

Briefly, 5×10^5 of chondrocyte cell line (CHON002) was subcultured into a T25 flask (SPL 70075, Hong Kong, China) and incubated in a 5% CO₂ incubator at 37 °C until they achieved 70% confluence. The medium was replaced and treated with recombinant IL1 β (Biolegend 511604, California, America) with 0 and 10 ng / mL concentrations within five days. Administration of SM-MSCs-CM and IGF-1-SM-MSCs-CM to IL-1 β -CHON002 was conducted and designed as follows: 1) CHON002 without IL-1 β induction and without administration CM (control); 2) IL-1 β -CHON002 without administration of CM; 3) IL1- β CHON002 with administration SM-MSCs-CM 15%; 4) IL-1 β -CHON002 with administration WJMSCs-CM 30%; 5) IL-1 β -CHON002 with the administration of IGF-1-SM-MSCs-CM 15%; 6) IL-1 β -CHON002 with the administration of IGF-1-SM-MSCs-CM 30%. CM of SM-MSCs and IGF-1-SM-MSCs were changed every two days. CM induction was carried out for 7 and 14 days (Wei et al., 2017, Adachi et al., 2012, de Sousa et al., 2014).

Levels of PGE₂, NF-kB, and RANTES

ELISA assays were done for PGE₂ (E-EL-M0052, Wuhan, China), NFkB (E-EL- H1388, Wuhan, China), and RANTES (E-EL-H0023, Wuhan, China) expression using Elabscience kits.

Post-treatment CHON002 RNA isolation

CHON002 cells were treated for 7 and 14 days with CM and were harvested using trypsin-EDTA for 3 minutes in a 37°C incubator and 5% CO₂. Complete medium was added to inactivate trypsin and then transferred into a 15 mL tube, followed by centrifugation at 1600 xg for 5 minutes. The supernatant was removed, and the pellet was added by 350 μ L lysis solution and 350 μ L 70% ethanol. RNA isolation was done using Aurum – RNA Solution Kit (BioRad, 7326820, California, America) following the manufacturer's instructions. RNA was then stored at -80°C until further analysis (Widowati et al., 2018, Afifah et al., 2019).

Synthesis of cDNA CHON002 post-treatment

The synthesis of cDNA was done using Kit I Script cDNA synthesis following the manufacturer's instructions (BioRad 1708890, California, America). The process started with making a reaction of master mix/sample which contains: 5x iScript 4 μ L, RT iScript 1 μ L, 50 ng RNA sample and nuclease-free water. The mixture was incubated in gradient PCR with stages as follows: priming at 25°C for 5 minutes; reverse at 42°C for 30 minutes; RT Inactivation at 85°C for 5 minutes; hold at 4°C for 5 minutes. The cDNA samples were stored at -80°C (Widowati et al., 2018, Afifah et al., 2019).

Levels of COL1 and ACAN

The RT-qPCR reaction was done for COL1 and ACAN using SsoFast Eva Green Supermix kits following the manufacturer's instructions (BioRad 172-5200, California, America) for COL1 and ACAN assays.

Statistical analysis

Statistical analysis of all experiments was done in triplicate using the SPSS program (Version 20.0). Analysis of variance (One Way ANOVA) was used to measure the significant differences between groups ($p < 0.05$). Besides significant differences, posthoc analysis was done using Tukey's HSD test and Bonferroni Post-hoc Test for gene expression.

RESULTS

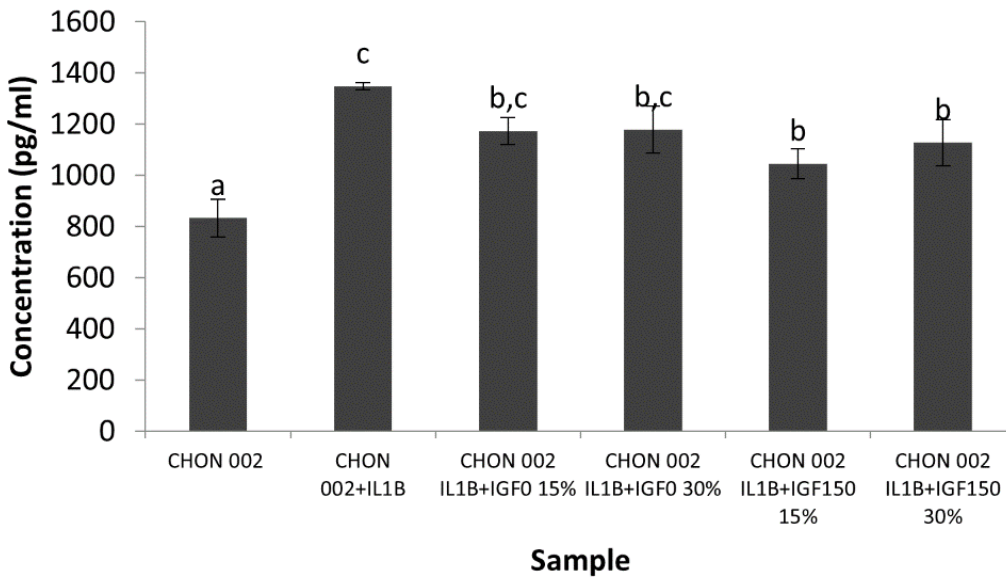
All of the data is represented as mean \pm standard deviation. The difference in letters (a, b, c, d) in the column equation is significant at $p < 0.05$. The test used for PGE₂, NF-kB, and RANTES is Tukey's post hoc test, while for COL1 and ACAN is the post hoc Bonferroni test.

Levels of PGE2, NFkB and RANTES

In Figure 1, the result of PGE₂ concentration was decreased from each treatment, meaning that CM SM-MSC and IGF-1 played an essential role in the recovery or cell regeneration in OA.

Figure 1

PGE₂ mean concentration

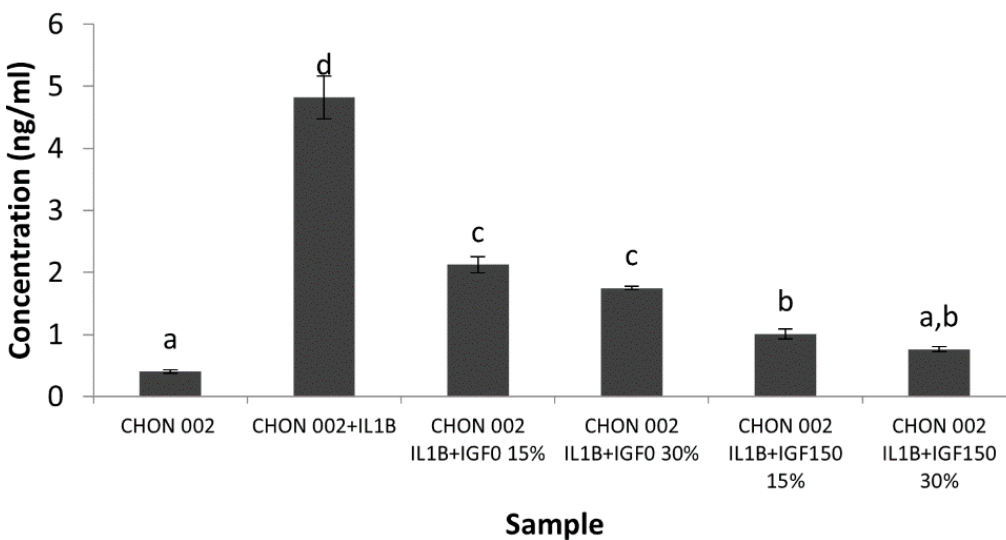


Note: Data is represented as mean ± standard deviation. The difference in letters (a, b, bc, c) in the column equation is significant at p<0.05.

In Figure 2, the result of NFkB concentration was decreased from each treatment, meaning that CM SM-MSC and IGF-1 played an essential role in the recovery or cell regeneration in OA.

Figure 2

NF-kB mean concentration

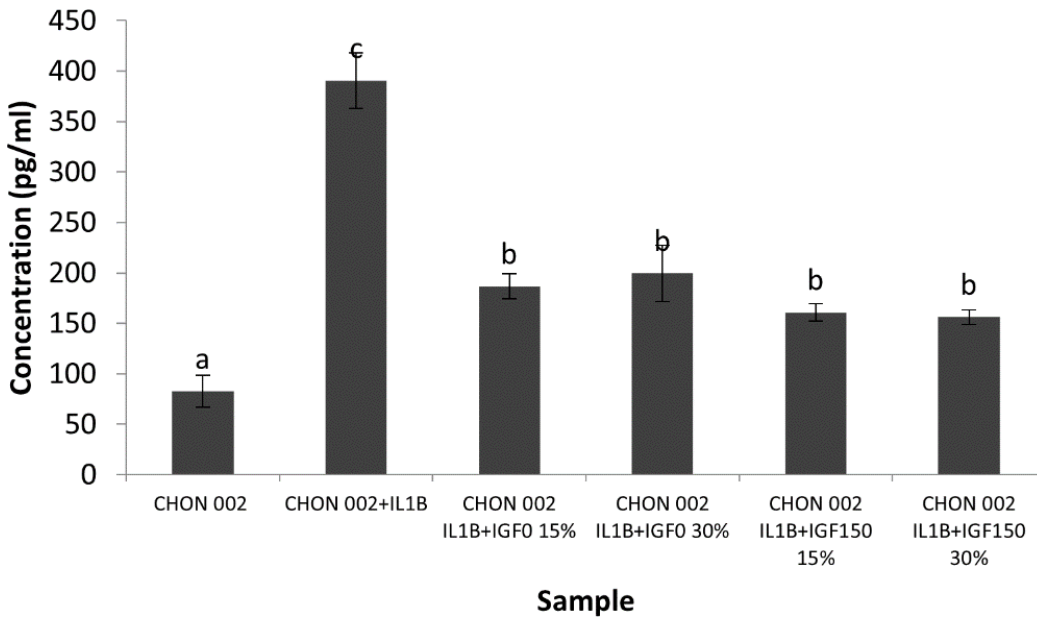


Note: Data is represented as mean ± standard deviation. The difference in letters (a, ab, b, c, d) in the column equation is significant at p<0.05.

In Figure 3, the result of RANTES mean concentration was decreased from each treatment, meaning that CM SM-MSC and IGF-1 played an essential role in the recovery or cell regeneration in OA.

Figure 3

RANTES mean concentration

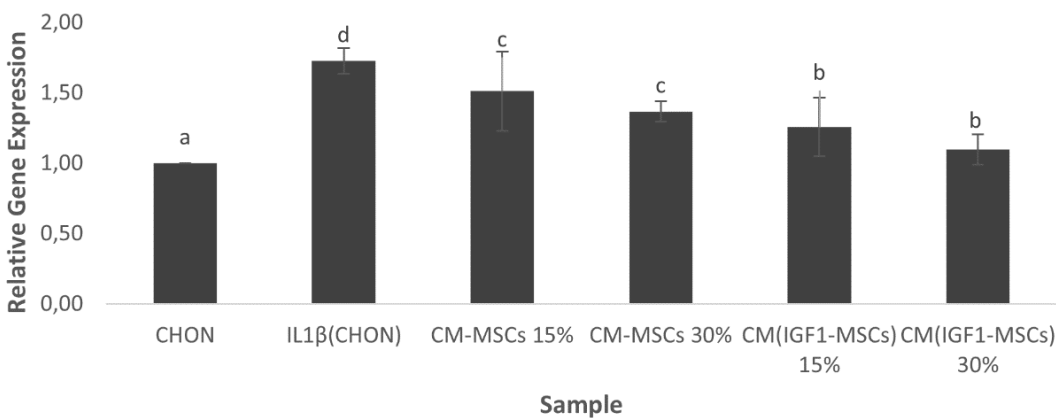


Note: Data is represented as mean ± standard deviation. The difference in letters (a, b, c) in the column equation is significant at $p < 0.05$.

In Figure 4, the result of COL1 mean relative gene expression was decreased from each treatment, which means that adding CM SM-MSC and IGF-1 influenced the reduction of inflammation caused by increased expression of COL1.

Figure 4

COL1 mean relative gene expression

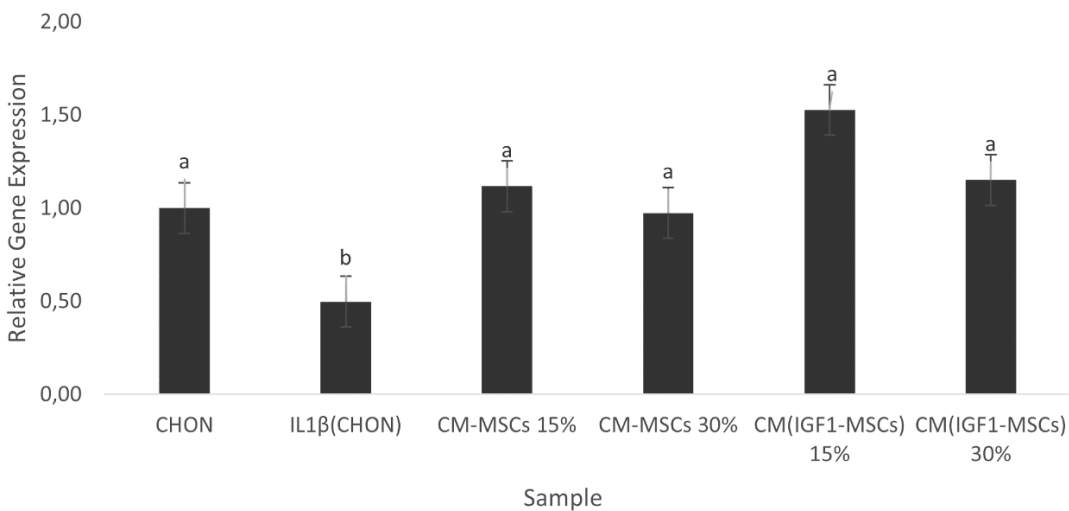


Note: Data is represented as mean ± standard deviation. The difference in letters (a, b, c, d) in the column equation is significant at $p < 0.05$.

As shown in Figure 5, the expression of ACAN mean relative gene expression increased from each treatment, as its role as an anti-inflammatory marker helps recovery or cell regeneration in OA therapy.

Figure 5

ACAN mean relative gene expression



Note: Data is represented as mean ± standard deviation. The difference in letters (a, b) in the column equation is significant at $p < 0.05$.

DISCUSSION

SM-MSCs have been considered to be more efficient compared to adipose-derived stem cells (ADSCs) or bone marrow stem cells (BMSCs) for chondrocyte differentiation (Sakaguchi et al., 2005). It means that SM-MSC is able for OA treatment since it triggered more growth factors to be produced. It has been reported that adding CM-MSC into OA tissue can increase the production of anti-inflammatory and anti-catabolic in the tissue (Maggio et al., 2013). Therefore, the application of these cells shows potential in addressing chronic degenerative conditions and safeguarding against cartilage deterioration in individuals with OA, owing to their regenerative and trophic characteristics (Widowati et al., 2018). Cytokines, including IL-1 β and TNF α , possessed a crucial part in this mechanism by promoting the secretion of proinflammatory mediators and proteases while inhibiting the synthesis of extracellular matrix components. This cascade of events ultimately results in the degradation of cartilage (Alaaeddine et al., 2001).

This study showed that IGF-1 affected the inflammatory marker concentration. The concentration of PGE₂, NF- κ B, RANTES, COL1 decreased significantly after inducing CM SM-MSC with 0 ng/mL and 150 ng/mL IGF-1 concentration. In contrast, ACAN concentration increased due to its marker as antiinflammatory, compared with CHON002+IL-1 β as the comparison. CHON002 shows the normal morphology of the chondrocyte without damage. After CHON002 induced by IL-1 β , the chondrocyte was triggered to produce pro-inflammatory markers and degradation matrix proteinase, which ended up with osteoarthritis condition and used as OA in vitro model. (Wojdasiewicz et al., 2014). IL-1 β has been associated with the progression of OA through multiple mechanisms. It can disrupt the balance of cartilage repair processes, trigger the generation of reactive oxygen species (ROS), and induce the production of inflammatory mediators like prostaglandin E₂ by enhancing the expression of inducible nitric oxide synthase (iNOS) and COX-2. These mechanisms contribute to the development of OA and the consequent deterioration of cartilage (Singh et al., 2002, Ahmed et al., 2002). Collected CM SM-MSC induced with IGF1 0 ng/ml and 150 ng/ml. IGF-1 serves as a growth factor to help CM SM-MSC to treat the OA model faster because IGF-1 contains growth factors that can help increase the chondrogenesis of OA to rebuild and renew damaged cells (Ahmed et al., 2002). IGF-1 is also an enhancer which responsible for the rate of gene expression (Madry et al., 2005, McQuillan et al., 1986 and Schoenle et al., 1982).

The elevation of PGE₂, an inflammatory factor, is primarily attributed to the activity of COX-2 (Ying et al., 2013). Therefore, the higher concentration of PGE₂ in a cell leads to a higher possibility of inflammation in cells, which can end the OA in the knees. This study compares the effect of induced and uninduced CM SM-MSC with IGF-1. As shown in Figure1, PGE₂ concentration was decreased significantly by inducing CM SM-MSC 15% and 30% with successively concentrations 1173,33 pg/ml and 1177,71 pg/ml. It decreases more in samples with IGF-1 150 ng/ml and CM SM-MSC 15% and 30% with concentrations 1044,88 pg/ml and 1127,13 pg/ml. But the most reduction was found to be affected by CM SM-MSC IGF-150 at 15%. Comparing the results with CHON002+IL-1 β as the comparison with concentration 1347,63 pg/ml.

Under normal conditions, NF κ B is retained in the cytoplasm by forming a complex with the inhibitory protein I κ B α . However, IL-1 β stimulation leads to the degradation of I κ B α , thereby activating NF- κ B. Upon activation, the p65 subunit of NF- κ B dissociates from I κ B α and moves to the nucleus. In the nucleus, NF κ B has the ability to trigger the transcription of specific target genes, such as iNOS and COX-2, which are involved in the production of inflammatory mediators (Miagkov et al., 1998). Therefore NF- κ B, in this case, should be reduced to prevent OA. As evidenced in Figure 2, NF- κ B concentration decreases significantly when given CM SM-MSC 15% and 30% with concentrations 2,12 ng/ml and 1,75 ng/ml, and the concentration decreases more in samples CM SM-MSC IGF-150 15% and 30% with concentration 1,01 ng/ml and 0,76 ng/ml. The most reduction was found affected by IGF-150 at 30% compared with CHON002+IL-1 β 4,82 ng/ml concentration as the comparison.

RANTES is synthesized by chondrocytes that have been activated by cytokines, as well as by cartilage affected by OA. The activation of chondrocytes by RANTES leads to the stimulation of functions related to joint inflammation and cartilage degradation. It is worth noting that under normal circumstances, RANTES is not naturally produced in normal cartilage or cultured chondrocytes under (Alaaeddine et al., 2001). Based on Figure 3, the concentration of RANTES in normal condition is 82,67 pg/ml, but after being induced by IL-1 β , the concentration increases significantly to 390,53 pg/ml, which causes inflammatory and OA conditions. But after adding CM SM-MSC 15% and 30%, the RANTES concentration decreased significantly to 186,69 pg/ml and 199,44 pg/ml. Inducing IGF-1 150 in CM SM-MSC 15% and 30% to samples also causing reduction, which is more significant than uninduced samples with IGF-1, 160,81 pg/ml and 156,14 pg/ml. Therefore, the most affecting samples which can reduce the RANTES concentration is CMMSC+IGF1 30% compared with CHON002+IL-1 β as the comparison.

COL1 is the major fibrous collagen that is produced by fibroblasts during the process of wound healing (Keen et al., 1994). COL1 is a fibroblastic marker expressed in differentiated chondrocytes. It was found that the expression of COL1 would significantly increase in the later stages of OA compared to the earlier stages of the condition (Ma et al., 2013). Therefore, this study uses CM SM-MSC to decrease the concentration of COL1, as seen in Figure 4. the relative gene expression of COL1 keeps decreasing with IGF-1 uninduced and induced samples. Uninduced samples with CM SM-MSC 15% and 30% show a reduction of COL1 into 1.51 and 1.36, but induced CM SM-MSC 15% and 30% with IGF-1 150 shows 1.25 and 1.09 concentrations.

The progressive degeneration of articular cartilage is characterized by decreased ACAN levels and the dysregulation of matrix components, including proteoglycans and collagen (Flugge et al., 1999). ACAN is a protein interacting with many non-protein molecules and linking proteins to form proteoglycans structure to be the main component of ECM cartilage. Chondroitin sulfate and keratan sulfate are the most common type of glycosaminoglycans that can be found in cartilage ECM and interact with aggrecan. Those components are important to generate compressive ability and maintaining cartilage functions (Koga et al., 2008). As shown in Figure 5, the relative gene expression of ACAN is increased at different concentrations from each sample. CM SM-MSC 15% and 30% succeeded in increasing the concentration of ACAN to 0,24 and 0,66 compared with CHON002+IL-1 β with 0.24 concentration as a comparison. However, samples contain IGF-1 150 with CM SM-MSC 15% and 30% show more increase at 0.66 and 0.56 concentration of ACAN.

Based on the results, it can be proven that CM SM-MSC induced with IGF-1 can be used as treatment of OA because it can decrease pro-inflammatory markers and increase anti-inflammatory marker due to its ability to produce more growth factors than uninduced CM SM-MSC with IGF-1.

CONCLUSION

CM from SM-MSCs cultured with IGF-1 with concentrations of 15% and 30% resulted in the most significant levels of PGE₂, NF- κ B, RANTES, COL1, and ACAN. Both concentration of treatment shows insignificant difference on each parameter. Therefore, the treatment has the potency to be used as OA therapy.

AUTHOR CONTRIBUTIONS

Marlina Marlina was involved with the conception and design of the research and final approval of the manuscript. Armenia Armenia was involved with the conception and design of the research. Rizki Rahmadian was involved with the conception and design of the research. Wahyu Setia Widodo was involved with research management, drafting and revising the manuscript. Syarifah Fadhlira Haddeline prepared the manuscript draft. Maharani Safitri was involved with the interpretation of data for the research and conducted data analysis. Nur Elida was involved with drafting the manuscript, while Wahyu Widowati was involved with the conception and design of the research.

ETHICS APPROVAL

This study was approved by ethics committee of the Faculty of Medicine, Andalas University (Number 563/KEP/FK/2019)

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CONFLICTS OF INTEREST

The authors declare no conflict of interest in this work.

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