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1- Figure 1: in vertical axis the title have to be: expression level of .... in reference to GAPDH (reference gene). It seems that the authors analyze the gene expression in related to GAPDH gene. So in the title of vertical axis it must be mentioned.

2- Material and methods: this part isn't comprehensive. All references for protocols are required and have to be described.

3- Scientific writing need to be improved (grammatically and ...).

4- About the manuscript title: in this study evaluation of proteins performed by ELISA and just one gene expression is evaluated (COL2) however in the title we see genes expression!!! It is suggested that the title change based on the method.

REVIEWER 2

Abstract does not have discussion.

Introduction: Please describe the necessity for using supernatant instead of the cell source.

Materials and methods:

The protocol for Real-Time PCR does not exist. need to add it in Methods.

Page 4, Line 84: Adding the reference for using the dose of 150ng/ml is recommended.

Discussion: Involving extracellular vesicles in the process of differentiation should be discussed.

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Dear Dr Widowati ,

Thank you very much for sending the revised manuscript. It will be sent for re-evaluation and you will be informed of the decision as soon as possible.

Best Regards

Haleh Kosari, MD

Executive Manager

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REVIEWER 1

1- Figure 1: in vertical axis the title have to be: expression level of .... in reference to GAPDH (reference gene). It seems that the authors analyze the gene expression in related to GAPDH gene. So in the title of vertical axis it must be mentioned.
Response: Thank you for your comment, the figure has been revised according to the advice.
2- Material and methods: this part isn't comprehensive. All references for protocols are required and have to be described.
Response: Thank you for your advice, all references protocols has been added and described
3- Scientific writing need to be improved (grammatically and ...)

Response: Scientific writing has been improved.

4- About the manuscript title: in this study evaluation of proteins performed by ELISA and just one gene expression is evaluated (COL2) however in the title we see genes expression!!! It is suggested that the title change based on the method.

Response: I apologize for the incompatibility of the title with the expression gene. The title has been changed according to your advice.

REVIEWER 2

1. **Abstract** does not have discussion.
   Response: Thank you for your advice, the discussion has been added into abstract.
2. **Introduction:** Please describe the necessity for using supernatant instead of the cell source.
   Response: The reason in this study using Condition Medium has been added in Introduction.
3. **Materials and methods:**
   The protocol for Real-Time PCR does not exist. need to add it in Methods.
   Page 4, Line 84: Adding the reference for using the dose of 150ng/ml is recommended.
   Response: The protocol for Real-Time PCR has been added in Materials and Method and reference for using the dose of 150ng/ml is recommended has been added in Materials and Method too.
4. **Discussion:** Involving extracellular vesicles in the process of differentiation should be discussed.
   Response: Thank you for your advice, discussion about Involving extracellular vesicles in the process of differentiation has been added in Discussion.

Dear Dr Widowati,

Thank you very much for submitting your manuscript entitled "**Efficiency of Conditioned Medium from IGF1-Induced Human Wharton's Jelly Mesenchymal Stem Cells (IGF1-hWJMSCs-CM) on Osteoarthritis**" for publication in the ***Avicenna Journal of Medical Biotechnology (AJMB)***.

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Efficiency of Conditioned Medium from IGF1-Induced Human Wharton's Jelly Mesenchymal Stem Cells (IGF1-hWJMSCs-CM) on Osteoarthritis

Abstract

Background: Osteoarthritis (OA) is a chronic disease that attacks joints and bones that can be caused by trauma or a result of other joint diseases (secondary). Stem cell and Conditioned Medium (CM) of stem cells are developed for OA therapy, which is minimally invasive, decrease inflammation, slow and repair prevent knee replacement surgery. This study aims to utilize human Wharton's Jelly-mesenchymal stem cells (hWJMSCs) conditioned medium as alternative OA therapy.

Method: CM from hWJMSCs induced by IGF1 was collected. The OA cells model (IL1β-CHON002) culture was treated as follows : 1) with hWJMSCs-CM 15%; 2) with hWJMSCs-CM 30%; 3) with IGF1-hWJMSCs (IGF1-hWJMSCs-CM) 15%; 4 with IGF1- hWJMSCs-CM 30%. Parameters including inflammatory cytokines (IL10 and TNFα), extracellular matrix degradation (MMP3 expression), and

chondrogenic marker (COL2 expression) were determined.

Results: The most significant increase in COL2 chondrogenic markers was found in the (IL1β-CHON002 treatment induced using 15% CM of hWJMSCs induced with IGF1. CM of hWJMSCs can reduce inflammatory cytokines (TNFα and IL10) and matrix degradation mediator MMP3. Better result was gained from IGF1-induced hWJMSCs-CM.

Discussion: hWJMSCs might secret some important such as anti-inflammatory cytokines that might lower inflammatory response in the injured knee and growth factors related to SOX9 upregulation resulted in matrix deposition of COL2 a chondrogenic marker as mark of successful joint repair while lowering MMP3 matrix degradation protein. hWJMSCs pre-conditioning using IGF1 could induce higher secretion of these factors resulted in better improvement of OA model.

Conclusion: CM of IGF1-hWJMSCs has successfully lower inflammation while repairing injured joint in the human chondrocyte OA model. Better result was gained through pre-conditioning of hWJMSCs using IGF1.

Keywords : Chondrocyte, IGF1, Osteoarthritis, Proinflammatory, Wharton's Jelly

Introduction

Disease related to calcification of the joints or osteoarthritis (OA) and rheumatoid arthritis (RA) are increasing due to the elderly population are also increasing [1]. Osteoarthritis is a chronic disease that attacks joints and bones caused by trauma as well as from other joint diseases. Commonly, synovial inflammation can cause joint homeostasis disorders related to OA [2]. Interleukin-1β (IL1β) is a cytokine that can trigger OA through a variety mechanism, such as trigger an imbalance cartilage repair process, triggering the formation of ROS including nitric oxide (NO), inflammatory mediators such as prostaglandin E2 (PGE2) through increased expression of inducible nitric oxide synthase (iNOS) and Cyclooxygenase-2 (COX2)[3]. The formation of free radicals and the lack of an antioxidant defense system can trigger oxidative stress causing damage to joints in OA and RA[4].

Clinical measures for OA therapy usually based on the main symptoms and the main focus is to reduce pain from inflammation using nonsteroidal anti-inflammatory drugs (NSAIDs) or total replacement of joints[5]. OA therapy is not intended to regenerate articular cartilage. Continuous use of NSAIDs has side effects that can cause kidney, digestive and cardiovascular disorders [6,7]. Chondrocyte which are part of the cartilage are most widely used for OA therapy [8], however treatment using autologous chondrocytes implantation has various disadvantages such as surgery performed twice that cause damage and degradation of the cartilage. MSCs) have the ability to differentiate into chondrocytes so that is appropriate for cartilage regeneration therapy. MSCs have a variety of abilities, one of which is modulating the microenvironment through anti-inflammatory and immunosuppressive functions. Diverse bioactive soluble factors excreted by MSCs can protect cartilage from damage and induce regeneration of remaining progenitor cells [9]. The ability of homing

possessed by MSCs causes MSCs to converge on the cartilage defect and can proliferate to regenerate articular cartilage, reduce the concentration of synovial fluid from prostaglandins [10], and decrease the progressive nature of OA [11].

MSCs were first isolated from cartilage (bone marrow mesenchymal stem cells/BMMSCs) after that MSCs can also be isolated from adipose tissue, placenta, umbilical cord, umbilical cord blood, dental pulp, and amnion [12] and Wharton's Jelly [13]. Various therapies for patients with OA are chondroprotective, reduce inflammation, and delay damage to the cartilage [14]. When compared with BMMSCs, Adipose tissue-MSCs (ADMSCs) have a lower chondrogenesis ability. Induction of Transforming Growth Factor-β2 (TGFβ2) dan Insulin like Growth Factor-1 (IGF1) in ADMSCs can produce chondrocyte markers comparable to BMMSCs, using chondrocyte markers which include Collagen-1A (COL1A), COL2A1, SRY-related HMG-box (SOX9)[15]. Plasmid-based overexpression from IGF1 in rabbit chondrocytes encapsulated using alginate and given *in vivo* shows the ability to repair cartilage and accelerate subchondral bone formation in osteochondral disorders [16].

OA treatment uses stem cells, especially human Wharton's Jelly mesenchymal stem cells (hWJ-MSCs) have potential that can be applied in the treatment of OA because of their high regeneration power and are easily obtained because they come from the umbilical cord. The previous study by Sanchooli (2017) shows Conditioned Medium from ADMSCs (ADMSCs-CM) has a high potential for bone healing. The effectiveness of MSCs-CM therapy is due to the presence of growth factors and cytokines which can inhibit apoptosis and increase cell proliferation and even stimulate mobilization and placement of stem cells to the site of injury[17]. Nevertheless, stem cells transplantation have obstacle such as differentiation and low cell endurance. These problems can be overcome by using CM obtained from stem cell culture. Analysis shows that CM of hWJMSCs contains various important proteins such as cytokines, growth factors, and angiogenic factors [18,19]. This study was conducted to evaluate the potential of CM of IGF1-

induced hWJMSCs (IGF1-hWJMSCs-CM) for OA therapy.

Materials and Methods

Cultivation of hWJMSCs and CM Colletion of hWJMSCs

The hWJMSCs were collected from the Stem Cell and Cancer Institute (Jakarta, Indonesia). The cells had been characterized by the cell multipotent differentiation and surface phenotype[13,20]. Informed consent was obtained from the Institutional Ethics Committee at the Stem Cell and Cancer Institute, Jakarta, Indonesia. Chondrocyte (CHON002, ATCC® CRL-2847™) obtained from Aretha Media Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia. The hWJMSCs at a density of 1 x 10^6/well were cultured in minimum essential medium-α (MEM-α) (Gibco, 12561056**)** supplemented with fetal bovine serum (20%) (FBS) (Gibco, 10270106) and 1% antibiotic and anti-mycotic (Gibco, 1772653). The cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 24 h. The medium was discarded and washed with Phosphate Buffered Saline (PBS) (Gibco, 1740576). The hWJMSCs at density 3 x 10^5 cells/well was maintained in a complete medium. The cells were treated with IGF-1 (Biolegend, 590904) at concentrations of 0 and 150 ng/ml, and incubated at 5% CO^2, 37 °C for 7 days, to obtain IGF1-induced hWJMSCs cells (IGF1-hWJMSCs) for measuring COL2 gene expression. After inducing IGF1, the hWJMSCs were harvested. The medium was collected and centrifuged at 3000 g for 4 min at room temperature, and the supernatant was filtered by a 0.22-mm (TPP, 99722) and used as CM of hWJMSCs (hWJMSCs-CM) and stored at -80^0C [19-21].

OA Model Treated with CM of IGF1-hWJMSCs

CHON002 cells (5x10^5 cells) were obtain from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia. The cells were seeded into T-25 flasks and incubated for 48 h. The medium was replaced and treated with recombinant IL1β (Biolegend,

579404) with concentrations of 0 and 10 ng/mL for 5 days in preparation for the OA model [20,21,22]. CHON002 was induced with IL1β for 5 days, then treated with hWJMSCs-CM with experiments as follows, 1) CHON002 without IL1β induction and without additions of CM (control); 2) IL1β-CHON002 without additions of hWJMSCs-CM; 3) IL1β-CHON002 treated with hWJMSCs-CM 15%; 4) IL1β-CHON002 treated with hWJMSCs-CM 30%; 5) IL1β-CHON002 treated with CM of IGF1-hWJMSCs (IGF1-hWJMSCs-CM) 15%; 6) IL1β-CHON002 treated with IGF1-hWJMSCs-CM 30%. CM of hWJMSCs and CM of IGF1-hWJMSCs were carried out by replacing the medium containing CM every 2 days. OA model using IL1β-CHON002 treated with hWJMSCs-CM and IGF1-hWJMSCs-CM for 7-14 days [21,23].

102102

Analysis of COL2 Gene Expression

RNA was extracted using the Aurum RNA kit (Bio-Rad, 7326820) based on the manufacturer's instructions. The concentration and purity of RNA of each sample was determined at 260/280 nm (Table 2). The cDNA synthesis was performed using cDNA synthesis kit (Bio-Rad, 1708841). Primer sequences can be seen at Table 1. The synthesis of cDNA from the RNA was carried out using iScript cDNA synthesis kit (Bio-Rad, 1708890) at 25°C temperature for 5 min, 42°C for 30 min, and 85°C for 5 min for the final step. The end-product was stored at -20°C. Quantitative gene expression was conducted using Thermo Scientific PikoReal Real-time PCR System (Thermo Fisher). PCR: pre-incubation cycle 95 °C for 5 min, 40 cycles of denaturation 95°C for 1 min, annealing 52°C for 40 sec, and extension 72°C for 1 min. The reaction mix that was used to perform qPCR was from an Evagreen master mix (Bio-Rad, 1725200). Table 1 shows the primers used in this research[21,23].

Quantification of IL10, TNFα, and MMP3 Level in OA Model Treated with hWJMSCs-CM

Secretion IL10, TNFα, MMP3 were assessed using ELISA Kit IL10 (Elabsci, E-EL-

H0103), TNFα (Elabsci, E-EL-H0109), and MMP3 (Elabsci, E-EL-H1446). The procedure was in accordance with manufacturer protocol. Sample absorbances were read at 450 nm using microplate reader (Multiskan GO, ThermoScientific). Color changes of samples are observed then read immediately at 450 nm wavelength and the IL10, TNFα, MMP3 concentration can be calculated based on a protein standard curve [19,21,24].

Results

COL2 Gene Expression Level

COL2 is a monomer protein that forms the main formation of the cartilage matrix and is the main target of tissue that is attacked by OA. The long process assembly of COL2 in the cytoplasm will eventually be transported out of the cell to form a cartilage matrix and its expression culminates in the ripening process of chondrocytes [25]. RNA concentration and purity can be seen in Table 2. COL2 expression as can be seen in Figure 1, has increased from the first week to the second week. The highest expression was found in the treatment given hWJMSCs-CM concentrating 15% of the IGF1-induced hWJMSCs 150 ng/mL on IL1β-induced CHON002 as the OA model.

Level of TNFα, IL10, and MMP3

TNFα, together with IL1β, is considered an inflammatory cytokine which is key in the pathophysiological process that occurs during OA. TNFα is secreted by the same cells as cells that synthesize IL1β [26,27]. Figure 2 showed the results of TNFα levels using the ELISA method. It can be seen that IL1β-CHON002 has the highest levels of TNFα among others, while addition CM of IGF1-hWJMSCs 15% shows a significant decrease in TNFα ($p < 0.05$). TNFα also experienced elevated levels in the treatment for 2 weeks.

IL10 is a cytokine that acts as an anti-inflammatory and it is one of the cytokines that shows a chondroprotective effect on OA[28]. The IL10 cytokines and IL10R receptors are expressed by chondrocytes [29]. IL10 works by stimulating antagonist proteins against IL1β, namely IL1Ra, metalloproteinase inhibitors (TIMP1), and also as growth factors.

The results of IL10 levels using the ELISA method can be seen in Figure 3. IL10 as an anti-inflammatory mediator that inhibits pro-inflammatory mediators was found with the highest levels of IL1β-CHON002 as OA cells model. While addition of hWJMSCs-CM with or without IGF1 induction show a decrease in IL10 levels compared to OA cells model.

The MMPs are expressed in joint tissues of patients with OA and RA. The MMP3 secreted from chondrocyte and synovial cells and MMP3 can reduce various of extracellular matrix. Figure 4 showed the result of MMP3 levels using ELISA method.

The lower MMP3 level was shown by treatment with the addition 15%, 30% of IGF1-hWJMSC-CM both 1 and 2 weeks incubation, while the lowest MMP3 levels was 2 weeks incubation of IGF1-hWJMSCs-CM 15%. This result shows that IGF1-hWJMSCs-CM can reduce levels of MMP3 which plays a role in matrix degradation.

Discussion

MSCs has been used as one of the candidates for tissue engineering which includes repair, replacement, and regeneration of cartilage tissue, because of its high proliferation and differentiation ability [29]. One of which originates from Wharton's Jelly [13,19]. Previous studies reported that hWJMSCs can differentiate into chondrocytes [21,24], skeletal muscle cells, heart muscle cells, osteoblasts, adipocytes, β cells on the islets of Langerhans, and endothelial cells *in vitro* [31]. Therefore, these cells can be used as alternatives in the treatment of chronic degenerative

160 disorders and prevent cartilage degradation in OA patients.

In this study, the induction of hWJMSCs using IGF1 increased COL2

161 expression. The COL2 gene which is the cartilage matrix gene has been indicated to be under the
162 regulation of transcription control of SOX9 [32,33], and is involved in the structure and function of
163 articular cartilage. This is in accordance with previous studies, where the inducing of IGF1 to
164 hWJMSCs increased the expression of the SOX9 gene which means that the expression of the
165 COL2 gene will also increase [21]. SOX9 is presented and is present in the presumed cartilage during
166 embryonic development. COL2 downregulation in OA is likely to contribute to cartilage
167 pathology. IGF1 can stabilizes the chondrocyte phenotype in pathological conditions, and also has
168 mitogenic properties in the articular cartilage of adults and strongly stimulates the production of
169 chondrocyte extracellular matrix components [21]. COL2 is a chondrogenic gene marker in the joint
170 cartilage, associated with extracellular matrix secretion (ECM). In OA, early changes in articular
171 cartilage are characterized by proteoglycan loss and a decrease in the expression of the
303 COL2 gene without altering the regularity of the articular tissue structure[34]. After
304 being treatment, as shown in **Figure 1**, there were significant improvement in COL2 gene expression
305 in OA model, indicated that there might be repairing process in the ECM. The highest expression of
306 COL2 showed by IGF1-hWJMSCs-CM 15% + IL1β-CHON002 which different significantly from other
307 treatments. It is known that the expression of COL2 is closely related to SOX9.

172 The effect of TNFα in many cases coincides with the action of IL1β, and there is a
173 relationship in many phenomena that occur during OA between two cytokines [28]. This effect is the
174 result of activating the same group of intracellular signaling pathways, which in turn triggers
175 effects that increase inflammation in joint tissues [35]. In contrast, IL10 is a cytokine that acts as an

anti-inflammatory and it is one of the cytokines that shows a chondroprotective effect on OA and works by stimulating antagonist proteins against IL1β as pro-inflammatory cytokine [28].

In the present study, the highest levels of TNFα were found in cells without treatment using CM of hWJMSCs and IGF1-hWJMSCs, the highest IL10 level was also found in cells without treatment of CM from hWJMSCs and IGF1-hWJMSCs. This is possible because the production of proinflammatory cytokines is directly proportional to the production of anti-inflammatory cytokines, when high levels of proinflammatory cytokines occur and the body adjusts to provide a regulatory response to levels of anti-inflammatory cytokines. Furthermore, hWJMSCs-CM and IGF1-hWJMSCs-CM in the OA cells model decrease TNF-α level which is responsible for OA inflammation. however, administration to IL10 did not show an increase in IL10 levels responsible for inhibiting pro-inflammatory cytokines. This is in line with the results of Al-Banna (2008) which states that the induction of inflammatory cytokines is followed by an increase in the level of anti-inflammatory cytokines [36].

Cell therapies can directly aid repair by forming new functional tissues, or support tissue repair through paracrine mechanisms, for instance by secreting growth factors, immunomodulatory molecules, and Extracellular Vesicles (EV). EV can mediate cell-cell communication and are involved in many processes, including immune signaling, angiogenesis, stress response, senescence, proliferation, and cell differentiation[36]. In vitro passaging of MSCs results in cell enlargement, differentiation, and decrease in proliferation within 10 passages, and causes a strong response to micro-environment stiffness, affecting cell morphology, and function [37,38]

Metabolic imbalances between degradation and synthesis of articular cartilage are the main reason for degeneration in OA sufferers. MMP is a protein that is responsible for the degradation of the extracellular matrix and basement membrane components[39]. MMP is a protein endopeptidase that depends on zinc ions which is localized in various connective tissues, can

degrade various components of the ECM [40]. In the present study, OA model (IL1β-CHON002) has high MMP3 levels, and after treatment using hWJMSCs-CM and IGF1-hWJMSCs-CM, showed a decrease in MMP-3 levels (Figure 4) and the lowest MMP-3 level was shown by treatment with the addition IGF1-hWJMSCs-CM 30%. this indicates that the administration of hWJMSCs-CM with the addition of IGF1 can reduce MMP3 levels which are the cause of the degradation of the extracellular matrix.

Conclusion

In conclusion, CM of IGF1-induced hWJMSCs increases higher COL2 gene expression compared with CM of IGF1-uniduced hWJMSCs. CM of IGF1-hWJMSCs reduce lower the levels of pro-inflammatory cytokines TNFα, IL10, MMP3 compared with CM of IGF1-uniduced hWJMSCs. CM of IGF1-hWJMSCs also increases chondrogenesis and can subsequently be an alternative for the treatment of OA. Further studies on animal models must be carried out for validation of IGF1-hWJMSCs.

Conflict of Interest

There are no conflict of interest.

Acknowledgement

This study was supported by the Grants-in-Aid Insinas Riset Pratama Individu 2019, from Ministry of Research, Technology and Higher Education of the Republic of Indonesia. The authors like to thank to Rr. Anisa Siwianti Handayani, Alya Mardhotillah Azizah, and Jenifer Kiem Aviani from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, West Java, Indonesia for their technical assistants.

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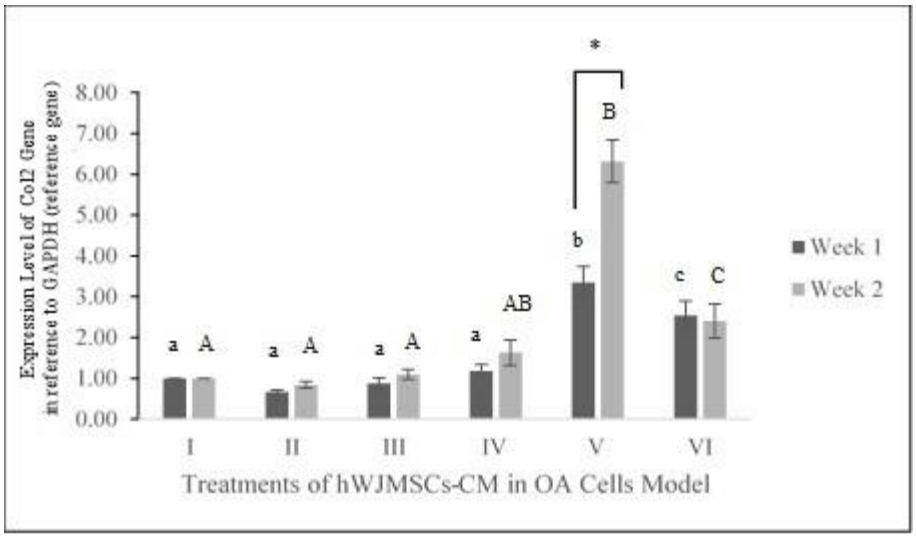
Table 1. RTPCR Details of COL2 and GAPDH Gene

Gene Symbols	Primer Sequence (5' to 3') Upper strand : sense Lower strand : antisense	Product Size (bp)	Annealing (^{0}C)	Cycle	References
COL2	5'-TTTCCCAGGTCAAGATGGTC-3' 5'-CTGCAGCACCTGTCTCACCA-3'	377	53	40	NM_001844.4
GAPDH	5'-GGGCTGCTTTTAACTCTGGT-3' 5'-TGGCAGGTTTTTCTAGACGG-3'	702	51	40	NM_001289745.1

Table 2. Concentration and Purity of RNA

Treatment	RNA Concentration (ng/ml)		RNA Purity (λ260/ λ280 nm)	
	Week 1	Week 2	Week 1	Week 2
Normal cell (CHON002)	38.32	147.00	2.18	2.29
IL1β-CHON002	39.36	162.38	2.13	2.26
hWJMSCs-CM 15% + IL1β-CHON002	36.76	134.22	2.10	2.25
hWJMSCs-CM 30% + IL1β-CHON002,	61.64	230.02	2.11	2.26
IGF1-hWJMSCs-CM 15% + IL1β-CHON002	61.04	233.00	2.13	2.29
IGF1-hWJMSCs-CM 30% + IL1β-CHON002	49.48	194.24	2.19	2.27



372 **Figure 1.** Effect of hWJMSCs-CM, IGF1-hWJMSCs-CM toward COL2 gene expression on OA
373 cells model. (I) Normal cell (CHON002), (II) IL1β-CHON002, (III) hWJMSCs-CM 15% + IL1β-
374 CHON002 (IV), hWJMSCs-CM 30% + IL1β-CHON002, (V) IGF1-hWJMSCs-CM 15% + IL1β-
375 CHON002, (VI) IGF1-hWJMSCs-CM 30% + IL1β-CHON002.

376 The histograms are presented as mean ± standard deviation, the treatment was done triplicate. The data were analyzed with
377 ANOVA and continued with Tukey post hoc test. Different letters (a,b,c) indicate significant differences among treatment of 1
378 week incubation (black colour) and different letters (A,AB,B,C) significant differences among treatment of 2 week
379 incubation (gray colour). The symbol (*) present significant difference between week 1 and week 2 based on paired t-test ($p<0.05$).

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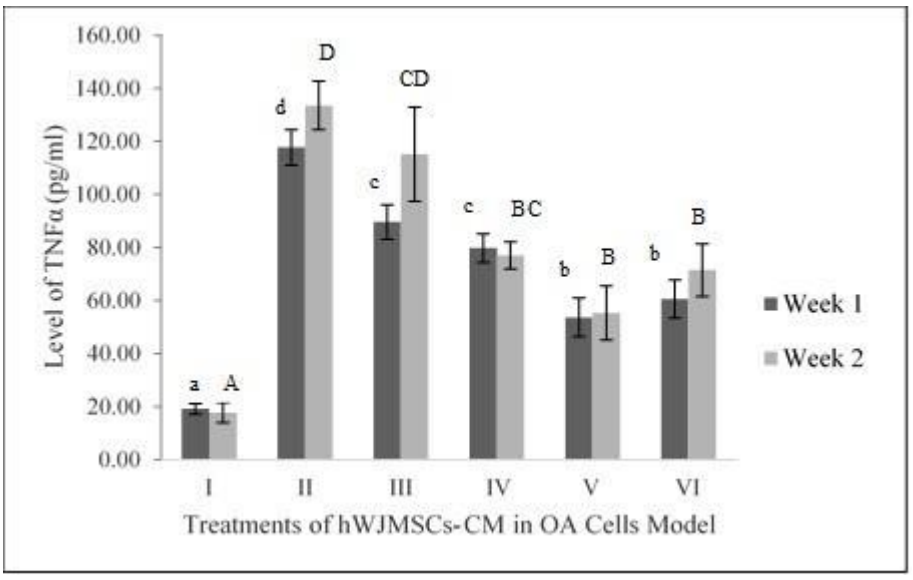

386346

387 **Figure 2.** Effect of hWJMSCs-CM, IGF1-hWJMSCs-CM toward TNFα on OA cells model. (I)

388 Normal cell (CHON002), (II) IL1β-CHON002, (III) hWJMSCs-CM 15% + IL1β-CHON002, (IV)

389 hWJMSCs-CM 30% + IL1β-CHON002, (V) IGF1-hWJMSCs-CM 15% + IL1β-CHON002, (VI)

390 IGF1-hWJMSCs-CM 30% + IL1β-CHON002.

391 The histograms are presented as mean ± standard deviation, the treatment was done triplicate. The data were analyzed with ANOVA

392 and continued with Tukey post hoc test. Different letters (a,b,c,d) indicate significant differences among treatment of 1 week

393 incubation (black colour) and different letters (A,B,BC,CD,D) significant differences among treatment of 2 week incubation (gray

394 colour)

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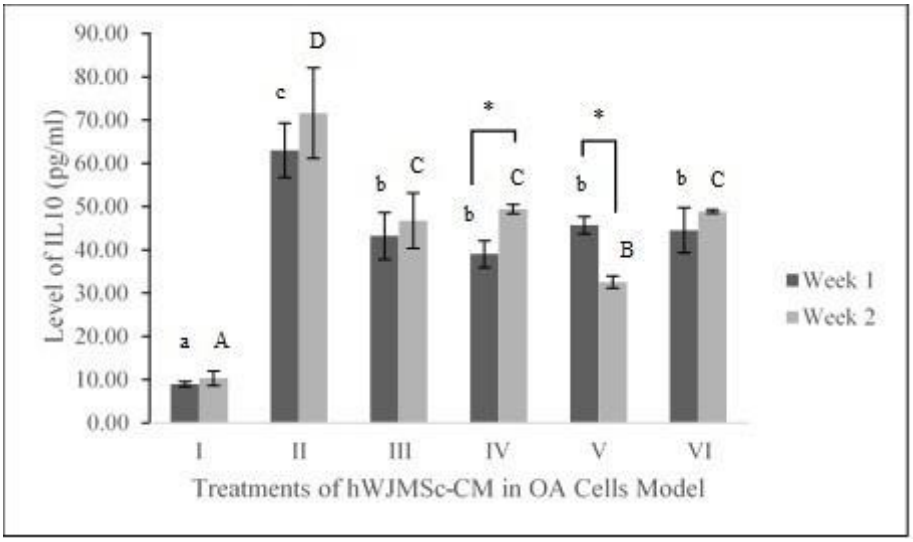

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397 **Figure 3.** Effect of hWJMSCs-CM, CM of IGF1-hWJMSCs toward IL10 level on OA cells

398 model. (I) Normal cell (CHON002), (II) IL1β-CHON002, (III) hWJMSCs-CM 15% + IL1β-

399 CHON002 (IV), hWJMSCs-CM 30% + IL1β-CHON002, (V) IGF1-hWJMSCs-CM 15% +

400 IL1β-CHON002, (VI) IGF1-hWJMSCs-CM 30% + IL1β-CHON002.

401 The histograms are presented as mean ± standard deviation, the treatment was done triplicate. The data were analyzed with

402 ANOVA and continued with Tukey post hoc test. Different letters (a,b,c) indicate significant differences among treatment of 1

403 week incubation (black colour) and different letters (A,B,C,D) significant differences among treatment of 2 week

404 incubation (gray colour). The symbol (*) present significant difference between week 1 and week 2 based on paired t-test

365 ($p<0.05$).

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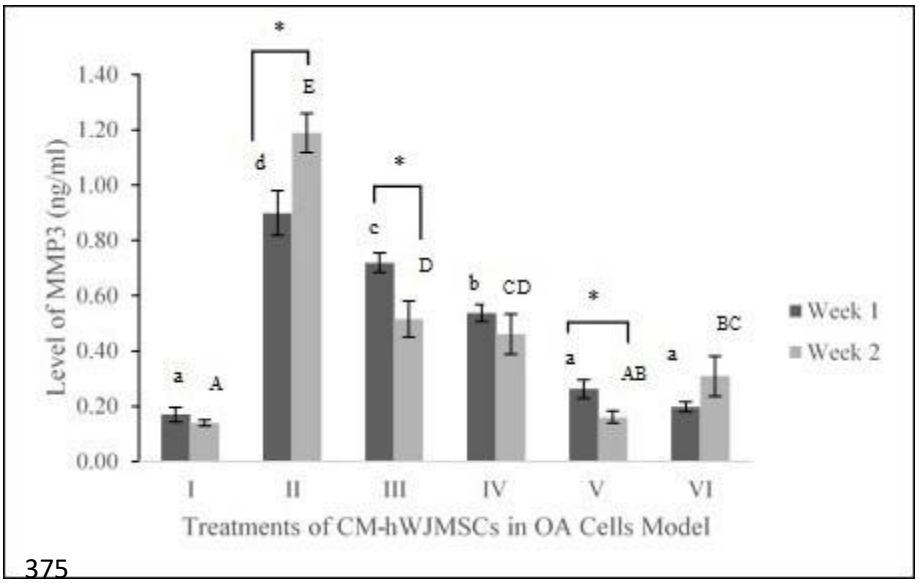

375

376 **Figure 4.** Levels of MMP3 in cells of OA models cultured in hWJMSCs-CM. (I) Normal cell

377 (CHON002), (II) IL1β-CHON002, (III) hWJMSCs-CM 15% + IL1β-CHON002 (IV), hWJMSCs-

378 CM 30% + IL1β-CHON002, (V) IGF1-hWJMSCs-CM 15% + IL1β-CHON002, (VI) IGF1-

379 hWJMSCs-CM 30% + IL1β-CHON002

380 The histograms are presented as mean ± standard deviation, the treatment was done triplicate. The data were analyzed with ANOVA

381 and continued with Tukey post hoc test. Different letters (a,b,c,d) indicate significant differences among treatment of 1 week

382 incubation (black colour) and different letters (A,AB,BC,C,CD,D,E) significant differences among treatment of 2 weeks

383 incubation (gray colour). The symbol (*) present significant difference between week 1 and week 2 based on paired t-test ($p<0.05$).

Effect of Conditioned Medium from IGF1-Induced Human Wharton's Jelly Mesenchymal Stem Cells (IGF1-hWJMSCs-CM) on Osteoarthritis

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Effect of Conditioned Medium from IGF1-Induced Human Wharton's Jelly Mesenchymal Stem Cells (IGF1-hWJMSCs-CM) on Osteoarthritis

Hanna Sari Widya Kusuma [1], Wahyu Widowati [2*], Rimonta Febby Gunanegara [2], Berry Juliandi [3], Nyoman Ehrich Lister [4], Seila Arumwardana [1], Dewani Tediana Yusepany [1], Dwi Surya Artie [1], Enden Dea Nataya [1], Kamila Yashfa Gunawan [1], Ika Adhani Sholihah [1], Ermi Girsang [4], Chrismis Novalinda Ginting [4], Indra Bachtiar [5], and Harry Murti [5]

1. Biomolecular and Biomedical Research Center, Aretha Medika, Utama, Bandung, West Java, Indonesia
2. Faculty of Medicine, Maranatha Christian University, Bandung, West Java, Indonesia
3. Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, IPB Darmaga Campus, Bogor, West Java, Indonesia
4. Universitas Prima Indonesia, Medan North Sumatera, Indonesia
5. Stem Cell and Cancer Institute, Jakarta, Indonesia

*** Corresponding author:**
Wahyu Widowati, Ph.D., Faculty of Medicine, Maranatha Christian University, Bandung, West Java, Indonesia
Tel: +62 81910040010
E-mail: wahyu_w60@yahoo.com
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Abstract

Background: Osteoarthritis (OA) is a chronic disease that attacks joints and bones which can be caused by trauma or other joint diseases. Stem cell and Conditioned Medium (CM) of stem cells are developed for OA therapy, which is minimally invasive. It can decrease inflammation and be a replacement for knee surgery. This study aimed to utilize human Wharton's Jelly-Mesenchymal Stem Cells (hWJMSCs) as an alternative OA therapy.

Methods: CM from hWJMSCs induced by IGF1 was collected. The OA cells model (IL1β-CHON002) culture was treated as follows: 1) with hWJMSCs-CM 15% (v/v); 2) with hWJMSCs-CM 30% (v/v); 3) with IGF1-hWJMSCs (IGF1-hWJMSCs-CM) 15% (v/v); 4) with IGF1-hWJMSCs-CM 30% (v/v). Parameters including inflammatory cytokines (IL10 and TNFα), extracellular matrix degradation (MMP3 expression), and chondrogenic marker (*COL2* expression) were determined.

Results: The most significant increase in *COL2* chondrogenic markers was found in IL1β-CHON002 treatment using 15% CM of hWJMSCs induced with IGF1. CM of hWJMSCs can reduce inflammatory cytokines (TNFα and IL10) and matrix degradation mediator MMP3. Better result was gained from IGF1-induced hWJMSCs-CM.

Conclusion: CM of IGF1-hWJMSCs reduce inflammation while repairing injured joint in the human chondrocyte OA model.

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Keywords: Chondrocyte, IGF1, Osteoarthritis, Proinflammatory, Wharton's jelly

Introduction

The prevalence of Osteoarthritis (OA) and Rheumatoid Arthritis (RA) are increasing in linear to the growth of elderly population [1]. Osteoarthritis is a chronic disease that attacks joints and bones which is caused by trauma as well as other joint diseases. Commonly, synovial inflammation can cause joint homeostasis disorders related to OA [2].

Interleukin-1β (IL1β) is a cytokine that can trigger OA through a variety of mechanisms, such as triggering an imbalance in cartilage repair process, triggering the formation of ROS including Nitric Oxide (NO), inflammatory mediators such as Prostaglandin E2 (PGE2) through increased expression of inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX2) [3]. The formation of free radicals and the lack of an antioxidant defense system can trigger oxidative stress causing damage to joints in OA and RA [4]. Clinical measures for OA therapy are usually based on the main symptoms and the main focus is to reduce pain from inflammation using NonSteroidal Anti-Inflammatory Drugs (NSAIDs) or total replacement of joints [5]. OA therapy is not intended to regenerate articular cartilage. Continuous use of NSAIDs has side effects that can cause kidney, digestive and cardiovascular disorders [6,7].

Chondrocytes which are part of the cartilage are

most widely used for OA therapy [8]; however, treatment using autologous chondrocytes implantation has various disadvantages such as involving two-step surgeries which causes damage and degradation of the cartilage. Mesenchymal Stem Cells (MSCs) have the ability to differentiate into chondrocytes so they are suitable candidates for cartilage regeneration therapy. MSCs have a variety of abilities, one of which is modulating the microenvironment through anti-inflammatory and immunosuppressive functions. Diverse bioactive soluble factors excreted by MSCs can protect cartilage from damage and induce regeneration of remaining progenitor cells [9]. The ability of homing possessed by MSCs causes MSCs to converge on the cartilage defect and they can proliferate to regenerate articular cartilage, reduce the concentration of synovial fluid from prostaglandins [10], and decrease the progressive nature of OA [11].

MSCs are first isolated from cartilage [Bone Marrow Mesenchymal Stem Cells (BMMSCs)] and after that they can also be isolated from adipose tissue, placenta, umbilical cord, umbilical cord blood, dental pulp, amnion [12] and Wharton's jelly [13]. Various therapies for patients with OA are chondroprotective and they reduce inflammation and delay damage to the cartilage [14]. When compared with BMMSCs, Adipose tissue-MSCs (ADMSCs) have lower chondrogenesis ability. Induction using Transforming Growth Factor-β2 (TGFβ2) and Insulin like Growth Factor-1 (IGF1) in ADMSCs can produce chondrocyte markers comparable to BMMSCs, which include collagen-1A (COL1-A), COL2A1, and SRY-related HMG-box (SOX9) [15]. Plasmid-based overexpression from IGF1 in rabbit chondrocytes encapsulated using alginate and given *in vivo* shows the ability to repair cartilage and accelerate subchondral bone formation in osteochondral disorders [16].

OA treatment uses stem cells, and especially human Wharton's Jelly Mesenchymal Stem Cells (hWJMSCs) have the potential to be applied in the treatment of OA because of their high regeneration power and are easily obtained because they come from the umbilical cord. The previous study by Sanchooli *et al* shows that conditioned medium from ADMSCs (ADMSCs-CM) has a high potential for bone healing. The effectiveness of MSCs-CM therapy is due to the presence of growth factors and cytokines which can inhibit apoptosis and increase cell proliferation and even stimulate mobilization and placement of stem cells to the site of injury [17].

Nevertheless, stem cells transplantation has some obstacles such as differentiation and low cell endurance. These problems can be overcome by using CM obtained from stem cell culture. Analysis shows that CM of hWJMSCs contains various important proteins such as cytokines, growth factors, and angiogenic factors [18,19]. This study was conducted to evaluate the potential of CM of IGF1-induced hWJMSCs (IGF1-hWJMSCs-CM) for OA therapy.

Materials and Methods

Cultivation of hWJMSCs and CM collection

hWJMSCs were collected from the Stem Cell and Cancer Institute (Jakarta, Indonesia). The cells had been characterized by the cell multipotent differentiation and surface phenotype [13,20]. Informed consent was obtained from the Institutional Ethics Committee at the Stem Cell and Cancer Institute, Jakarta, Indonesia. hWJMSCs at a density of 1×10^6/well were cultured in Minimum Essential Medium-α (MEM-α) (Gibco, 12561056) supplemented with Fetal Bovine Serum (20%) (FBS) (Gibco, 10270106) and 1% antibiotic and antimycotic (Gibco, 1772653). The cells were incubated in a humidified atmosphere with 5% CO_2 at 37 ℃ for 24 *hr*. The medium was discarded and washed with Phosphate Buffered Saline (PBS) (Gibco, 1740576). hWJMSCs at density of 3×10^5 cells/well were maintained in a complete medium. The cells were treated with IGF-1 (Biolegend, 590904) at concentrations of 0 and 150 *ng/ml*, and incubated at 5% CO_2, 37℃ for 7 days, to obtain IGF1-induced hWJMSCs cells (IGF1-hWJMSCs) for measuring *COL2* gene expression. After inducing IGF1, hWJMSCs were harvested. The medium was collected and centrifuged at 3000 *g* for 4 *min* at room temperature, and the supernatant was filtered by a 0.22-*mm* filter (TPP, 99722) and used as CM of hWJMSCs (hWJMSCs-CM) and stored at -80 ℃ [19-21].

OA model treated with CM of IGF1-hWJMSCs

Human chondrocyte CHON002 cell line (ATCC RL-2847) (5×10^5 cells) were obtained from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia. The cells were seeded into T-25 flasks and incubated for 48 *hr*. The medium (hWJMSCs-CM) was replaced and the cells treated with recombinant IL1β (Biolegend, 579404) with concentrations of 0 (no treatment) and 10 *ng/ml* for 5 days in preparation for the OA model [20-22].

The experiment were conducted with 6 different groups as follow: 1) CHON002 without IL1β induction and without additions of CM (control); 2) IL1β-CHON002 without additions of hWJMSCs-CM; 3) IL1β-CHON002 treated with hWJMSCs-CM 15% (v/v); 4) IL1β-CHON002 treated with hWJMSCs-CM 30% (v/v); 5) IL1β-CHON002 treated with CM of IGF1-hWJMSCs (IGF1-hWJMSCs-CM) 15% (v/v); 6) IL1β-CHON002 treated with IGF1-hWJMSCs-CM 30% (v/v). The medium were replaced every 2 days. The experiment were carried for 1 and 2 weeks [21,23].

Analysis of COL2 gene expression

RNA was extracted using Aurum RNA kit (Bio-Rad, 7326820) based on the manufacturer's instructions. The concentration and purity of RNA of each sample was determined at 260/280 *nm* (Table 1). Primer sequences can be seen in table 2. The synthesis of cDNA from the RNA was carried out using iScript cDNA synthesis kit (Bio-Rad, 1708890) at 25℃ for 5

Table 1. Concentration and purity of RNA

Treatment	RNA concentration (*ng/ml*)		RNA purity (λ260/λ280 *nm*)	
	Week 1	Week 2	Week 1	Week 2
Normal cell (CHON002)	38.32	147.00	2.18	2.29
IL1β-CHON002	39.36	162.38	2.13	2.26
hWJMSCs-CM 15%+IL1β-CHON002	36.76	134.22	2.10	2.25
hWJMSCs-CM 30%+IL1β-CHON002,	61.64	230.02	2.11	2.26
IGF1-hWJMSCs-CM 15%+IL1β-CHON002	61.04	233.00	2.13	2.29
IGF1-hWJMSCs-CM 30%+IL1β-CHON002	49.48	194.24	2.19	2.27

Table 2. Primer sequence of *COL2* and *GAPDH* gene

Gene symbols	Primer sequence (5' to 3') upper strand: sense lower strand: antisense	Product size (*bp*)	Annealing (℃)	Cycle	References
COL2	5'-TTTCCCAGGTCAAGATGGTC-3' 5'-CTGCAGCACCTGTCTCACCA-3'	377	53	40	NM_001844.4
GAPDH	5'-GGGCTGCTTTTAACTCTGGT-3' 5'-TGGCAGGTTTTTCTAGACGG-3'	702	51	40	NM_001289745.1

min, 42 ℃ for 30 *min*, and 85 ℃ for 5 *min* for the final step. The end-product was stored at -20 ℃. Quantitative gene expression was conducted using Thermo Scientific PikoReal Real-time PCR System (Thermo Fisher). PCR included pre-incubation cycle at 95 ℃ for 5 *min*, 40 cycles of denaturation at 95 ℃ for 1 *min*, annealing at 53 ℃ for 40 *s*, and extension at 72 ℃ for 1 *min*. The reaction mix that was used to perform qPCR was from Evagreen master mix (Bio-Rad, 1725200). Table 2 shows the primers used in this research [21,23].

Quantification of IL10, TNFα, and MMP3 level in OA model treated with hWJMSCs-CM

Secretion of IL10, TNFα, MMP3 was assessed using ELISA Kit IL10 (Elabsci, E-EL-H0103), TNFα (Elabsci, E-EL-H0109), and MMP3 (Elabsci, E-EL-H1446). The procedure was in accordance with manufacturer's protocol. Sample absorbances were read at 450 *nm* using microplate reader (Multiskan GO, ThermoScientific). IL10, TNFα, MMP3 concentration were calculated based on a protein standard curve [19,21,24].

Results

COL2 gene expression level

COL2 is a monomer protein that forms the main formation of the cartilage matrix and is the main target of tissue that is attacked by OA. The long assembly process of *COL2* in the cytoplasm will eventually be transported out of the cell to form a cartilage matrix and its expression culminates in the ripening of chondrocytes [25]. *COL2* expression can be seen in figure 1 and has increased from the first week to the second week. The highest expression was found in the IGF1-hWJMSCs-CM 15% treated group.

Level of TNFα, IL10, and MMP3

TNFα, together with IL1β, is considered an inflammatory cytokine which is a key in the pathophysiological process that occurs during OA. TNFα is secreted by the same cells as cells that synthesize IL1β [26,27]. Figure 2 shows TNFα levels using the ELISA method. It can be seen that IL1β-CHON002 has the highest level of TNFα among others, while addition of CM of IGF1-hWJMSCs 15% shows a significant decrease in TNFα ($p<0.05$). TNFα also experienced elevated levels in the treatment for 2 weeks.

IL10 is a cytokine that acts as an anti-inflammatory agent and it is one of the cytokines that shows a chondroprotective effect on OA [28]. The IL10 cytokines and IL10R receptors are expressed by chondrocytes [29].

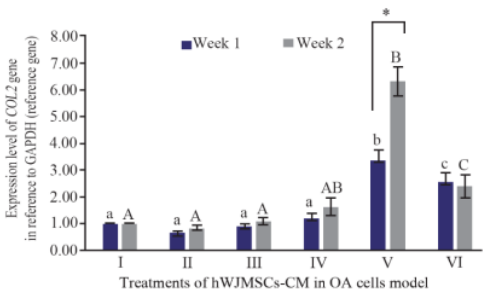

Figure 1. Effect of hWJMSCs-CM, IGF1-hWJMSCs-CM toward *COL2* gene expression on OA cells model. (I) Normal cell (CHON002), (II) IL1β-CHON002, (III) hWJMSCs-CM 15% + IL1β-CHON002 (IV), hWJMSCs-CM 30% + IL1β-CHON002, (V) IGF1-hWJMSCs-CM 15% +IL1β-CHON002, (VI) IGF1-hWJMSCs-CM 30%+IL1β-CHON002. The histograms are presented as mean± standard deviation, the treatment was done triplicate. The data were analyzed with ANOVA and Tukey post hoc test. Different letters (a, b, c) indicate significant differences in 1 week incubation (Blue color) and different letters (A, AB, B, C) indicate significant differences in 2 week incubation (Gray color). The symbol (*) presents significant differences between week 1 and week 2 based on paired t-test ($p<0.05$).

IL10 works by stimulating antagonist proteins against IL1β, namely IL1Ra, metalloproteinase inhibitors (TIMP1), and also as growth factors. IL10 levels can be seen in figure 3. IL10 as an anti-inflammatory mediator was found with the highest levels in IL1β-CHON002. However, addition of hWJMSCs-CM with or without IGF1 induction shows a decrease in IL10 levels compared to OA cells model.

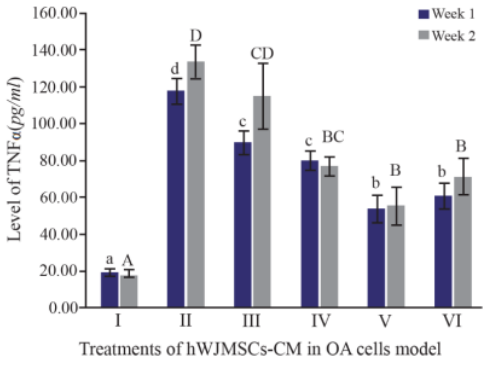

Figure 2. Effect of hWJMSCs-CM, IGF1-hWJMSCs-CM toward TNFα on OA cells model. (I) Normal cell (CHON002), (II) IL1β-CHON002, (III) hWJMSCs-CM 15%+IL1β-CHON002, (IV) hWJMSCs-CM 30%+IL1β-CHON002, (V) IGF1-hWJMSCs-CM 15%+IL1β-CHON002, (VI) IGF1-hWJMSCs-CM 30%+IL1β-CHON002. The histograms are presented as mean±standard deviation, the treatment was done triplicate. The data were analyzed with ANOVA and Tukey post hoc test. Different letters (a, b, c, d) indicate significant differences in 1 week incubation (Blue color) and different letters (A, B, BC, CD, D) indicate significant differences in 2 week incubation (Gray color).

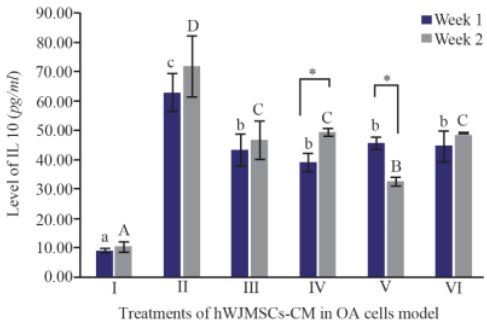

Figure 3. Effect of hWJMSCs-CM, CM of IGF1-hWJMSCs toward IL10 level on OA cells model. (I) Normal cell (CHON002), (II) IL-1β-CHON002, (III) hWJMSCs-CM 15%+IL1β- CHON002 (IV), hWJMSCs-CM 30%+IL1β-CHON002, (V) IGF1-hWJMSCs-CM 15%+IL1β-CHON002, (VI) IGF1-hWJMSCs-CM 30%+IL1β-CHON002. The histograms are presented as mean±standard deviation, the treatment was done triplicate. The data were analyzed with ANOVA and Tukey post hoc test. Different letters (a, b, c) indicate significant differences in 1 week incubation (Blue color) and different letters (A, B, C, D) indicate significant differences in 2 week incubation (Gray color). The symbol (*) presents significant differences between week 1 and week 2 based on paired t-test ($p<0.05$).

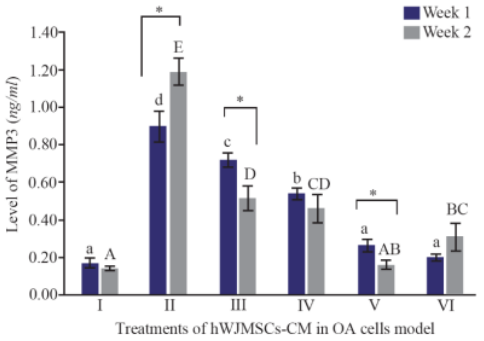

Figure 4. Levels of MMP3 in cells of OA models cultured in hWJMSCs-CM. (I) Normal cell (CHON002), (II) IL1β-CHON002, (III) hWJMSCs-CM 15%+IL1β-CHON002 (IV), hWJMSCs-CM 30%+IL1β-CHON002, (V) IGF1-hWJMSCs-CM 15%+IL1β-CHON002, (VI) IGF1-hWJMSCs-CM 30%+IL1β-CHON002. The histograms are presented as mean ± standard deviation, the treatment was done triplicate. The data were analyzed with ANOVA and Tukey post hoc test. Different letters (a, b, c, d) indicate significant differences in 1 week incubation (Blue color) and different letters (A, AB, BC, C, CD, D, E) indicate significant differences in 2 week incubation (Gray color). The symbol (*) presents significant differences between week 1 and week 2 based on paired t-test ($p<0.05$).

The MMPs are expressed in joint tissues of patients with OA and RA. The MMP3 is secreted from chondrocyte and synovial cells and MMP3 can reduce various extracellular matrix. Figure 4 shows MMP3 levels using ELISA method. The lower MMP3 level was shown by treatment with the addition of 15%, 30% of IGF1-hWJMSC-CM during 1 and 2 weeks of incubation, while the lowest MMP3 levels was during 2 weeks of incubation of IGF1-hWJMSCs-CM 15%. This result shows that IGF1-hWJMSCs-CM can reduce levels of MMP3 which plays a role in matrix degradation.

Discussion

MSCs have been used as one of the candidates for tissue engineering which have the ability in repair, replacement, and regeneration of cartilage tissue, because of their high proliferation and differentiation [29]. One type of them originates from Wharton's jel[13,19]. Previous studies reported that hWJMSCs can differentiate into chondrocytes [21,24], skeletal muscle cells, heart muscle cells, osteoblasts, adipocytes, β cells on the islets of Langerhans, and endothelial cells *in vitro* [30,31]. Therefore, these cells can be used as alternatives in the treatment of chronic degenerative disorders and prevent cartilage degradation in OA patients. In this study, the induction of hWJMSCs using IGF1 increased *COL2* expression. The *COL2* gene which is the cartilage matrix gene has been indicated to be regulated by SOX9 [32,33], and is involved in the structure and function of articular cartilage. This is in accordance with previous studies, where the inducing of IGF1 in hWJ-

MSCs increased the expression of the *SOX9* gene which means that the expression of the *COL2* gene will also increase [21]. *SOX9* is present in the presumed cartilage during embryonic development. *COL2* downregulation in OA is likely to contribute to cartilage pathology. IGF1 can stabilize the chondrocyte phenotype in pathological conditions, and also has mitogenic properties in the articular cartilage of adults and strongly stimulate the production of chondrocyte extracellular matrix components [21]. *COL2* is a chondrogenic gene marker in the joint cartilage, associated with Extracellular Matrix Secretion (ECM). In OA, early changes in articular cartilage are characterized by proteoglycan loss and a decrease in the expression of the *COL2* gene without altering the regularity of the articular tissue structure [34]. After treatment, as shown in figure 1, there were significant improvements in *COL2* gene expression in OA model, indicating that there might be repairing process in the ECM. The highest expression of *COL2* is by IGF1-hWJMSCs-CM 15%+IL1β-CHON-002 which is significantly different from other treatments. It is known that the expression of *COL2* is closely related to *SOX9*.

The effect of TNFα in many cases coincides with the action of IL1β, and a relationship can be detected that occurs during OA between two cytokines [28]. This effect is the result of activating the same group of intracellular signaling pathways, which in turn trigger effects that increase inflammation in joint tissues [35]. In contrast, IL10 is a cytokine that acts as an anti-inflammatory agent and it is one of the cytokines that shows a chondroprotective effect on OA and works by stimulating antagonist proteins against IL1β as pro-inflammatory cytokine [28].

In the present study, the highest levels of TNFα were found in OA cells without treatment using CM of hWJMSCs and IGF1-hWJMSCs. This is possible because the production of proinflammatory cytokines is directly proportional to the production of anti-inflammatory cytokines, when high levels of proinflammatory cytokines occur and the body adjusts to provide a regulatory response to levels of anti-inflammatory cytokines. Furthermore, hWJMSCs-CM and IGF1-hWJMSCs-CM in the OA cells model decrease TNFα level which is responsible for OA inflammation; however, administration of them to IL10 did not show an increase in IL10 levels responsible for inhibiting pro-inflammatory cytokines. This is in line with the results of Al-Banna *et al* who state that the induction of inflammatory cytokines is followed by an increase in the level of anti-inflammatory cytokines [36].

Cell therapies can directly aid repair by forming new functional tissues, or support tissue repair through paracrine mechanisms, for instance by secreting growth factors, immunomodulatory molecules, and Extracellular Vesicles (EVs). EVs can mediate cell-cell communication and are involved in many processes, including immune signaling, angiogenesis, stress response, senescence, proliferation, and cell differentiation [36]. *In vitro* passaging of MSCs results in cell enlargement, differentiation, and decrease in proliferation within 10 passages, and causes a strong response to micro-environment stiffness, affecting cell morphology, and function [37,38].

Metabolic imbalances between degradation and synthesis of articular cartilage are the main reason for degeneration in OA sufferers. MMP is a protein that is responsible for the degradation of the extracellular matrix and basement membrane components [39]. MMP is a endopeptidase that is connected with zinc ions and is localized in various connective tissues; it can degrade various components of the ECM [40]. In the present study, OA model (IL1β-CHON002) has shown high MMP3 levels, and after treatment using hWJMSCs-CM and IGF1-hWJMSCs-CM, showed a decrease in MMP3 levels (Figure 4) and the lowest MMP3 level was shown by treatment with the addition of IGF1-hWJMSCs-CM 30%. This indicates that the administration of hWJMSCs-CM with the addition of IGF1 can reduce MMP3 levels which are the cause of the degradation of the extracellular matrix.

Conclusion

In conclusion, CM of IGF1-induced hWJMSCs increases *COL2* gene expression compared with CM of IGF1-uniduced hWJMSCs. CM of IGF1-hWJMSCs actively reduce the levels of pro-inflammatory cytokines of TNFα, IL10, MMP3 compared with CM of IGF1-uniduced hWJMSCs. CM of IGF1-hWJMSCs also increases chondrogenesis and can subsequently be an alternative for the treatment of OA. Further studies on animal models must be carried out for validation of IGF1-hWJMSCs effect.

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Conflict of Interest

There are no conflict of interest.

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46. Effect of Conditioned Medium from IGF1-Induced Human Wharton's Jelly Mesenchymal Stem Cells (IGF1-hWJMSCs-CM) on Osteoarthritis

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