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The Effect of Mesenchymal Stem Cell Wharton's Jelly on Nuclear Factor Kappa Beta and Interleukin-10 Levels in Osteoarthritis Rat Model

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Abstract

BACKGROUND: Osteoarthritis (OA) is a degenerative joint disease in one or more joints characterized by changes in pathological structures such as cartilage, hypertrophy, and remodeling of the subchondral bone and secondary inflammation of the synovium membrane, causing changes in joint components such as cells, matrices, and molecular production. At the molecular level, an imbalance between catabolic and anabolic activities in joint cartilage results in OA. Nuclear factor kappa beta (NFκβ) is a cytokine that plays an important role in the signaling pathway of the pathogenesis of OA in causing an inflammatory reaction, whereas interleukin (IL)-10 is an anti-inflammatory cytokine that is involved in the pathogenesis of OA.

AIM: This study aims to prove the influence before and after administration mesenchymal stem cells from Wharton's jelly on the serum NFκβ and IL-10 levels in OA rat models.

MATERIALS AND METHODS: This research is an experimental study with the design of post-test-only control group design. The sample consisted of 16 OA rats as a control group and 16 OA rats treated with MSC-WJ as a treatment group. OA induction is done by injection of monosodium iodoacetate (MIA) into the intra-articular right knee. Giving MSC-WJ is done in the 3rd week after MIA induction. The serum NFκβ and IL-10 levels were measured after 3 weeks treated with MSC-WJ using the ELISA method. The statistical test used is an independent t-test. $p < 0.05$ was said to be statistically significant.

RESULTS: From the research results obtained, serum levels of knee OA of rat knee OA treated with mesenchymal stem cell Wharton jelly are lower than serum NFκβ levels of knee OA of the rat that is not treated, but the difference in levels of NFκβ is not significant ($p > 0.05$). The serum IL-10 level of rat OA of knee treated with mesenchymal stem cell Wharton jelly was higher than the serum IL-10 level of rat OA of the knee that was not treated, difference in levels of IL-10, is significant ($p < 0.05$).

CONCLUSION: This study concluded that MSC-WJ significantly decreased the serum NFκβ levels of OA rats and not significantly increased the serum IL-10 levels of OA rats.

Introduction

Osteoarthritis (OA) is a degenerative joint disease in one or more joints characterized by changes in pathological structures such as cartilage, hypertrophy, and remodeling of the subchondral bone and secondary inflammation of the synovium membrane [1]. OA is no longer considered a degenerative disease, but age remains one of the risk factors. As we get older, the possibility of OA increases. OA is the most common arthritis in the world. OA is a cause of disability in millions of patients, including about 60–70% of people over the age of 60 years. Overtime, the challenges in the right treatment for OA will be even greater because of the increasing population of the elderly population; therefore, in handling the problem of OA, a variety of therapies are needed [2].

At the molecular level, an imbalance between catabolic and anabolic activity in joint cartilage results in OA. The main catabolic factors are played by interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) stimulated by nuclear factor kappa beta (NFκβ) which contribute to the inflammatory process in OA. The anabolic factors are played by IL-4, IL-10, and transforming growth factor-beta. IL-10 is a pro-inflammatory cytokine that has a positive effect on the inflammatory reaction of OA. A study conducted by Driessler, in 2004, showed that IL-10 can inhibit NFκβ through inhibition of p65 so that the inflammatory process can be suppressed by increasing IL-10 and preventing the degradation process more quickly. Degradation and synthesis of normal cartilage matrix molecules are controlled by chondrocytes constantly [3].

Until now, therapies that can cure OA with satisfactory results have not been found. Management of OA is primarily intended to relieve pain, improve motion and joint function [4]. Pharmacological therapy in OA has many undesirable effects resulting from long-term use of drugs, such as the use of long-term

nonsteroidal anti-inflammatory drugs, resulting in peptic ulcers and impaired kidney function. For patients who are not responsive to pharmacological therapy, a total knee replacement surgery will be performed. The effort to maintain the joints is done in several ways, namely, injection with hyaluronic acid, platelet-rich plasma, and cell-based therapy such as mesenchymal stem cell.

This research uses experimental animals, male white rats. Rats were injected intra-articular with monosodium iodoacetate (MIA) into the rat knee joint. The development of joint pathology in this way can be controlled with the injected monoiodoacetate dose, thus providing a useful model system for evaluating potential OA modulators [6].

Based on the background description above, where the use of stem cells can be used as an alternative for OA therapy, the authors are interested in conducting *in vivo* research related to the effect of mesenchymal stem cells from Wharton's jelly on decreasing NFκβ and increasing IL-10 concentrations in rat serum OA of the knee.

Materials and Methods

Animal and experimental design

Male white rats (*Rattus norvegicus*) with a weight ranging from 200 to 250 g as experimental animals placed in clean, disinfected, and pathogen-free cages and given standard food in the form of pellets and drinking in *ad libitum*. Trial animals adapted first for 1 week before treatment. Induction of OA conducted with 300 μg intra-articular injection of MIA (Sigma-Aldrich, USA) in 50 μl of saline solution (0.9% NaCl) sterile [7] singly into the right knee joint rats anesthetized by intraperitoneal injection of xylazine 10 mg/kg and ketamine 20 mg/kg uses insulin syringe with a needle 27G [8]. Thirty-two OA male white rats (3 weeks after MIA induction) were divided into two treatment groups (n = 16): Control group and MSC-WJ group. MSC-WJ group is given 50 μl MSC-WJ with a dose of 1 × 10^6 cells into the right knee joint and a control group given 50 μl complete medium after anesthetized. Rats were sacrificed after 3 weeks of treatment. Serum and knee joint were taken and then analyzed.

Analysis of flow cytometry

Mesenchymal stem cell Wharton jelly was obtained from the Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, University of Indonesia. Based on the analysis of flow cytometry, MSC-WJ used for this therapy had CD73-APC cell surface expression 99.8%, CD105-PerCP-Cys5.5 95%, and CD90-FITC 99.9%. Photocell was taken use Nikon Ti-S microscope, scale bar: 500 μm (Figure 1).

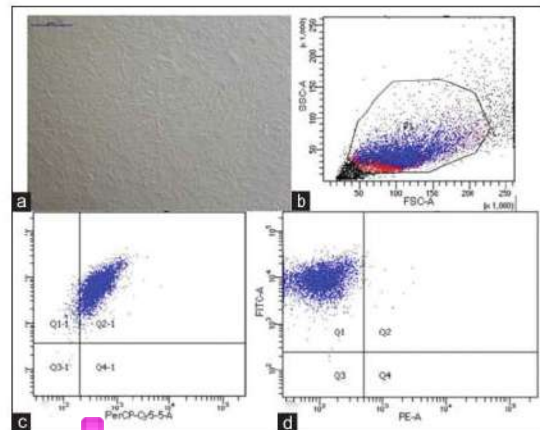


Figure 1: Data on characteristics of mesenchymal stem cells Wharton MHOO\□D□&HOOV□6&□-□□UHDFK□FRQIXHQFH□6FDOH□EDU□□□ Photographs of cells taken using a Nikon Ti-S microscope; (b) Data &ZE\WRPHWU□RUZDUG□FDWWHU□SORW□DQG□LGH□FDWWHU□SORW□RX JDWHG□HYHQWV□□□□&HOO□XUIDFH□PDUNHUV□H[SUHVVLRQ□ &'□□&□DQG□&'□□HU&3&\□□□&HOO□XUIDFH□ PDUNHUV□H[SUHVVLRQ□&'□□7&□DQG□□Q□□□

Measurement of serum NFκβ and IL-10 by ELISA

Blood was taken from sinus periorbital and centrifuged at 3000 rpm for 15 min. The collected serum was stored at −80°C until measurement. Serum NFκβ and IL-10 levels were measured by an ELISA kit (Bioassay Technology Laboratory, China). All samples are measured in duplicate.

Examination of levels NFκβ (work protocol based on rat NFκβ ELISA kit)

Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature. Determine the number of strips required for the assay. Insert the strips in the framers for use. The unused strips should be stored at 2–8°C. Add 50 μL standard well. Add 40 μL sample to sample wells and then add 10 μL anti-NFκβ antibody to sample wells, then add 50 μL streptavidin-HRP to sample wells and standard wells (not blank control well). Mix well. Cover the plate with a shaker. Incubate 60 min at 37°C. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 s to minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material. Add 50 μL substrate solution A to each well and then add 50 μL substrate solution B to each well. Incubate plate covered with a new sealer for 10 min at 37°C in the

dark. Add 50 μL stop solution to each well; the blue color will change into yellow immediately. Determine the optical density (OD) value of each well immediately using a microplate reader set a 450 nm within 30 min after adding the stop solution.

Examination of IL-10 levels (work protocol based on rat IL-10 ELISA kit)

Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature. Determine the number of strips required for the assay. Insert the strips in the framers for use. The unused strips should be stored at 2–8°C. Add 50 μL standard well. Add 40 μL sample to sample wells and then add 10 μL anti-IL-10 antibody to sample wells, then add 50 μL streptavidin-HRP to sample wells and standard wells (not blank control well). Mix well. Cover the plate with a shaker. Incubate 60 min at 37°C. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 s to minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material. Add 50 μL substrate solution A to each well and then add 50 μL substrate solution B to each well. Incubate plate covered with a new sealer for 10 min at 37°C in the dark. Add 50 μL stop solution to each well; the blue color will change into yellow immediately. Determine the OD value of each well immediately using a microplate reader set a 450 nm within 30 min after adding the stop solution.

Research ethics

This study was already passed the ethics clearance and has been approved by the Ethics Committee of the Faculty of Medicine, Andalas University, Padang, with registration number: 192/KEP/FK/2019.

Statistical analysis

Data are presented in mean and elementary forms. The statistical analysis used is SPSS 18.0. The statistical test used is an independent t-test. $p < 0.05$ was said to be statistically significant.

Results

OA rats were divided into two groups, namely, the OA group and the group treated with MSC-WJ. Examination of the levels of NFκβ and IL-10 was carried out in the serum of rats by ELISA.

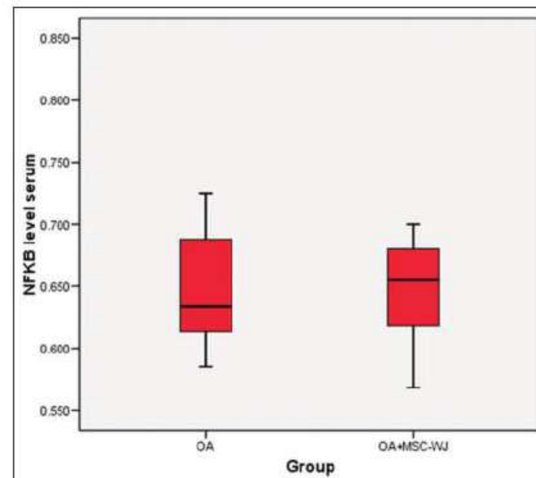
ELISA examination

The blood obtained from the centrifuged animal is then obtained serum. Serum before analysis was stored in a refrigerator temperature of −80°C. The serum obtained was determined by NFκβ and IL-10 levels, carried out in the Biomedical Laboratory, Faculty of Medicine, Andalas University.

The results of the measurement of NFκβ and IL-10 levels were carried out in normal rat, and the mean levels of NFκβ and IL-10 were 0.58 ng/mL and 6.05 pg/mL. Based on the results of the normality test, the data show that the two research variables, namely, NFκβ and IL-10 are normally distributed ($p > 0.05$). Thus, the parametric test (independent t-test) can then be carried out.

Effect of MSC-WJ on NFκβ levels in serum of OA rats

The results of the measurement of NFκβ levels by ELISA method showed that the serum NFκβ levels of OA rats treated with MSC-WJ were lower than those not treated which are shown in Figure 2.



FigXUH [SOR graph of rat serum nuclear factor kappa beta levels

The difference in NFκβ levels between the serum of rats treated with MSC-WJ and not treated bivariate test is shown in Table 1.

Table 1 and Figure 3 show that there are differences in levels of NFκβ based on treatment. Decreased levels of NFκβ in the group treated with MSC-WJ from the control group. Statistically, the differences were not significant ($p>0.05$).

Table 1: Mean differences in NFκβ levels by group

Group	NFκβ levels (ng/mL) Mean±SD	p value
OA	0.658±0.06	
OA+MSC-WJ	0.647±0.04	0.53

Effect of MSC-WJ on IL-10 levels in serum of OA rats

The results of the measurement of IL-10 levels by ELISA method showed that the serum IL-10 levels of OA rats treated with MSC-WJ were higher than those not treated with bivariate tests which are shown in Figure 4.

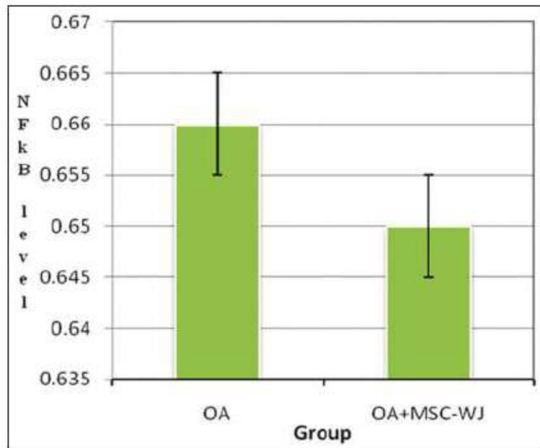
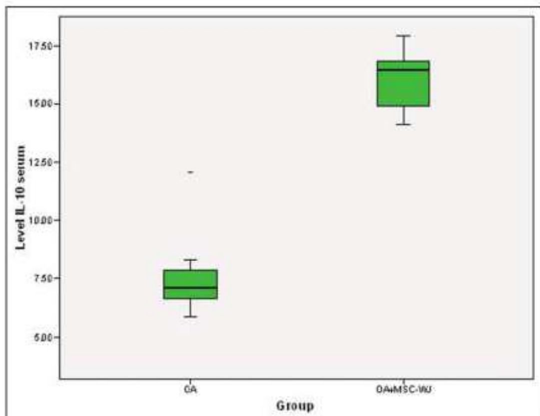


Figure 3: Histogram of rat serum nuclear factor kappa beta levels

The difference in IL-10 levels between the serum of rats treated with MSC-WJ and not treated bivariate test is shown in Table 2.



)LJXUHDWHFWRIWKH06&EXQ,ÃHYHOVLQWKHUXPRI2$UDWV

Table 2 and Figure 5 show that there are differences in levels of IL-10 based on treatment. Increased levels of IL-10 in the group treated with MSC-WJ from the control group. Statistically, the differences were significant ($p < 0.05$).

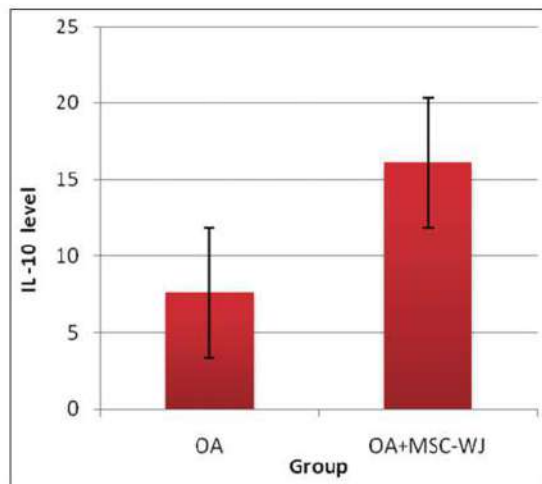
Table 2: Mean differences in IL-10 levels by group

Group	IL-10 levels (pg/mL) Mean±SD	p value
OA	7.604±0.06	
OA+MSC-WJ	16.144±0.04	0.00

Discussion

Formation of OA in rat

The formation of an OA mouse model in this study refers to the method used by Janusz *et al.*, van Buul *et al.*, and Javanmard *et al.* [6], [7], [8]. The dosage of MIA by following that used by van Buul *et al.*, which is 300 μg in a volume of 50 μL MIA is given intra-articularly. MIA is an inhibitor of glyceraldehyde-3-phosphate



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dehydrogenase which results in reduced glycolysis and causes changes in articular cartilage associated with histological and morphological features of OA by inhibiting the integration of subchondral structures and inducing chondrocyte cell death. The OA model induced by MIA is regularly used to measure behavior pain and drug therapy to overcome pain in animals. This model might be more stressful the efficacy of drugs than other pain models used to test OA drugs. Thing this is commonly used in rats and rat [9]. Surgically induced OA models may be more clinically relevant than model ones chemically induced related to the pathophysiology of OA. However, there are some disadvantages surgery-induced OA models, including the need for surgical manipulation for inducing OA and difficulty in reaching the severity of arthritis which can reproduce. Therefore, the OIA model that is used by MIA is used and confirmed reproducibility grade and stage of OA in rat [10]. Furthermore, MIA causes the development of recurrent synovial hyperplasia and infiltration of inflammatory cells, destroys articular cartilage, and induces bone loss and chondral deformation. MIA injection into the rat knee joint is considered a suitable

model and resembles the phenomenon observed in human OA [11].

Research by Matsumoto *et al.* [10] has also researched OA models on a rat. Sixty female rats were 10 weeks old (NIH-Whn NIHRNU-M; Taconic, Germantown, NY) which are used. The animal was given anesthetic 3% isoflurane and O2 gas (1.5 L/min) through inhalation mask OA-like arthritis induced by a single injection intrajoint MIA (Aldrich Chemical, Milwaukee, WI) (0.3 mg/150 mg body weight) into both rat knee joints. A chronic OA model with progressive manifestations was found 2 weeks after MIA injection which was compared with subacute OA models which manifested 1 week after MIA injection. MIA injection in the rat's knee shows an increase in activity enzymes collagenase and gelatinase. In a study by Janusz *et al.* found increased levels of MMP and aggrecanase as well as proteoglycans, starting 7th day post-MIA injection until the 21st day and the administration of anti-MMP from day 7 can significantly inhibit cartilage damage [6].

The results showed that the induction of MIA in rat with a single dose of 300 µg after 3 weeks showed depletion of cartilage thickness. The results of this study are also the same as those conducted by Janusz *et al.* [6] concerning the effect of MIA on rat cartilage, where his research also occurred depletion of cartilage after MIA induction. This depletion of cartilage results from the loss of proteoglycans that build the matrix. Besides, the results of this study also showed a reaction in the form of proliferation of reactive chondroblast cells in rat induced by MIA so that a higher density of chondrocyte cells was found in the cartilage of OA rat compared to normal rat [12].

NFκβ

The NF-κβ molecules are a family of ubiquitously expressed transcription factors involved in immunity, stress responses, inflammatory diseases, cell proliferation, and cell death. The OA chondrocytes express a variety of NF-κβ-mediated catabolic cytokines and chemokines, such as TNF-α, IL-1, IL-6, receptor activator of NF-κβ (RANK) RANK ligand (RANKL), and IL-8 that increase the production of MMPs, decrease collagen and proteoglycan synthesis, and act in a positive feedback loop to augment NF-κβ activation.

In articular joint, the NF-κβ pathway is activated in chondrocytes and synoviocytes on stimulation by mechanical stress or cytokines (TNF-α and IL-1). Subsequently, the activated joint cells produce several MMPs, ADAMPTS, apoptotic molecules (cyclooxygenase 2 [COX2], nitric oxide [NO], and prostaglandin E2 [PGE2]), chemokines (IL-8), and cytokines (TNF-α, IL-1, and IL-6) which induce cartilage destruction and synovium membrane inflammation (synovitis). In addition, the cytokines TNF-α, IL-1, and IL-6 secreted by chondrocytes and synoviocytes activate osteoblasts to produce the RANKL cytokine which induces osteoclast-mediated bone resorption [13].

The activation of stress- and inflammation-induced signaling and transcriptional and post-transcriptional events results in release of the chondrocytes from growth arrest, imbalanced homeostasis, and chondrocyte activation with aberrant expression of inflammation-related genes, including NO synthase-2, COX2, and catabolic genes such as MMP-1, 3, and 13 and ADAMTS-4 and 5 [5]. The signaling kinases activated by mechanical and inflammatory stimuli include the stress- and mitogen-activated ERK, p38, and JNK, which coordinately activate AP-1, ETS, Runx2, and C/EBP transcription factors, and IKKα and IKKβ, which activate, respectively, non-canonical (RelA/p52) and canonical NF-κB (p65/p50) pathways [14].

In addition, the OA chondrocytes express a variety of NFκβ-mediated catabolic cytokines and chemokines, such as TNF-α, IL-1, IL-6, receptor activator of NFκβ, and IL-8 that increase the production of MMPs, decrease collagen and proteoglycan synthesis, and act in a positive feedback loop to augment NFκβ activation. Finally, the NFκβ molecules enhance the articular damage through induction of NO, COX2, NO synthase, and PGE2, which promote the synthesis of catabolic factors, cartilage inflammation, and apoptosis of OA chondrocytes [15].

From the results of the t-test independent statistical analysis using SPSS version 18 software obtained significance value 0.531>0.05. This shows that there is no significant difference in the level of NFκβ between the OA rat groups that are not treated with OA rat treated by MSC-WJ. This is because NFκβ is a regulatory cytokine that functions to regulate the process of transcription of genes that play a role in inflammatory reactions. NFκβ is a transcription factor in mammals that control many important genes in the process of immunity and inflammation. NFκβ is found in all cell types involved in cellular responses to stimuli such as stress, cytokines, chemokines, free radicals, and others [16].

From the research results obtained related to NFκβ levels, some previous research results are relevant to the results of this study. The results of Chen's *et al.* study, stated that NFκβ plays an important role in the process of differentiation from periodontal ligament stem cells to differentiate into bone (osteogenic). NFκβ is involved in regulating the inflammatory process and plays a role in increasing the ability of stem cells in the treatment of bone diseases [17].

Several studies to date have shown that NFκβ signaling plays an important role in certain parts of the activity of osteoclasts, osteoblasts, and chondroblasts. Some of these functions appear to be important during embryonic development, such as the role of NFκB 1 and 2 in osteoclastogenesis. NFκβ activation seems to play an important role in endochondral ossification to prevent cartilage damage in animal models of rheumatoid arthritis

and OA. In this case, there is an opportunity in the development of drugs to promote or inhibit the activation of NFκB to treat or prevent common bone diseases, but to date, no one has been in clinical trials [18].

Research conducted by Guercio *et al.*, in 2012, the administration of MSC by intra-articular injection in dogs with OA models showed a significant improvement in humeroradial joint compared to controls. Cell therapy is very potential before the onset of injury to OA [19]. The opposite regarding NFκβ was obtained from Chang *et al.*'s study in 2013 which found an association between pro-inflammatory cytokines such as TNF-α and IL-17 stimulated by Ikβ kinase (IKK)-NFκβ with failure of the MSC differentiation process to osteogenic on the contrary, IKK-NFκβ inhibition can significantly increase the ability of MSC in the process of bone formation [20]. During the differentiation process of MSC, there will be an increase in NFKβ gene activity and expression. The strength of pF unit NFKβ gene expression (RELA) causes the differentiation process of MSC [21].

IL-10

From the results, MSC-WJ can increase serum IL-10 levels in OA rat model, of the independent t-test obtained a significance value of 0.00 < 0.05. This shows that there are significant differences in IL-10 levels between groups of OA mice that are not treated with OA mice treated by MSC-WJ. The results of this study are in line with the study conducted by Yang *et al.*, in 2009, which states that the administration of MSC can increase IL-10 levels which can suppress the proliferation of suppressor T cells that play a role in the occurrence of inflammatory reactions [22]. Research conducted by Jang, in 2019, states that MSC administration in mice irritable bowel disease model with IL-10 knock out can reduce the production of reactive oxygen species (ROS) which are oxidative stress agents and increase the production of antioxidant agents. Inflammation and ROS are closely related to tissue damage [23]. From a study conducted by Maiti *et al.*, in 2019, it was stated that concomitant administration of MSC and IL-10 can significantly stimulate the activation of autophagy, mitophagy, and other cell survival markers and can reduce markers from cell death and neuroinflammation. The administration of MSC and IL-10 together is possible as an effective therapeutic strategy to prevent neuronal damage in traumatic brain injury mice [24]. Research conducted by Liu *et al.*, in 2013, states that the administration of MSC can inhibit the maturation of dendritic cells which are agents of antigen-presenting cells which play an important role in triggering an immune reaction. MSC inhibits the maturation of dendritic cells through the secretion of IL-10. MSC can stimulate the release of IL-10 which is one of the anti-inflammatory cytokines [25].

The results obtained from this study were also supported this research conducted by Choi *et al.*, in 2008, *in vitro* coculture MSC and IL-10 were able to secrete IL-10 in large quantities. Intra-articular injection of IL-10 and MSC can significantly reduce the severity of arthritis. IL-10 is a contributing factor as a neuroprotective in MSC transplants after ischemic stroke [26]. From the results of the immunological analysis, experiments showed that transplantation of MSC and IL-10 can significantly inhibit microglial activation and expression of pro-inflammatory cytokines. Overexpression of IL-10 can increase the neuroprotective effect after administration of MSC through the mechanism of anti-inflammatory modulation and thus can prevent the occurrence of more extensive nerve damage during the onset of acute ischemia [27]. IL-10 is the main anti-inflammatory cytokine in the immune system that it plays a role for stop the excessive inflammatory response through the inactivation of macrophages and T cells. These cytokines are local and systemic inflammatory mediators that the body can produce in large quantities, so they are easily detected in serum [28].

This study can explain the effect of mesenchymal stem cells Wharton jelly on OA model mice through the parameters of serum levels of NFκβ and IL-10. In general, MSC-WJ can reduce serum NFκβ levels and increase serum IL-10 levels which are pro-inflammatory cytokines in OA. Mesenchymal stem cells Wharton jelly can increase IL-10 levels, which, in turn, can improve the damage to synovial tissue in OA, because IL-10 is an anti-inflammatory cytokine that plays an important role in protecting the joint cartilage matrix. The results of this study can also be useful as a reference for the use of stem cells, especially MSC-WJ as a promising OA therapy in future.

Conclusion

Based on the results of research that has been done can be concluded as follows, the NFκβ serum levels of knee OA rat treated with mesenchymal stem cell Wharton jelly are lower than the NFκβ serum levels of knee OA of the knee that is not treated, but the difference in NFκβ levels is not significant. IL-10 serum levels of knee OA rat treated with mesenchymal stem cell Wharton jelly are higher than IL-10 serum levels of knee OA rat that is not treated, the difference in IL-10 levels is significant.

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