The Effect of secretome-hypoxia mesenchymal-stem-cells and vitamin-d3 in type-2 diabetes-mellitus induced periodontitis rats

Isa Anshori Muchaeroni, Yayun Siti Rochmah**, Agung Putra****

**Magister of Biomedical Program, Medical Faculty, Sultan Agung Islamic University, Semarang, Indonesia*
***Dental Faculty, Sultan Agung Islamic University, Semarang, Indonesia*
****Stem Cell and Cancer Research Institute, Medical Faculty, Sultan Agung Islamic University, Semarang, Indonesia*

Correspondence: yayun@unissula.ac.id

Received 17 November 2022; 1st revision 8 May 2023; 2nd revision 14 June 2023; Accepted 26 July 2023; Published online 31 July 2023

Keywords:

T2DM; Periodontitis; SOD; IL-10; TNF-α

ABSTRACT

Background: *A systemic metabolic disorder by insulin resistance, type-2 diabetes mellitus (T2DM), is characterised by an increased level of reactive oxygen species (ROS) and decreased superoxide dismutase (SOD), which is associated with the activation of proinflammatory cytokine pathways such as tumor necrosis (TNF)-α. This condition usually stimulates inflammation of the periodontal tissue (periodontitis). Vitamin-D3 can inhibit the release of inflammatory mediator and reduce the risk of chronic periodontitis. Purpose this study to determine the effect of the secretome-hypoxia mesenchymal-stem-cells (SH-MSCs)-Vitamin-D3 combination on the expression of SOD, IL-10, and TNF-α genes in T2DM periodontitis-induced rats.*

Method: *30 rats were randomly divided into five groups; normal sham condition, positive control, SH-MSCs group, vitamin-D3 group, and combination of SH-MSCs and vitamin D3 group. SH-MSCs were injected at doses of 150 uL in the gingival. In addition, vitamin D3 5000 IU 2.25 mcg were administrated orally. After 35 days, all rats were sacrificed, and qRT-PCR from gingival tissue was performed to identify the expression of SOD, IL-10 and TNF-α genes*

Results: *The results a significant increase in SOD and IL-10 gene expression (p<0.000) and a significant decrease in TNF-α gene expression (p<0.000) compared to control.*

Conclusion: *The combination of SH-MSCS with vitamin D3 significantly reduce TNF-α and increa sed SOD and IL-10 genes' expression.*

doi: http://dx.doi.org/10.30659/odj.10.1.125-131
2460-4119 / 2354-5992 ©2022 National Research and Innovation Agency
This is an open access article under the CC BY-SA license (https://creativecommons.org/licenses/by-sa/4.0/)
Odonto : Dental Journal accredited as Sinta 2 Journal (https://sinta.kemdikbud.go.id/journals/profile/3200)
How to Cite: Muchaeroni *et al*. The Effect of secretome-hypoxia mesenchymal-stem-cells and vitamin-d3 in type-2 diabetes-mellitus induced periodontitis rats. Odonto: Dental Journal, v.10, n.1, p. 125-131, July 2023

INTRODUCTION

Type-2 diabetes mellitus (T2DM) is a systemic metabolic disorder characterized by insulin resistance. Under this condition, insulin is produced in excess1 but the body's cells cannot adequately respond to the hormone insulin.[2] This condition causes the body's cells to be unable to absorb glucose, thus failing energy production.[3] As a result, glucose accumulates in the blood and causes hyperglycemia.[4] Increased level of advanced glycation end products (AGEs) in plasma and tissues usually found in T2DM patients.[5,6] AGEs and receptors for AGEs (RAGE) play an essential role in periodontal disease . Increased AGEs in patients with diabetes mellitus lead to stimulating ROS activity and activating proinflammatory cytokine pathways such as TNF-α, IL-1, and IL-6 which damage connective tissue and bone.[7] Proinflammatory cytokines and other mediators will then stimulate osteoclasts to carry out excessive alveolar bone resorption resulting in destructive periodontal tissue leading to periodontitis in T2DM patients.[8] The prevalence of T2DM in Indonesia in 2019 was 11,3%. This figure is higher than in 2013 (2.1%). A total of 31 provinces (93.9%) showed a significant increase in the prevalence of diabetes mellitus. In general, almost 85% of the prevalence of diabetes mellitus is type 2 diabetes mellitus.[9,10]

Previous research reported the prevalence of periodontitis severity in a sample of 45 people with T2DM and 45 people without T2DM. It is known that there is a difference in the mean index of periodontal tissue conditions in patients with T2DM and non-DM patients. In patients with T2DM (2.11), it was higher than in patients with non-T2DM (1.77), and this difference was statistically significant ($p<0.05$).[11] Bone graft treatment has shown promising results for chronic periodontitis with alveolar bone destruction. Still, this therapy has a disadvantage: the lack of growth factors and cells that can accelerate alveolar bone remodelling.[12] Conditioned medium mesenchymal stem cells (MSCs) can improve the condition of endothelial damage, inflammation and oxidative stress in a rat model of T2DM with periodontitis by decreasing levels of IL-6, TNF-α, ROS and increasing eNOS.[13] SH-MSCs are secretions produced by MSCs that are rich in anti-inflammatory soluble molecules such as IL-10, IL-1 and growth factors such as transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF).[14,15] IL-10 released by MSCs can downregulate the production of inflammatory cytokines and inhibit T cell activation.[16,17] IL-10 is also a potent inhibitor of Th1 proinflammatory cytokines such as IL-2.[18]. Vitamin D is known to be useful in chronic periodontitis disease to inhibit the release of proinflammatory mediators such as TNF-α, IL-1β, IL-6, IL-8 which contributes to periodontal destruction also regenerates damaged alveolar bone.[19,20] This study aimed to determine the combination of SH-MSCs with Vitamin D3 on the expression of SOD, IL-10, and TNF-α genes in T2DM and periodontitis rat models.

RESEARCH METHOD

Diabetes Induction

Wistar (Rattus Novergicus) male white rats were two months old after completing the adaptation period for seven days, then 110 mg/kg BW nicotinamide (NA) was injected, then after 15 minutes 45 mg/kg BW streptozotocin (STZ) was injected intraperitoneally. STZ was dissolved in sodium citrate solution, pH 4.5 and NA was dissolved using water for injection. On day 14, the average GDP of rats was 340 mg/dL. The procedure for making mouse models is based on

standard protocols to obtain experimental animal models of type 2 diabetes.[21]

Periodontitis Induction

On day 14, Wistar rats were anaesthetized by intramuscular injection of ketamine HCl in the back muscle at a dose of 0.2 ml/200 g/BW before induction of periodontitis. Then the induction was done by tying the thread of silk (silk ligature) size 3.0 in the subgingival area around the mandibular anterior incisor. Seven days after ligation, clinical signs of periodontitis were seen. The color of the gingival margin was reddish, the contour of the gingival margin was rounded, and there was a decrease in the gingival margin (gingival recession). Ligation aims for the accumulation of dental plaque.[22,23]

MSCs Isolation

The umbilical cord of female white rats at 19 days of gestation was washed with PBS solution. The umbilical cord was chopped finely, placed in a 25 T flask, and allowed to stand for 3 minutes until the tissue adhered to the surface of the flask. A complete medium consisting of DMEM, fungizone, penstrep, and FBS was added slowly to cover the tissue. The explants were incubated in an incubator at a temperature of 370 C, and 5% CO2. Cells would appear approximately 14 days from the start of the culture process. The medium was replaced every three days by removing half of the medium and replaced with a freshly prepared medium. Cell maintenance was performed to reach 80% compliance.[24]

Induction of hypoxic condition on MSCs

MSCs that have reached 80% confluency is added with a complete medium up to 10 mL then the flashed flask containing MSCs is put into the hypoxic chamber. Nitrogen gas is channelled through the inlet valve, and an oxygen meter is placed in the sensor hole to measure the oxygen concentration in the chamber until it shows a 5% oxygen concentration. The chamber filled with the flask was incubated for 24 hours at 370C. After 24 hours, the culture media was taken and filtered using Tangential flow filtration (TFT) to obtain SH-MSCs, which were then mixed with a water-based gel according to the dose of the treatment group.

Stimulation of SH-MSCs in vivo

Wistar model of T2DM and periodontitis were divided into five groups (n=6/group). The control group was given (1% Na-CMC), and group P1 was injected with SH-MSCs around the gingiva at a dose of 150 microliters and vitamin-D3 5000 IU at a dose of 2.25 mcg orally. Group P2 was given vitamin-D3 5000 IU at a dose of 2.25 mcg orally. Group P3 was injected with SH-MSCs around the gingiva at a dose of 150 microlites. Sham group was given standard distilled water. After 35 days, rats were sacrificed using a lethal dose cocktail of 10 mL ketamine 50 mg/kgBW, xylazine 10 mg/kgBW, and acepromazine mg/kgBW intramuscularly. The rats were then euthanized, and the gingival organs were isolated for RT-PCR.

Data analysis

Data analysis was carried out by statistical methods of normality test of data with Shapiro Wilk test, then continued with the Levene Homogeneity test. Finally, homogeneous data continued with the One Way Annova parametric test to determine the differences between treatment groups ($p < 0.05$), followed by the Post Hoc LSD test.

Table 1. Gene primer sequen

No	*Gene*	Forward primer sequence	Reverse primer sequence
1	*SOD*	5'-AGCCCAGCCTGCGTAGA-3¢	5'-GGTACTTCTCCTCGGTGACG
2	*IL-10*	5'-TCAAACAAAGGACCAGCT GGACAACATACTG-3'	5'-CTGTCTAGGTCCTGGAGT CCAGCAGACTCAA-3'
3	*TNF-α*	5'- AGGCAATAGGTTTTGAGGGCCAT-3	5'-TCCTCCCTGCTCCGATTCCG-3'

RESULTS

Characteristics of MSCs

Identification of the characteristics of MSCs was carried out by CD73 and CD105 markers immunocytochemistry (Figure 1).

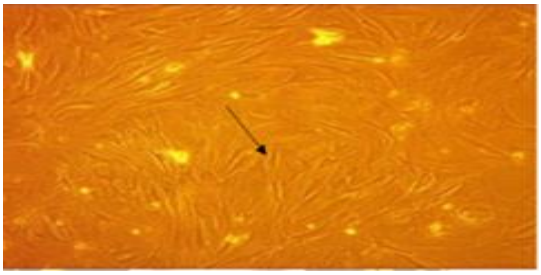

Figure 1. Characteristics of MSCs cultured with spindle-like shape (pointed by arrows) at 100x. magnification

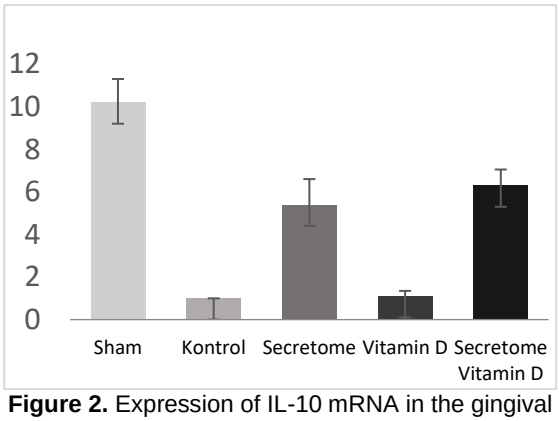

Figure 2. Expression of IL-10 mRNA in the gingival tissue

mRNA expression was analyzed from gingival tissue samples on day 35. The procedure was carried out according to the applicable protocol. The results that the mRNA expression of the IL-10 gene in the combination of SH-MSCs and vitamin D3 group and SH-MSCs group gave a significant difference in increasing IL-10 compared to the control group (p=0.000) (Figure 2).

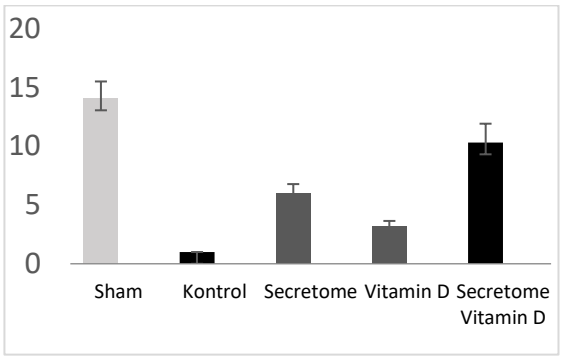

Figure. 3 Expression of SOD mRNA in the gingival tissue

The results showed that the mRNA expression of the *SOD* gene was not significantly different from the SH-MSCs group (p=0.21) (Figure 3). These proves that the administration of SH-MSCs and vitamin D3 can increase the expression of SOD gene mRNA equivalent to healthy conditions.

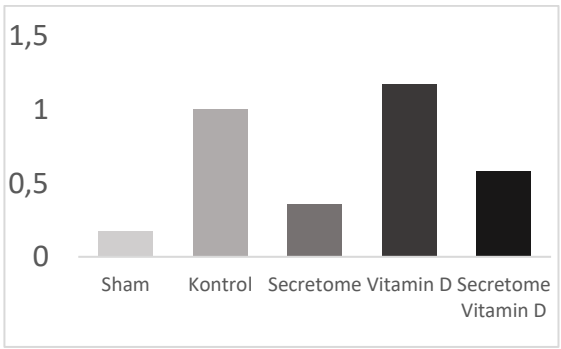

Figure 4. *TNF-α* gene mRNA expression in the gingival tissue

The results showed that the mRNA expression of the *TNF-α* gene in the SH-MSCs and vitamin D3 groups was not significantly different from the SH-MSCs group (p=0.845) (Figure 4).

This proves that the administration of SH-MSCs and Vitamin D3 can reduce the expression of *TNF-α* gene mRNA equivalent to healthy condition.

DISCUSSION

T2DM with poor glycemic control will result in an increased inflammatory response in the periodontal tissue and stimulate receptor activation of NF-kB, followed by osteoclastogenesis and alveolar bone destruction and alveolar bone resorption are largely irreversible.[25] Diabetes increases inflammation of the periodontal tissues with higher levels of inflammatory mediators such as TNF-α.[26] The accumulation of ROS, oxidative stress, and interactions between AGEs in periodontal tissue and RAGE will contribute to increased inflammation in periodontal tissues in people with diabetes.[27,28] Previous study have shown that MSCs contain anti-inflammatory cytokines that can repair pancreatic endothelial damage, inflammation and oxidative stress in a rat model of diabetes mellitus.[29]

This study showed a significant increase in IL-10 expression in treating SH-MSCs and vitamin D3. IL-10 contained in SH-MSCs can trigger type 1 macrophages (M1) into type 2 macrophages (M2) which are anti-inflammatory in T2DM.[30]. Vitamin D3 also has an anti-inflammatory effect that works by inhibiting the production of proinflammatory cytokines. In addition, vitamin D3 increased the expression of IL-10 from activated cells, binding to the vitamin D receptor to IL-10 and modulating calcium-dependent signalling to a lesser extent.[31]
Based on the study's results, there was an increase in the expression of the SOD gene due to the administration of SH-MSCs and Vitamin D3. Regulation of nuclear factor-related (Nrf)2 in the cytoplasm depends on kelch-like ECH-associated protein (Keap)1. Under high oxidative stress, Nrf2 dimerizes with Maf and binds to ARE genes such as H0-1. Then the regulated H0-1 catalyzes heme to CO and will activate the NF-kB pathway to prevent the formation of antioxidant enzymes and induce proinflammatory cytokine expression.[32] IL-10 in SH-MSCs can inhibit the release of Nrf2 from Kaep-1 to increase SOD expression.[33]

Based on the study's results, there was a significant decrease in TNF-α gene expression due to the administration of SH-MSCs and vitamin D3. SH-MSCs as inflammatory sensors will create proinflammatory and anti-inflammatory effects when interacting with innate immune system cells or exposed to various cytokines.[34] Previous studies have reported that SH-MSCs respond to the inflammatory environment by either polarizing into type 2 MSCs with an immunosuppressive receptor or MSCs type 1 with a proinflammatory profile depending on the kind of activation of Toll-Like Receptors (TLRs).[35–37] The significant decrease in TNF-α gene expression is because SH-MSCs have immunosuppressive properties capable of releasing IL-10 cytokines. IL-10 has been known to decrease the expression of TNF-α released by inflammatory cells, which is associated with the immune response of T2DM and periodontitis.[38]

TNF-α, IL-1β, and IFN-γ are cytokines that can act synergistically during cell inflammation, leading to the destruction of pancreatic cells.[39] In a previous study, the secretome stimulated a significant increase in cellular insulin release. Insulin-producing islets were induced with 1 g/ml IFN-γ and 100 ng/ml TNF-α for 24 h before acute exposure to glucose.[40] Inhibition of IFN-γ expression by IL-10 as an antiinflammatory agent contributes to the inflammation caused by occurs in pancreatic islets.[41,42]

CONCLUSION

This study showed that SH-MSCs and vitamin D3 compounds have anti-T2DM and periodontitis activity in rat models by preventing cellular oxidative stress by increasing SOD gene expression, increasing antiinflammatory cytokine IL-10 and preventing inflammation through decreasing TNF-α gene expression. This evidence suggests that SH-MSCs and Vitamin D3 have the potential to be developed as targeted therapy in T2DM with periodontitis.

ACKNOWLEDMENT

The author would like to thank the Stem Cells and Cancer Research (SCCR) laboratory of FK Unissula, along with the staff and the Magister of Biomedical Study Program of the Medical Faculty of Unissula for the permission and supporting facilities in this research.

REFERENCES

1. Goyal R JI. Diabetes Melitus Type 2. StatPearls Publishing : January 2022; 2021. 1–1 p.
2. l Mansour MA. The prevalence and risk factors of type 2 diabetes mellitus (DMT2) in a semi-urban Saudi population. Int J Environ Res Public Health. 2020 Jan 1;17(1).
3. Galicia-Garcia U, Benito-Vicente A, Jebari S, Larrea-Sebal A, Siddiqi H, Uribe KB, et al. Pathophysiology of type 2 diabetes mellitus. Vol. 21, International Journal of Molecular Sciences. MDPI AG; 2020. p. 1–34.
4. Olokoba AB, Obateru OA, Olokoba LB. Type 2 Diabetes Mellitus: A Review of Current Trends.
5. Preshaw PM, Alba AL, Herrera D, Jepsen S, Konstantinidis A, Makrilakis K, et al. Periodontitis and diabetes: A two-way relationship. Vol. 55, Diabetologia. 2012. p. 21–31.
6. Li DX, Deng TZ, Lv J, Ke J. Advanced glycation end products (AGEs) and their receptor (RAGE) induce apoptosis of periodontal ligament fibroblasts. Brazilian Journal of Medical and Biological Research. 2014;47(12):1036–43.
7. van Dyke TE. The Management of Inflammation in Periodontal Disease. J Periodontol. 2008 Aug;79(8s):1601–8.
8. Albarakat M, Guzu A. Prevalence of type 2 diabetes and their complications among home health care patients at Al-Kharj military industries corporation hospital. J Family Med Prim Care. 2019;8(10):3303.
9. Wahyu D, Jurusan H∗, Kesehatan I, Keolahragaan I, Disetujui D, D. Faktor-Faktor Yang Berhubungan Dengan Kepatuhan Dalam Pengelolaan Diet Pada Pasien Rawat Jalan Diabetes Mellitus Tipe 2 Di Kota Semarang [Internet]. Vol. 2, JHE. 2017. Available from: http://journal.unnes.ac.id/sju/index.php/jhealthedu/
10. Infodatin-2020-Diabetes-Melitus.
11. Chee B, Park B, Bartold PM. Periodontitis and type II diabetes: A two-way relationship. Vol. 11, International Journal of Evidence-Based Healthcare. 2013. p. 317–29.
12. Nugraha AP, Rezkita F, Puspitaningrum MS, Luthfimaidah MS, Narmada IB, Prahasanti C, et al. Gingival mesenchymal stem cells and chitosan scaffold to accelerate alveolar bone remodelling in periodontitis: A narrative review. Vol. 13, Research Journal of Pharmacy and Technology. Research Journal of Pharmacy and Technology; 2020. p. 2502–6.
13. A. Letourneau P. Human Bone Marrow-Derived Mesenchymal Stem Cells Regulate Leukocyte-Endothelial Interactions and Activation of Transcription Factor NF-Kappa B. J Tissue Sci Eng. 2011;01(S3).
14. Khammissa RAG, Ballyram R, Jadwat Y, Fourie J, Lemmer J, Feller L. Vitamin D Deficiency as It Relates to Oral Immunity and Chronic Periodontitis. Vol. 2018, International Journal of Dentistry. Hindawi Limited; 2018.
15. Dokka S, Shi X, Leonard S, Wang L, Castranova V, Rojanasakul Y, et al. Interleukin-10-mediated inhibition of free radical generation in macrophages [Internet]. Vol. 280, Am J Physiol Lung Cell Mol Physiol. Available from: http://www.ajplung.org
16. Pankajakshan D, Agrawal DK. Mesenchymal Stem Cell Paracrine Factors in Vascular Repair and Regeneration.
17. Gao F, Chiu SM, Motan DAL, Zhang Z, Chen L, Ji HL, et al. Mesenchymal stem cells and immunomodulation: Current status and future prospects. Vol. 7, Cell Death and Disease. Nature Publishing Group; 2016.
18. Gao X, Song L, Shen K, Wang H, Qian M, Niu W, et al. Bone marrow mesenchymal stem cells promote the repair of islets from diabetic mice through paracrine actions. Mol Cell Endocrinol. 2014 May 5;388(1–2):41–50.
19. Machado V, Lobo S, Proença L, Mendes JJ, Botelho J. Vitamin D and periodontitis: A systematic review and meta-analysis. Vol. 12, Nutrients. MDPI AG; 2020. p. 1–17.
20. Calton EK, Keane KN, Newsholme P, Soares MJ. The impact of Vitamin D levels on inflammatory status: A systematic review of immune cell studies. PLoS One. 2015 Nov 3;10(11).

21. Ghasemi A, Khalifi S, Jedi S. Streptozotocin-nicotinamide-induced rat model of type 2 diabetes (review). Vol. 101, Acta Physiologica Hungarica. Akademiai Kiado ZRt.; 2014. p. 408–20.
22. Chriestedy Prasetya R, Purwanti N, Haniastuti T, Kedokteran F, Universitas Jember G, Universitas G, et al. Infiltrasi Neutrofil pada Tikus dengan Periodontitis setelah Pemberian Ekstrak Etanolik Kulit Manggis.
23. Kumar V, Abbas, Fausto N. Pathologic Basis Of Disease. New York: Elsevier; 2006. 48–69 p.
24. Basic Molecular Stem Cell.
25. González-Moles MÁ, Ramos-García P. State of evidence on oral health problems in diabetic patients: A critical review of the literature. J Clin Med. 2021 Nov 1;10(22).
26. Naguib G, Al-Mashat H, Desta T, Graves DT. Diabetes prolongs the inflammatory response to a bacterial stimulus through cytokine dysregulation. Journal of Investigative Dermatology. 2004;123(1):87–92.
27. Vlassara H. The AGE-receptor in the pathogenesis of diabetic complications. Vol. 17, Diabetes/Metabolism Research and Reviews. 2001. p. 436–43.
28. Lalla E, Lamster IB, Stern DM, Schmidt AM. Receptor for Advanced Glycation End Products, Inflammation, and Accelerated Periodontal Disease in Diabetes: Mechanisms and Insights Into Therapeutic Modalities. Ann Periodontol.
29. The Effect of Mesenchymal Stem Cells on The Endothelial Cells [Internet]. Available from: https://www.researchgate.net/publication/329698656
30. Saqib U, Sarkar S, Suk K, Mohammad O, Baig MS, Savai R. Phytochemicals as modulators of M1-M2 macrophages in inflammation [Internet]. Vol. 9, Oncotarget. 2018. Available from: www.oncotarget.com
31. Heine G, Niesner U, Chang HD, Steinmeyer A, Zügel U, Zuberbier T, et al. 1,25-dihydroxyvitamin D3 promotes IL-10 production in human B cells. Eur J Immunol. 2008 Aug;38(8):2210–8.
32. Ahmed SMU, Luo L, Namani A, Wang XJ, Tang X. Nrf2 signaling pathway: Pivotal roles in inflammation. Vol. 1863, Biochimica et Biophysica Acta - Molecular Basis of Disease. Elsevier B.V.; 2017. p. 585–97.
33. Saha S, Buttari B, Panieri E, Profumo E, Saso L. An Overview of Nrf2 Signaling Pathway and Its Role in Inflammation. Vol. 25, Molecules (Basel, Switzerland). NLM (Medline); 2020.
34. Putra A, Ridwan FB, Putridewi AI, Kustiyah AR, Wirastuti K, Sadyah NAC, et al. The role of tnf-α induced mscs on suppressive inflammation by increasing tgf-β and il-10. Open Access Maced J Med Sci. 2018;6(10):1779–83.
35. Keating A. Mesenchymal stromal cells: New directions. Vol. 10, Cell Stem Cell. 2012. p. 709–16.
36. Prockop DJ, Youn Oh J. Mesenchymal stem/stromal cells (MSCs): Role as guardians of inflammation. Vol. 20, Molecular Therapy. Nature Publishing Group; 2012. p. 14–20.
37. Raicevic G, Rouas R, Najar M, Stordeur P, Id Boufker H, Bron D, et al. Inflammation modifies the pattern and the function of Toll-like receptors expressed by human mesenchymal stromal cells. Hum Immunol. 2010;71(3):235–44.
38. Kedokteran Hewan Sri Pantja Madyawati J, Pantja Madyawati S, Agung Budianto Achmad dan. Efektivitas Terapi Rat Bone Marrow Mesenchymal Stem Cell Pada Tikus (Rattus Norvegicus) Model Teratogenik Particulate Matter Terhadap Ekspresi Tnf-α , Bax, Dan Bcl-2 Plasenta Effectiveness of Rat Bone Marrow Mesenchymal Stem Cell Therapy on Rats (Rattus norvegicus) as Teratogenic Models of Particulate Matter on Expression of TNF-α, Bax, and Bcl-2 Placenta.
39. Hindinger C, Bergmann CC, Hinton DR, Phares TW, Parra GI, Hussain S, et al. IFN-γ signalling to astrocytes protects from autoimmune-mediated neurological disability. PLoS One. 2012 Jul 27;7(7).
40. Al-Azzawi B, McGuigan DH, Koivula FNM, Elttayef A, Dale TP, Yang Y, et al. The Secretome of Mesenchymal Stem Cells Prevents Islet Beta Cell Apoptosis via an IL-10-Dependent Mechanism. Open Stem Cell J. 2020 Mar 20;6(1):1–12.
41. Xu A, Zhu W, Li T, Li X, Cheng J, Li C, et al. Interleukin-10 gene transfer into insulin-producing β cells protects against diabetes in non-obese diabetic mice. Mol Med Rep. 2015 Sep 1;12(3):3881–9.
42. YEKTI, Nirmala; ROCHMAH, Yayun Siti; MUJAYANTO, Rochman. Analisa Profil Kadar C-Reactive Protein Pada Status Kesehatan Periodontal Pasien Diabetes Melitus Tipe 2 (Studi di Rumah Sakit Islam Sultan Agung Semarang). **Odonto : Dental Journal**, [S.l.], v. 1, n. 2, p. 19-24, dec. 2014. ISSN 2460-41