RESEARCH ARTICLE OPEN ACCESS

Regulatory Effect of Secretome-Hypoxia Mesenchymal Stem Cells on TNF-α Level in Streptozotocin-induced Rats

Nazzala Luthfin Amellia[1*], Arini Dewi Antari[2], Mohammad Ariq Nazar[3], Sofian Azalia Husain[4], Naufal Ardjivani Arifin[3]

*Correspondence:
lnazzala@yahoo.com

[1]Undergraduate student in the Medical Study Program, Faculty of Medicine, Sultan Agung Islamic University (UNISSULA), Semarang
[2]Department of Pathological Anatomy, Faculty of Medicine, Sultan Agung Islamic University (UNISSULA), Semarang
[3]Stem Cell and Cancer Research (SCCR) Laboratory, Semarang
[3]Graduated student of Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Gadjah Mada (UGM), Yogyakarta

Received 02 January 2023
Accepted 11 January 2023
Available online on 30 January 2023

ABSTRACT

Background: Type 1 Diabetic Mellitus (T1DM) is a well-known autoimmune disease that is characterized by a specific adaptative immunity against β-cell antigens. Mesenchymal stem cells (MSC) have emerged as potential immunomodulators in a paracrine manner via their bioactive soluble molecules that involve inflammation-related diseases, including T1DM. **Objective:** This study aims to investigate the effect of SHMSCs on regulating IL-10 concentrations in STZ-induced rats. **Materials and Methods:** This study uses a post-only control group design and randomized system. To induce T1DM-like rats, an intraperitoneal injection (65 mg/kg BW) of streptozotocin (STZ) was inducted. 20 male Wistar rats were subdivided into the following groups: Sham, STZ, STZ with 0,5 cc SHMSCs (Low-dose), and STZ with 1 cc SHMSCs (High-dose). The animals received an intraperitoneal injection of SHMSCs once a week for up to 4 weeks. On day 28, the animals were terminated and TNF-α concentrations were measured by ELISA. **Results:** After SHMSCs administration, the level of TNF-α in the treated group was increased in either low-dose or high-dose groups compared with the T1DM group. **Conclusion:** Administration of secretome-hypoxia MSCs may regulate IL-10 concentrations in STZ-induced Rats.

Keywords: Secretome, Mesenchymal Stem Cells, TNF-α, T1DM Rat

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder primarily mediated by the adaptive immune response against several islet cell autoantigens, which eventually leads to the destruction of pancreatic β cells and in turn severe insulin deficiency[1,2]. In T1DM, chronic pancreatic beta cell damage results from T-cells attacking these insulin-producing cells leading to high expression of pro-inflammatory cytokines, including IFN-γ, TNF-α, and IL-1β, and controlled by anti-inflammatory cytokines activities[1–3]. The effect of anti-inflammatory cytokines may inhibit TNF-α which is one of the initiated inflammatory cytokines and suppressing this factor may prevent tissue damage[4,5]. Inflammatory mediators thought to play a role in pancreatic cell inflammation are synergistic actions from IFN-γ, TNF-α, and IL-1β[6,7]. Therefore, anti-inflammatory agents such as IL-10, TGFβ1, and IL-35 are needed to prevent inflammatory process development. In recent years, mesenchymal stem cells (MSC) have emerged as potential immunomodulators that act by secreting a large number of bioactive molecules into their medium, namely MSC-Conditioned Medium (CM) or Secretome, that involve inflammation-related diseases[8–11], including T1DM. In autoimmune diabetes,

have been observed in insulitis lesions, the former seems to promote and the latter to regulate beta-cell destruction[3,12,13]. Hence, an imbalance between inflammatory and anti-inflammatory cytokines may play a significant role both in autoimmunity and chronic inflammation that probably leads to complications in T1DM.

Hypoxia MSCs, mesenchymal stem cells under hypoxic culture conditions, may act in a paracrine manner via secreting growth factors, cytokines, and exosomes, which have been extensively applied in the research of various diseases[14–16]. A previous study also revealed the administration of MSCs in T1DM rat models may increase anti-inflammatory and immunosuppressive factors such as interleukin-10 (IL-10), hepatocyte growth factor (HGF), transforming growth factor-β1 (TGFβ1), and prostaglandin E2 (PGE2)[4,17]. However, there is a lack of standardization regarding the administration routes used and the number of applications required to produce a beneficial effect. Furthermore, cell therapy has some limitations, as there is a need for in vitro expansion to have an adequate cell quantity, and this can cause genomic instability and cell senescence[18]. Malignant transformation in situ and immunological rejection can also occur[19,20]. An alternative method would be to use the MSC-derived secretome instead of the stem cells themselves since the secretion produced by these cells is responsible for most of the observed beneficial effect. The Secretome of Hypoxia MSCs (SHMSCs), obtained according to the modified hypoxic culture of MSCs, contained relatively high concentrations of cytokines and growth factors. The potential benefits of SHMSCs methods over traditional cell-based therapies are that cell-free therapies based on SHMSCs may overcome side effects associated with the use of transplanted cells, such as immune rejection[21].

A previous study showed that conditional medium hypoxia of bone marrow-mesenchymal stem cells (BM-MSC) cultured at 2% oxygen increased the release of vascular endothelial growth factor (VEGF), Interleukin 6 (IL-6), and Interleukin 8 (IL-8)[22]. Also, another studies revealed that MSC-derived extracellular vesicles (EVs) significantly reduced the production of IFN-γ, IL-12, and TNF-α, suggesting that MSC-derived EVs suppress cytokines produced by Th1 development[23,24]. Thus, the MSC-derived EV treatment significantly increased the concentrations of IL-10 in type 1 diabetic mice[25]. Recent study also shown that Secretome-MSCs regulating IL-10 plasma level in STZ rats[26]. In another hand, an in vitro study demonstrated that a conditioned medium from bone marrow MSC (CM-BMSCs) was able to reverse cytokine-induced apoptosis in pancreatic islet cell lines by an IL-10-dependent mechanism[3]. Another previous report also demonstrated that Adipose-derived stem cell conditioned medium (ASC-CM) contains anti-apoptotic factors, including VEGF, which could be responsible for the protective effects of islet functions[27].

However, the precise mechanisms of action related to the effect of the SHMSC on regulating TNF-α level in STZ-induced rats are limited. Therefore, this study is designed to investigate the regulatory effect of SHMSCs on TNF-α level in STZ-induced rats.

MATERIAL AND METHODS

Research Design

This post-test-only control group design study was conducted in the Stem Cell and Cancer Research Laboratory, Faculty of Medicine, Universitas Islam Sultan Agung (UNISSULA) from August to November 2022.

Isolation and Culture of MSC

The umbilical cord of a female rat at 19 days of pregnancy was washed in PBS. The umbilical blood vessels were removed, then the umbilicus was cut into 2-5 mm lengths using a sterile scalpel and the sections distributed evenly on T25 flask. The medium used was Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% PBS, 100 IU/ml

penicillin/streptomycin (GIBCO, Invitrogen), then incubated at 37ºC with 5% CO_2. The medium was refreshed every 3 days.

MSCs characterization and differentiation

MSCs were analyzed by flow cytometric analysis in the fourth passage. The cells were subsequently incubated in the dark with fluorescein isothiocyanate (FITC)-conjugated, Allophycocyanin (APC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies, including CD105, CD90, and CD73. FITC- APC- and PE-conjugated isotypes were used as negative controls. Analysis was performed using BD Pharmigen™ (BD Bioscience, Franklin Lakes, NJ, USA) at 4°C for 30 min. The cells were washed twice with 1% BSA/PBS, resuspended in 200 µL 1% BSA/ PBS, and analyzed by a flow cytometer (BD Biosciences, San Jose, CA, USA).

The MSCs were grown in the well plate at densities of $5x10^3$ and $1x10^4$ cells/well, to which was added osteogenic induction medium containing 10 mmol/L β-glycerophosphate, 10^7 mol/L/0.1 µM dexamethasone, 50µmol/L ascorbate-2-phosphate (Sigma-Aldrich, Louis St, MO) and 10 % fetal bovine serum (FBS) in DMEM. After 21 days of induction, the cells were rinsed in PBS and fixed with cold 70% ethanol (v/v) for 1 hour at room temperature, then rinsed three times with distilled water. A volume of 1 ml 2% Alizarin Red solution (w/v) (pH 4.1-4.3) was added and the cells were incubated for 30 minutes at room temperature, then rinsed four times in distilled water. Osteogenic differentiation was observed by Alizarin Red staining to find calcium deposits.

Secretome Hypoxia MSCs Preparation

MSCs cultured in serum-free complete medium were incubated under hypoxia conditions in the hypoxic chamber maintaining a gas mixture composed of 5% O_2, 5% CO_2, and balanced N_2 at 37 °C for 24 h. MSCs conditioned medium was then collected after 24-hour incubation. The collected MSCs conditioned medium was centrifuged at 2000 rpm for 5 minutes to remove cell debris and passed through a 0.22-µm filter membrane (Corning, NY, USA) to remove the remaining cell debris. The secretome hypoxia MSCs (SHMSCs) collection, especially for 10-50 kDa using tangential flow filtration (TFF)[21]. The SHMSCs were kept at 2-8℃ temperature until the treatment. Before being used for treatment, SHMSCs content was analyzed using ELISA.

Streptozotocin-induced rats and treatment with SHMSCs

For the induction of T1DM, twenty male Wistar rats approximately 2 months old were used to carry out the experiments. Animals were kept in boxes (maximum of 5 animals per box), under standard 12-h light/dark cycle conditions, with a temperature of 23C, receiving filtered water and standard commercial food suitable for rodents ad libitum. On day 6 after acclimatization, animals received an intraperitoneal injection (65 mg/kg BW) of streptozotocin (Sigma-Aldrich) diluted in citrate buffer (50 mM, pH 4.5) for 4 consecutive days[28]. On 28 days after the first streptozotocin injection, animals with blood glucose >250 or statistically higher than the control group were subdivided into the following groups: STZ, STZ with 0,5cc SHMSCs (Low dose), and STZ with 1cc SHMSCs (High dose). The animals received an intraperitoneal injection of SHMSCs once a week for up to 4 weeks. The 28 days after treatment, the periorbital-venous blood was collected for the next analysis.

Plasma TNF-α level measurement by ELISA

The blood of rats was harvested via periorbital venous plexus bleeding under general anaesthesia on day 28 and the plasma was collected by centrifugation. The TNF-α concentrations were measured by enzyme-linked immunosorbent assay (ELISA) kits, based on the manufacturer's

instructions (Fine Test, Wuhan, China) and according to a standard curve constructed for each assay. The colorimetric absorbance was recorded at a wavelength of 450 nm.

Statistical Analysis

Statistical analyzes were accomplished with the software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard deviation (SD). The data obtained were collected, compiled, and tested for normality with the *Shapiro-Wilk* test and the homogeneity test with the *Lavene* test. Data analysis used *one-way* ANOVA and continued with the Least Significant Difference (LSD) post hoc test using a *p*-value <0.05.

RESULTS

Morphological, differentiation ability and surface marker expression, of umbilical cord-derived cells provide similar characteristics to MSC

MSC was collected from the cultured umbilical cord MSC of pregnant rats that had reached the 4th passage. The appearance of cell growth from cultures was routinely monitored and recorded using an inverted light microscope (Leica, Germany). The results of the morphology characterization of the MSCs culture have obtained an image of adherent cells with spindle-like cell morphology, abundant cytoplasm, and large nuclei under microscopic observation (Figure 1 A). In this study, we performed in vitro differentiation capacity of MSCs under osteogenic induction. Osteogenic differentiation of MSCs using Alizarin red staining displayed morphologic changes leading into bone-forming cells indicated by the red calcium deposits in the MSC population (Figure 1 B), and accumulation of lipid droplets in the cytoplasm after adipogenic differentiation visualized by Oil Red staining (Figure 1C). In line with the osteogenic ability of MSCs, the results of isolated MSCs were validated using flow cytometry to show that MSCs were able to express several MSCs surface markers. The validation results showed that MSCs were able to express CD29 (91.60%), CD29 (83.20%) and lack express of CD45 (2.20%) and CD31 (5.00%) (Figure 1 D).

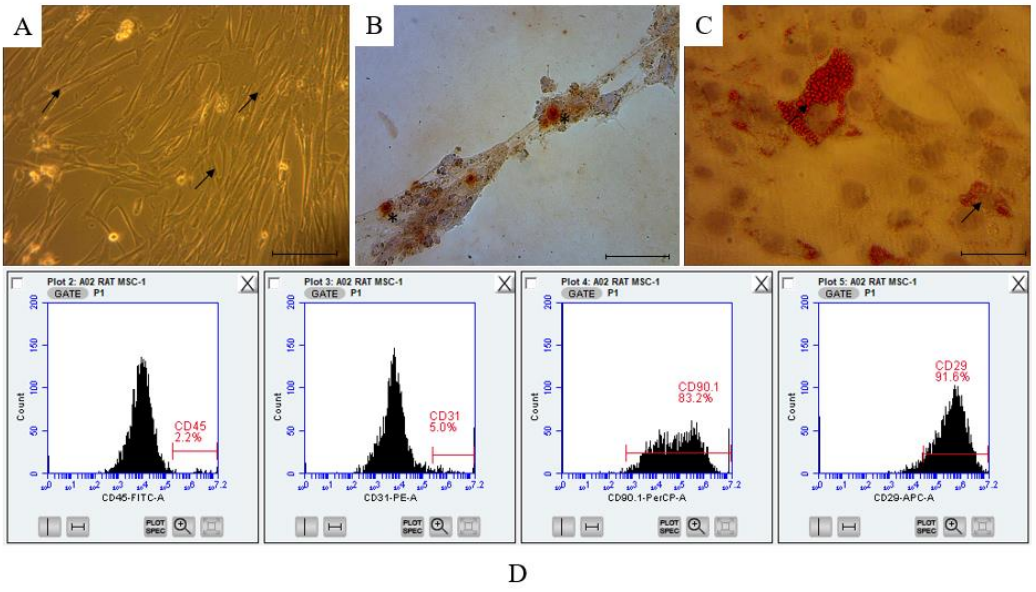

Figure 1. MSCs Characterization Profile. (A) Morphological characteristic of MSCs with 80% confluent showed spindle-like cells (pointed by arrows) at 100x magnification. (B) Osteogenic differentiation capacity using Alizarin Red staining appears in the MSC population at 100x magnification. (C) Oil droplets could be observed in MSCs after differentiation analysis in adipogenic induction medium for 21 days after Oil Red staining. (D) Surface marker phenotyping analysis of the MSCs showing the expressions of CD45, CD31, CD90, and CD29. Scale bars represent 100µm.

Analysis of the Soluble Molecular Content of SHMSCs

The SHMSCs represent all the molecules and factors that are primarily secreted in the extracellular space by the stem cells. MSCs were shown to produce and secrete various growth factors, chemokines, cytokines, soluble proteins, free nucleic acids, lipids, and extracellular vesicles. To identify and analyze the factors secreted by MSCs, 0.2 µm-filtered culture supernatant was collected and analyzed by using ELISA Kit (BIOENZY, Indonesia). Based on the result that showed in Table 1, it was observed that SHMSCs contained exhibited Interleukin-10 (395,10±17,34 pg/mL), PDGF-BB (943,06±44,59 pg/mL), TGF-β1 (302,13±10,23 pg/mL), and VEGF (1012,62±57,07 pg/mL).

Table 1. SHMSCs Content analyzed by ELISA

Content	Mean ± SD (pg/mL)
IL-10	395,10±17,34
PDGF-BB	943,06±44,59
TGF-β1	302,13±10,23
VEGF	1012,62±57,07

SHMSCs regulating TNF-α Concentration on Streptozotocin-induced rats

The concentration of TNF-α was determined by ELISA. As shown in Figure 3, at 28 days after SHMSCs administration, the concentration of TNF-α in the treated groups was increased in low-dose but significantly decreased in high-dose groups compared with the STZ group. Interestingly, TNF-α concentrations in the high-dose group were significantly lower than in the low-dose group. This result suggested that SHMSCs may alleviate TNF-α concentration in a dependent-dose manner.

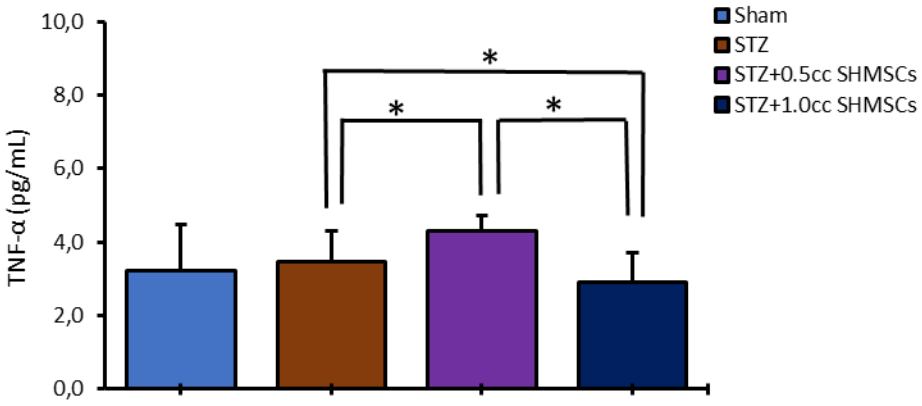

Figure 3. Regulating effect of SHMSCs on TNF-α level (n=5±SD). The level of TNF-α in plasma is decreased in the T1DM group and significantly increased after being treated with SHMSCs, either low dose or high dose. *p<0.05.

DISCUSSION

In this study, the result showed that administration of secretome-hypoxia MSC may increase TNF-α concentration in the blood serum of T1DM rats. Recent data demonstrated that secretome's paracrine signaling can be considered the primary mechanism by which MSCs contribute to healing processes[29–31]. Other investigations evidenced the presence of several molecules in secretome BM-MSC such as VEGF-A, IL-6, IL-8, IL-10, PDGF-AA, HGF, TGF-β1, and VEGF[32,33]. In line with previous reports, the present study also shows that SHMSCs highly contains several bioactive factors such as IL-10, PDGF-BB, TGF-β1, and VEGF. IL-10 plays the role of anti-inflammatory cytokines[34]. PDGF are

key growth factors essential for cell proliferation in the wound healing process[35]. TGF-β1 has the activity to regulate cell proliferation, differentiation, adhesion, and migration[36]. While, VEGF is an angiogenic potent that is responsible for vascular permeability, angiogenesis, endothelial cell growth, and apoptosis inhibition[37].

T1DM is a local autoimmune disorder that involves various factors and immune cells[3,38]. In innate response, T cells play an important role in the induction of T1DM[39,40]. The inflammatory infiltrate in islets consists mostly of T lymphocytes, but the data on the predominance of CD4+ (Th4 or helper) or CD8+ (Th8 or cytotoxic)[41]. CD4+ T cells have been subdivided into different subsets based on their cytokine secretion profiles: Th1, Th2, Th17, and Treg (regulatory T cells)[42]. Cytokines produced by Th1 are mediators in cellular immunity, whereas cytokines produced by Th2 are stimulators of humoral immune responses and antibody production[43]. Th17 cells may play a role in the induction of autoimmune tissue injury[42]. Th1 cells are responsible for aggressive disease, while Th2 cells infiltrate more slowly and do not induce diabetes[40]. The inflammation generated by Th1 cells might attract T cells that would not normally accumulate in the islets[44]. Treg cells are primary controllers of immune responsiveness and peripheral immunological tolerance which control natural killer (NK) cells in an insulitis lesion. These cells also regulate several organ-specific autoimmune diseases such as T1DM.

One strategy for treating T1DM may include the use of specific cytokines, chemokines, and growth factors that might modulate the inflammatory microenvironment. Previous studies revealed that MSC-CM therapy led to a significant decrease in blood glucose, and reconstruction of pancreatic islets in type 1 and type 2 diabetes mice[3,45,46]. The regeneration capacity of MSC-CM or SHMSCs may relate to their anti-inflammatory properties[29,30]. MSCs, especially under hypoxia condition, produce robust highly anti-inflammatory cytokines and trophic factors that modulate the inflammatory microenvironment and prevent prolong inflammatory responses[14]. Several studies also reported that soluble mediators secreted by MSC, especially anti-inflammatory cytokines such as IL-10, IL-4, and TGF- β1, may decrease chronic inflammatory conditions by inhibiting Th1 and Th17 and increasing Th2 and Treg activation[18,47,48]. Also, another case demonstrated that MSC-CM administration may decrease the number of inflammatory cytokines such as IL-17 and IFN-γ[19]. The current study further demonstrated that SHMSCs may provide a protective and immunomodulatory effect on STZ-induced rats by decreasing pro-inflammatory cytokine, mainly TNF-α. However, this study did not analyze other inflammatory microenvironment factors such as TGF- β1, IL-4, IL-10, IL-17, IFN-γ, and immune cells such as Th1, Th2, Th17, and Treg. Thus, this study did not analyze the effect of SHMSCs on pancreatic cell's regeneration in STZ-induced rats.

CONCLUSION

In conclusion, the administration of secretome-hypoxia MSC may control TNF-α concentrations in STZ-induced Rats. This finding might provide valuable information regarding the potential therapy of SHMSCs on T1DM. For clinical therapeutic applications, it is very necessary to conduct additional in vitro as well as in vivo experiments to establish the finding discussed in this report.

FUNDING

None

ACKNOWLEDGEMENTS

The authors are grateful to the researchers, managers, and technicians, who contributed and collaborated in this research. The authors would also like to recognize Stem Cell and Cancer Research (SCCR) Indonesia for their support of investigations.

AUTHORS' CONTRIBUTIONS

NLA: Conceptualization, Methodology, Investigation, Data analysis, Formal analysis, Writing – original draft, Preparation. **ADA**: Supervision, Conceptualization, Review & editing, Project administration, Resources Funding acquisition. **MAN**: Methodology, Investigation, Data interpretation. **SAH**: Methodology, Investigation, Data interpretation. **NAA**: Methodology, Investigation, Data interpretation.

COMPETING INTERESTS

The authors declare that there is no conflict of interest.

REFERENCES

1. Nojehdehi S, Soudi S, Hesampour A, Rasouli S, Soleimani M, Hashemi SM. Immunomodulatory effects of mesenchymal stem cell–derived exosomes on experimental type-1 autoimmune diabetes. *J Cell Biochem*. 2018;119(11):9433-9443. doi:10.1002/JCB.27260
2. Saberzadeh-Ardestani B, Karamzadeh R, Basiri M, et al. Type 1 Diabetes Mellitus: Cellular and MolecularPathophysiology at A Glance. *Cell J*. 2018;20(3):294. doi:10.22074/CELLJ.2018.5513
3. Al-Azzawi B, McGuigan DH, Koivula FNM, et al. The Secretome of Mesenchymal Stem Cells Prevents Islet Beta Cell Apoptosis via an IL-10-Dependent Mechanism. *Open Stem Cell J*. 2020;6(1):1-12. doi:10.2174/1876893802006010001
4. Nugraha A, Putra A. Tumor necrosis factor-α-activated mesenchymal stem cells accelerate wound healing through vascular endothelial growth factor regulation in rats. *Universa Med*. 2018;37(2):135. doi:10.18051/univmed.2018.v37.135-142
5. Xianyuan L, Wei Z, Yaqian D, et al. Anti-renal fibrosis effect of asperulosidic acid via TGF-β1/smad2/smad3 and NF-κB signaling pathways in a rat model of unilateral ureteral obstruction. *Phytomedicine*. 2019;53:274-285. doi:10.1016/j.phymed.2018.09.009
6. Marwaha AK, Tan S, Dutz JP. Targeting the IL-17/IFN-γ axis as a potential new clinical therapy for type 1 diabetes. *Clin Immunol*. 2014;154(1):84-89. doi:10.1016/J.CLIM.2014.06.006
7. Katuchova J, Harvanova D, Spakova T, et al. Mesenchymal Stem Cells in the Treatment of Type 1 Diabetes Mellitus. doi:10.1007/s12022-015-9362-y
8. Giacoppo S, Thangavelu SR, Diomede F, et al. Anti-inflammatory effects of hypoxia-preconditioned human periodontal ligament cell secretome in an experimental model of multiple sclerosis: a key role of IL-37. *FASEB J*. 2017;31(12):5592-5608. doi:10.1096/FJ.201700524R/-/DC1
9. Go YY, Lee CM, Ju WM, Chae SW, Song JJ. Extracellular vesicles (Secretomes) from human trophoblasts promote the regeneration of skin fibroblasts. *Int J Mol Sci*. 2021;22(13). doi:10.3390/ijms22136959
10. Kim ES, Jeon HB, Lim H, et al. Conditioned media from human umbilical cord blood-derived mesenchymal stem cells inhibits melanogenesis by promoting proteasomal degradation of MITF. *PLoS One*. 2015;10(5):1-11. doi:10.1371/journal.pone.0128078
11. Kim R, Song BW, Kim M, et al. Regulation of alternative macrophage activation by MSCs derived hypoxic conditioned medium, via the TGF-β1/Smad3 pathway. *BMB Rep*. 2020;53(11):600. doi:10.5483/BMBREP.2020.53.11.177
12. Alnek K, Kisand K, Heilman K, Peet A, Varik K, Uibo R. Increased blood levels of growth factors, proinflammatory cytokines, and Th17 cytokines in patients with newly diagnosed type 1 diabetes. *PLoS One*. 2015;10(12):1-16. doi:10.1371/journal.pone.0142976
13. Alzamil H. Elevated Serum TNF- α Is Related to Obesity in Type 2 Diabetes Mellitus and Is Associated with Glycemic Control and Insulin Resistance. *J Obes*. 2020;2020. doi:10.1155/2020/5076858
14. Hamra NF, Putra A, Tjipta A, Amalina ND, Nasihun T. Hypoxia mesenchymal stem cells accelerate wound closure improvement by controlling α-smooth muscle actin expression in the full-thickness animal model. *Open Access Maced J Med Sci*. 2021;9:35-41. doi:10.3889/oamjms.2021.5537
15. Yustianingsih V, Sumarawati T, Putra A. Hypoxia enhances self-renewal properties and markers of mesenchymal stem cells. 2019;38(3):164-171. doi:10.18051/UnivMed.2019.v38.164-171
16. Putra A, Ignatius R, Suharto, Taat P, Indra W. Typhonium flagelliforme extract induce apoptosis in breast cancer stem cells by suppressing survivin. *J Cancer Res Ther*. 2018;14(7):1525-1534. doi:10.4103/jcrt.JCRT
17. Agung Putra MSM. *Basic Molecular Stem Cell*. Vol 1.; 2019.
18. Ikhsan R, Putra A, Munir D, et al. Mesenchymal Stem Cells Induce Regulatory T-cell Population in Human SLE. *Bangladesh J Med Sci*. 20AD;19. doi:10.3329/bjms.v19i4.46635
19. Ho CH, Lan CW, Liao CY, Hung SC, Li HY, Sung YJ. Mesenchymal stem cells and their conditioned medium can enhance the repair of uterine defects in a rat model. *J Chinese Med Assoc*. 2018;81(3):268-276.

doi:10.1016/j.jcma.2017.03.013
20. Rossignol J, Boyer C, Thinard R, et al. Mesenchymal stem cells induce a weak immune response in the rat striatum after allo or xenotransplantation. *J Cell Mol Med*. 2009;13(8 B):2547-2558. doi:10.1111/j.1582-4934.2008.00657.x
21. Putra A, Widyatmoko A, Ibrahim S, et al. Case series of the first three severe COVID-19 patients treated with the secretome of hypoxia-mesenchymal stem cells in Indonesia. *F1000Research 2021 10228*. 2021;10:228. doi:10.12688/f1000research.51191.3
22. Jeon SH, Zhu GQ, Bae WJ, et al. Engineered mesenchymal stem cells expressing stromal cell-derived factor-1 improve erectile dysfunction in streptozotocin-induced diabetic rats. *Int J Mol Sci*. 2018;19(12):1-18. doi:10.3390/ijms19123730
23. Wang L-T, Liu K-J, Sytwu H-K, Yen M-L, Yen BL. Advances in mesenchymal stem cell therapy for immune and inflammatory diseases: Use of cell-free products and human pluripotent stem cell-derived mesenchymal stem cells. *Stem Cells Transl Med*. Published online 2021. doi:10.1002/SCTM.21-0021
24. Casado-Díaz A, Quesada-Gómez JM, Dorado G. Extracellular Vesicles Derived From Mesenchymal Stem Cells (MSC) in Regenerative Medicine: Applications in Skin Wound Healing. *Front Bioeng Biotechnol*. 2020;8(March):1-19. doi:10.3389/fbioe.2020.00146
25. Hu J, Liu X, Chi J, et al. Resveratrol Enhances Wound Healing in Type 1 Diabetes Mellitus by Promoting the Expression of Extracellular Vesicle-Carried MicroRNA-129 Derived from Mesenchymal Stem Cells. *J Proteome Res*. 2022;21(2):313-324. doi:10.1021/ACS.JPROTEOME.1C00248/SUPPL_FILE/PR1C00248_SI_001.PDF
26. View of Secretome Hypoxia-Mesenchymal Stem Cells Regulate IL-10 Concentrations in STZ-induced Type 1 Diabetes Rats. Accessed January 27, 2023. https://cbsjournal.com/cbs/article/view/18/13
27. Dias I, Pinheiro D, Ribeiro Silva K, et al. Secretome effect of adipose tissue-derived stem cells cultured two-dimensionally and three-dimensionally in mice with streptozocin induced type 1 diabetes. *Curr Res Pharmacol Drug Discov*. 2021;2:100069. doi:10.1016/J.CRPHAR.2021.100069
28. (PDF) The Effect of Mesenchymal Stem Cells on The Endothelial Cells. Accessed January 27, 2023. https://www.researchgate.net/publication/329698656_The_Effect_of_Mesenchymal_Stem_Cells_on_The_Endothelial_Cells
29. Gunawardena TNA, Rahman MT, Abdullah BJJ, Kasim NHA. Conditioned media derived from mesenchymal stem cell cultures: The next generation for regenerative medicine. *J Tissue Eng Regen Med*. 2019;13(4):569-586. doi:10.1002/TERM.2806
30. Katagiri W, Watanabe J, Toyama N, Osugi M, Sakaguchi K, Hibi H. Clinical Study of Bone Regeneration by Conditioned Medium from Mesenchymal Stem Cells after Maxillary Sinus Floor Elevation. *Implant Dent*. 2017;26(4):607-612. doi:10.1097/ID.0000000000000618
31. Arutyunyan I, Fatkhudinov T, Kananykhina E, et al. Role of VEGF-A in angiogenesis promoted by umbilical cord-derived mesenchymal stromal/stem cells: in vitro study. *Stem Cell Res Ther 2016 71*. 2016;7(1):1-13. doi:10.1186/S13287-016-0305-4
32. Kumar PL, Kandoi S, Misra R, Shanker Verma R. The mesenchymal stem cell secretome: A new paradigm towards cell-free therapeutic mode in regenerative medicine. Published online 2019. doi:10.1016/j.cytogfr.2019.04.002
33. Chang C, Yan J, Yao Z, Zhang C, Li X, Mao HQ. Effects of Mesenchymal Stem Cell-Derived Paracrine Signals and Their Delivery Strategies. *Adv Healthc Mater*. 2021;10(7):2001689. doi:10.1002/ADHM.202001689
34. Restimulia L, Ilyas S, Munir D, et al. The CD4+CD25+FoxP3+ Regulatory T Cells Regulated by MSCs Suppress Plasma Cells in a Mouse Model of Allergic Rhinitis. *Med Arch*. 2021;75(4):256. doi:10.5455/MEDARH.2021.75.256-261
35. Hwang B, Song JH, Park SL, Kim JT, Kim WJ, Moon SK. Carnosine impedes pdgf-stimulated proliferation and migration of vascular smooth muscle cells in vitro and sprout outgrowth ex vivo. *Nutrients*. 2020;12(9):1-18. doi:10.3390/nu12092697
36. Lv S, Liu G, Sun A, et al. Mesenchymal stem cells ameliorate diabetic glomerular fibrosis in vivo and in vitro by inhibiting TGF-β signalling via secretion of bone morphogenetic protein 7. *Diabetes Vasc Dis Res*. 2014;11(4):251-261. doi:10.1177/1479164114531300
37. Chen L, Xu Y, Zhao J, et al. Conditioned medium from hypoxic bone marrow-derived mesenchymal stem cells enhances wound healing in mice. *PLoS One*. 2014;9(4). doi:10.1371/journal.pone.0096161
38. Astari L, Cahyono H, Widjajanto E. Correlation of Interleukin-10, Superoxide Dismutase (SOD), and Malondialdehyde (MDA) Levels with HbA1c in Pediatric Type 1 Diabetes Mellitus. *J Trop Life Sci*. 2017;7(3):286-292. doi:10.11594/jtls.07.03.15
39. Yang SH, Park MJ, Yoon IH, et al. Soluble mediators from mesenchymal stem cells suppress T cell proliferation by inducing IL-10. *Exp Mol Med*. 2009;41(5):315-324. doi:10.3858/emm.2009.41.5.035
40. Swinkels M, Rijkers M, Voorberg J, Vidarsson G, Leebeek FWG, Jansen AJG. Emerging concepts in immune thrombocytopenia. *Front Immunol*. 2018;9(APR):880. doi:10.3389/FIMMU.2018.00880/BIBTEX
41. Szablewski L. Role of immune system in type 1 diabetes mellitus pathogenesis. *Int Immunopharmacol*. 2014;22(1):182-191. doi:10.1016/J.INTIMP.2014.06.033
42. Barygina V, Becatti M, Lotti T, Moretti S, Taddei N, Fiorillo C. Treatment with low-dose cytokines reduces

oxidative-mediated injury in perilesional keratinocytes from vitiligo skin. *J Dermatol Sci*. 2015;79(2):163-170. doi:10.1016/J.JDERMSCI.2015.05.003

43. Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-β: The role of T regulatory cells. *Immunology*. 2006;117(4):433-442. doi:10.1111/j.1365-2567.2006.02321.x
44. Ekasaputra VM, Mughni A, Putra A, et al. Combination of Sleeve Gastrectomy and Mesenchymal Stem Cells Improves Insulin Resistance by Modulating Pro-inflammatory Milieu in Obese Type 2 Diabetic Rat Model. *Open Access Maced J Med Sci*. 2022;10(B):158-163. doi:10.3889/oamjms.2022.8211
45. Jackson WM, Nesti LJ, Tuan RS. Concise Review: Clinical Translation of Wound Healing Therapies Based on Mesenchymal Stem Cells. *Stem Cells Transl Med*. 2012;1(1):44-50. doi:10.5966/SCTM.2011-0024
46. Kouhkheil R, Fridoni M, Piryaei A, et al. The effect of combined pulsed wave low-level laser therapy and mesenchymal stem cell-conditioned medium on the healing of an infected wound with methicillin-resistant Staphylococcal aureus in diabetic rats. *J Cell Biochem*. 2018;119(7):5788-5797. doi:10.1002/JCB.26759
47. Darlan DM, Munir D, Putra A, Jusuf NK. MSCs-released TGFβ1 generate CD4+CD25+Foxp3+ in T-reg cells of human SLE PBMC. *J Formos Med Assoc*. 2021;120(1):602-608. doi:10.1016/J.JFMA.2020.06.028
48. Kustiyah AR, Putra A, Nasihun T, Ramasamy R. The Normal Ratio of Th17 and Th1 Post-mesenchymal Stem Cells Coculture with PBMCs of Systemic Lupus Erythematosus Patients. *Open Access Maced J Med Sci*. 2021;9(A):169-176. doi:10.3889/oamjms.2021.5604