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# Secretome Hypoxia-Mesenchymal Stem Cells Regulate IL-10 Concentrations in STZ-induced Type 1 Diabetes Rats

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## 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 S-HMSC on regulating IL-10 concentrations in STZ-induced T1DM rats.

**Materials and Methods:** This study uses a post-only control group design and randomized system. To induce T1DM rats, an intraperitoneal injection (65 mg/kg BW) of streptozotocin (STZ) was inducted. 20 Wistar rats were subdivided into the following groups: T1DM, DM with 0,5cc S-HMSC (Low-dose), and DM with 1cc S-HMSC (High-dose). The animals received an intraperitoneal injection of S-HMSC once a week for up to 4 weeks. On day 28, the animals were terminated for IL-10 concentration measurement by ELISA.

**Results:** After S-HMSC administration, the concentration of IL-10 in the treated group was increased in either low-dose or high-dose groups compared with the T1DM group.

**Conclusion:** Administration of S-HMSC may regulate IL-10 concentrations in STZ-induced Type 1 Diabetes Rats.

**Keywords:** Mesenchymal Stem Cell-Conditioned Medium, Secretome, Interleukin-10, T1DM

## 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[1–3]. 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 activity[3–5]. 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[6–8]. Inflammatory mediators thought to play a role in pancreatic cell inflammation are synergistic actions from IFN-γ, TNF-α, and IL-1β[4,9]. 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 (MSCs) 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, including T1DM[10–13]. In autoimmune diabetes,

although both T helper type 1 (IFN-γ, IL-2) and type 2 (e.g., IL-4, IL-10)-associated cytokines have been observed in insulitis lesions, the former seems to promote and the latter to regulate beta-cell destruction[5,14,15]. 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, MSCs 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[16–18]. 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-β (TGFβ1), and prostaglandin E2 (PGE2)[19]. 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[20]. Malignant transformation in situ and immunological rejection can also occur[21]. 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[10,20]. The Secretome of Hypoxia MSC (S-HMSC), obtained according to the modified hypoxic culture of MSC, contained relatively high concentrations of cytokines and growth factors[22,23]. The potential benefits of S-HMSC methods over traditional cell-based therapies are that cell-free therapies based on S-HMSC may overcome side effects associated with the use of transplanted cells, such as immune rejection[11,24].

A previous study showed that conditional medium hypoxia of bone marrow-mesenchymal stem cells (BM-MCS) cultured at 2% oxygen increased the release of vascular endothelial growth factor (VEGF), Interleukin 6 (IL-6), and Interleukin 8 (IL-8)[25]. Also, another study revealed that MSC-derived extracellular vesicles (EVs) significantly reduced the production of IFN-γ, IL-12, and TNF-α, suggesting that MSC-derived extracellular vesicles MSC-derived EVs suppress cytokines produced by Th1 development[24,26]. In addition, MSC-derived EVs significantly suppressed the production of IL-6, a key cytokine for the lineage commitment of pathogenic IL-17-producing Th17 cells[4]. Thus, the MSC-derived EV treatment significantly increased the concentrations of IL-10 in type 1 diabetic mice[15]. In another hand, an in vitro study demonstrated that a conditioned medium from bone marrow MSC was able to reverse cytokine-induced apoptosis in pancreatic islet cell lines by an IL-10-dependent mechanism[4]. Another previous report also demonstrated that Adipose-derived Stem Cells (ASC) conditioned medium contains anti-apoptotic factors, including VEGF, which could be responsible for the protective effects of islet functions[27]. However, the precise mechanism of action related to the effect of the S-HMSC on regulating IL-10 concentrations in STZ-induced T1DM rats has not yet been studied. Therefore, this study is designed to investigate the effect of S-HMSC on regulating IL-10 concentrations in STZ-induced T1DM 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 March to July 2022. The animal research was approved by the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Islam Sultan Agung with number 251/III/2022/Komisi Bioetik.

### *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 were distributed evenly on a 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 37$^{o}$C with 5% $CO_2$. The medium was refreshed every 3 days.

### *MSC characterization and differentiation*

MSCs were analyzed by flow cytometric analysis at 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 Pharmingen$^{TM}$ (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 twice- 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 twice- distilled water. Osteogenic differentiation was observed by Alizarin Red staining to find calcium deposits.

### *Secretome Hypoxia-MSC Preparation*

MSCs cultured in serum-free complete medium were incubated under hypoxia conditions in the hypoxic chamber maintaining a gas mixture composed of 5% $O_2$ 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 S-HMSC collection, especially for 10-50 kDa using tangential flow filtration (TFF). The S-HMSCs were kept at 2-8℃ temperature until the treatment. Before being used for treatment, S-HMSC content was analyzed using ELISA

### *Streptozotocin-induced T1DM rats and treatment with S-HMSC*

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. 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: T1DM, DM with 0,5cc S-HMSC (Low dose), and DM with 1cc S-HMSC (High dose). The animals received an intraperitoneal injection of S-HMSC once a week for up to 4 weeks. The 28 days after treatment, the periorbital-venous blood was collected for the next analysis.

### *IL-10 concentration measurement by ELISA*

The blood of rats was harvested via periorbital venous plexus bleeding under general anesthesia on day 28 after the first S-HMSC injection and the serum was collected by centrifugation. The IL-10 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, and Surface Marker Expression of UC-Derived Cells Demonstrate MSC-Like Characteristics*

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 MSC 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 MSC 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). Furthermore, the culture cells were also analyzed by Oil red O staining to investigate the ability to differentiate into adipose cells. Based on the results, the culture cells showed an accumulation of lipid droplets in the cytoplasm after adipogenic differentiation (visualized by Oil Red staining) (Figure 1 C). In line with the differentiation ability of MSCs, the results of isolated MSC cells were validated using flow cytometry to show that MSCs were able to express several MSC surface markers. The validation results showed that MSCs were able to express CD90 (82.0%), CD29 (95.9%) and lack express of CD45 (2.6%) and CD31 (6.4%) (Figure 1 C).

**Table 1.** S-HMSC Content analyzed by ELISA

| Content | Mean ± SD (pg/mL) |
|---|---|
| IL-10 | 415,02±7,14 |
| PDGF-BB | 1043,06±24,49 |
| TGF-β1 | 282,83±6,28 |
| VEGF | 1228,86±27,71 |

### *S-HMSC Profiling*

The secretome represents 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 UC-MSCs, filtered culture supernatant was collected and analyzed

by using ELISA Kit. Based on the result that showed in Table 1, it was observed that S-HMSC containing exhibited Interleukin-10 (415,02±7,14 pg/mL), PDGF-BB (1043,06±24,49 pg/mL), TGF-β1 (282,83±6,28 pg/mL), and VEGF (1228,86±27,71 pg/mL).

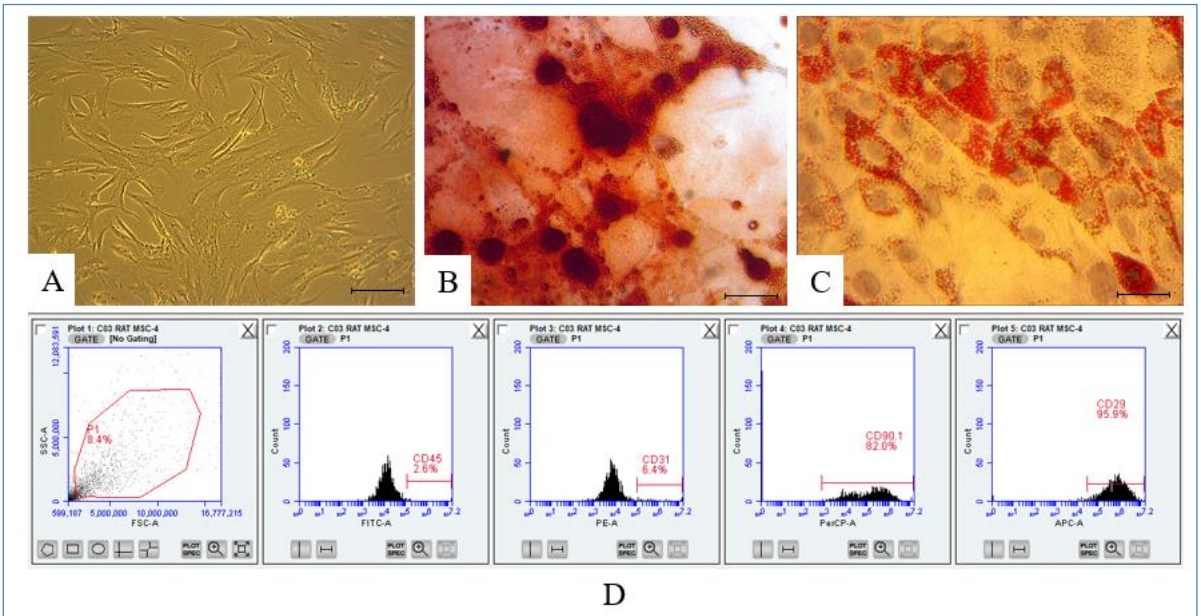


**Figure 1.** Isolation and Characterization of MSC. (A) Isolated MSCs with 80% confluent showed spindle-like cells (pointed by arrows) at 100x magnification. (B) Osteogenic differentiation using Alizarin Red staining appears in the MSC population at 100x magnification. (C) adipose deposition also appears in red after Oil red O staining. (D) Flow cytometry analysis of the expression of CD45, CD31, CD90, and CD29. Bar scale : 100 µM.

### *S-HMSC regulating IL-10 Concentration on Streptozotocin-induced T1DM rats*

The concentration of IL-10 was determined by ELISA. As shown in Figure 3, at 28 days after S-HMSC administration, the concentration of IL-10 in the treated groups was increased in either low-dose or high-dose groups compared with the T1DM group. Interestingly, IL-10 concentrations in the high-dose group were significantly higher than in the low-dose group. This result suggested that S-HMSC may alleviate IL-10 concentration in a dependent-dose manner.

## DISCUSSION

In this study, the result showed that administration of secretome-hypoxia MSC may increase IL-10 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[10,28,29]. 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[30,31]. In line with previous reports, the present study, also shows that S-HMSC highly contains several bioactive factors such as IL-10, PDGF-BB, TGF-β1, and VEGF. IL-10 plays the role of anti-inflammatory cytokines[32]. PDGF are key growth factors essential for cell proliferation in the wound healing process[33]. TGF-β1 has the activity to regulate cell proliferation, differentiation, adhesion, and migration[34]. While, VEGF is an angiogenic potent that is responsible for vascular permeability, angiogenesis, endothelial cell growth, and apoptosis inhibition[35].

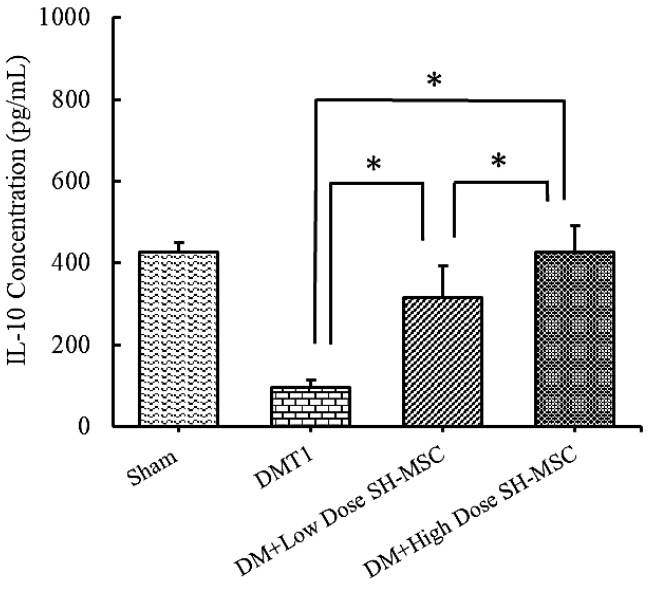


**Figure 3.** Effect of S-HMSC on IL-10 concentration (n=5±SD). The concentration of IL-10 was decreased in the T1DM group and significantly increased after being treated with S-HMSC, either low dose or high dose. *p<0.05.

T1DM is a local autoimmune disorder that involves various factors and immune cells[4,9]. In innate response, T cells play an important role in the induction of T1DM[1,36]. 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)[37]. CD4+ T cells have been subdivided into different subsets based on their cytokine secretion profiles: Th1, Th2, Th17, and Treg (regulatory T cells)[38]. Cytokines produced by Th1 are mediators in cellular immunity, whereas cytokines produced by Th2 are stimulators of humoral immune responses and antibody production[39]. Th17 cells may play a role in the induction of autoimmune tissue injury[38]. Th1 cells are responsible for aggressive disease, while Th2 cells infiltrate more slowly and do not induce diabetes[1]. The inflammation generated by Th1 cells might attract T cells that would not normally accumulate in the islets[40]. Treg cells are primary controllers of immune responsiveness and peripheral immunological tolerance which control natural killer (NK) cells in an insulitis lesion[41]. These cells also regulate several organ-specific autoimmune diseases such as T1DM[37].

One strategy for treating T1DM is 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[4,12,13]. The regeneration capacity of MSC-CM or S-HMSC may relate to their anti-inflammatory properties. MSCs produce robust highly anti-inflammatory cytokines and trophic factors that modulate the inflammatory microenvironment and prevent prolong inflammatory responses[42–44]. 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[45–47]. Also, another case demonstrated that MSC-CM administration may decrease the number of inflammatory cytokines such as IL-17 and IFN-γ[4]. The current study further demonstrated that S-HMSC may provide a protective and immunomodulatory effect on STZ-induce T1DM rats by increasing anti-inflammatory cytokine, mainly IL-10. However, this study did not analyze other inflammatory microenvironment factors such as TGF- β1, IL-4, IL-17, IFN-γ, and immune cells such as Th1, Th2, Th17, and Treg. Thus, this study did not analyze the effect of S-HMSC pancreatic cell regeneration in STZ-induced T1DM.

## CONCLUSION

In summary, the administration of S-HMSC may regulate IL-10 concentrations in STZ-induced Type 1 Diabetes Rats. This finding might provide valuable information regarding the potential therapy of S-HMSC 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.

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**AUTHORS' CONTRIBUTIONS**

IPS: Conceptualization, Methodology, Investigation, Data analysis, Formal analysis, Writing – original draft, Preparation. ADA: Methodology, Investigation, Writing – review & editing. AP: Supervision, Conceptualization, Review & editing, Project administration, Resources Funding acquisition. RCSI: Methodology, Investigation, Data interpretation. FEH: Methodology, Investigation, Data interpretation.

**COMPETING INTERESTS**

The authors declare that there was no conflict of interest.

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