ORIGINAL ARTICLE

Hypoxic secretome mesenchymal stem cells inhibiting interleukin-6 expression prevent oxidative stress in type 1 diabetes mellitus

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ABSTRACT

Aim Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by the chronic inflammation of the pancreatic islets of Langerhans. Hyperglycaemia leads to suppressed antioxidant enzyme and increased inflammation in the pancreatic cell, resulting in pancreatic cell death. Hypoxic secretome mesenchymal stem cells (HS-MSCs) are soluble molecules secreted by MSCS that have the antiinflammation ability by secreting various cytokines including IL-10 and TGF-β which potent as a promising therapeutic modality for T1DM. This study aims to investigate the role of HS-MSCs in regulating superoxide dismutase (SOD) and caspase-3 gene expression in T1DM model.

Methods Twenty male Wistar rats (6 to 8 weeks old) were randomly divided into four groups (sham, control, HS-MSCs 0.5 mL and HS-MSCs 1 mL intraperitoneal treatment group). Streptozotocin (STZ) 60mg/kgBB was conducted once on day 1, HS-MSCs 0.5mL (T1) and HS-MSCs 1 mL (T2) were administrated intraperitoneally on day 7, 14, and 21 after STZ administration. The rats were sacrificed on day 28; the gene expression of SOD and IL-6 was analysed by qRT-PCR.

Results This study showed that the ratio of SOD significantly increased in HS-MSCs treatment associated with suppression of IL-6 gene expression.

Conclusion HS-MSCs administration suppresses oxidative stress and inflammation by up regulating SOD and inhibiting IL-6 to control T1DM.

Key words: hypoxic, IL-6, secretome mesenchymal stem cells, SOD, type 1 diabetes mellitus

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INTRODUCTION

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by β-Langerhans cell destruction, associated with cellular infiltration and inflammatory response in the islets of Langerhans (1,2). Cytokines play an important role in the development and activation of immune cells and involved in the inflammatory response associated with insulin-resistant states associated with permanent damage of pancreatic β cells (3,4). Hyperglycaemic condition in T1DM leads to increased formation of reactive oxygen species (ROS) and inflammation (5). Previous studies reported that antioxidant enzymes such as superoxide dismutase (SOD) showed to protect cells from oxidative stress by eliminating ROS through conversion of anion to hydrogen peroxide (6,7). Increased ROS levels are also associated with adaptive immune cell activation through the release of proinflammatory cytokines such as interleukin-6 (IL-6), interleukin-1 (IL-1), and tumour necrosis factor alpha (TNF-α) involved in insulin resistance T1DM (8). The effectiveness of regulating inflammatory cytokine expression and suppressing ROS levels could enhance T1DM pathogenicity (9). In addition, the release of various proinflammatory cytokines by macrophages and CD4+ T cells activates nuclear factor kappa-light-chain enhancer of activated B cells (NF-kβ), inhibiting cytochrome c oxidase, NADH dehydrogenase, and the cycle enzyme aconitase (TCA), resulting in pancreatic β cell death (10,11). The proinflammatory cytokine IL-6 also stimulates the immune response during inflammation by forming the IL-6R/gp130/STAT3 complex for differentiation of T helper 17 (Th17) cells, inhibiting the development of regulatory (Treg) and resistance of effector T (Teff) leading to hyperresponsiveness of the immune system (12). Thus, preventing proinflammatory cytokine secretion and oxidative stress might be the crucial factor to treating T1DM.

In the first line therapy of T1DM, insulin decreases blood glucose level (13). However, long-term therapy causes several negative side effects including insulin resistance and intolerance (14). Therefore, a novel therapeutic strategy of T1DM is urgently needed. Recently, hypoxic secretome mesenchymal stem cells (HS-MSCs) have received increased attention for their immunomodulatory and anti-inflammatory properties in controlling T1DM, indicating HS-MSCs as a promising approach to control T1DM (15,16). The anti-inflammatory properties of HS-MSCs are supported by various specific mechanisms, such as the activation and generation of Treg cells followed by the release of several anti-inflammatory cytokines, including interleukin-10 (IL-10) (17,18). HS-MSCs also generated several growth factors such as transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF*),* and hepatocyte growth factor (HGF) (19,20) that increased the proliferation of pancreatic islets. On the other hand, HS-MSCs stimulated proliferation and angiogenesis of pancreatic cells through Akt and Erk phosphorylation (21). IL-10 released by HS-MSCs reduced the production of inflammatory cytokines in T1DM leading to lower blood sugar levels (22). All those mechanisms suggested that HS-MSCs have anti-inflammatory and immunomodulatory properties to control T1DM (23). Therefore, exploring the role of HS-MSCs in regulating superoxide dismutase (SOD) and IL-6 is crucial to the future management of T1DM. However, the mechanism of HS-MSCs in T1DM especially regulating SOD and IL-6 in pancreatic cells is still unclear.

The aim of this study was to analyse the role of HS-MSCs in regulating SOD and IL-6 in T1DM mouse model.

MATERIAL AND METHODS

Material and study design

This post-test only control group study design was conducted in the Stem Cell and Cancer Research (SCCR) Laboratory, Faculty of Medicine, Sultan Agung Islamic University, Semarang, Indonesia, from April to June 2022. The study was approved by the Ethic Committee of Sultan Agung Islamic University (No. 56/ II/2022/Komisi Bioetik).

Methods

MSCs culture and isolation. Rat MSCs were isolated from a 19-day pregnant female rat. Briefly, donor rats were anesthetized, and the abdomens were dissected out. Under sterile conditions, the umbilical cord (UC) was collected and

washed in phosphate-buffered saline (PBS). The UC artery and vein were removed, then the UC was cut into lengths of 2-5 mm using a sterile scalpel. The sections were then distributed evenly in T25 flasks using Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% PBS, 100 IU/ml penicillin/streptomycin (GIBCO, Invitrogen), and incubated at 37 °C with 5% CO_2. The medium was renewed every 3 days, and the cells were passaged after reaching 80% confluence. UC-MSCs at passages 4–6 were employed for the following experiments (17,23).

Osteogenic and adipogenic differentiation assay of MSCs. The MSCs were grown in a 24 well plate (1.5 x 10^4 cells/well) with a standard medium containing DMEM (Sigma-Aldrich, Louis St, MO), enriched with 10% FBS (Gibco Invitrogen, NY, USA) and 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) (GibcoInvitrogen, NY, USA) at 37 °C, 5% CO_2 and ≥ 95% humidity. After 80% confluent, the osteogenic and adipogenic protocol was initiated. For osteogenic differentiation, the standard medium was aspirated and replaced with an osteogenic differentiation medium containing Human MesenCult Osteogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore), augmented with 20% Human MesenCult Osteogenic Differentiation 5X Supplement (Stem Cell Technologies, Singapore) and 1% L-Glutamine (Gibco Invitrogen, NY, USA). The differentiation medium was renewed every 3 days. After bone matrix formation occurred, the osteogenic differentiation was visualized by staining with 1 ml of 2% alizarin red solution. For adipogenic differentiation, the growth medium was switched to Human MesenCult Adipogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore). The medium was changed every other day and at day 35 the cultures were stained with Oil Red O and observed under microscope.

Characterization of MSCs surface marker. MSCs were analysed for specific surface markers expression by flow cytometry. Briefly, the cultured cells were incubated in the dark with primary antibodies mouse anti-human CD29, mouse anti-human CD90, and mouse anti-human Lin negative (CD45/CD31) followed by secondary conjugated antibody. MSCs were stained with a specific antibody for 30 minutes at 4 °C, examined with a BD Accuri C6 Plus flow cytometer and analysed with BD Accuri C6 Plus software (BD Biosciences, San Jose, CA, USA) (23,24).

Hypoxic secretome MSCs (HS-MSCs) isolation and characterization. Once MSCs reached 70% confluence in 75 cm^2 flask containing complete medium, they were washed twice with 5 mL PBS and incubated in DMEM (Gibco, USA), and then placed in a hypoxic chamber (Anaerobic Environment; ThermoForma, Waltham, MA, USA) containing 15 mL DMEM for 12 h. The airtight humidified hypoxic chamber was maintained at 37 °C and continuously supplied with 5% CO_2,10% H2 , and 85% N_2. The oxygen level in the chamber was ~0.5%. After incubation, hypoxic secretom was collected and centrifuged twice at 1,500 rpm for 3 min at 4 °C to eliminate debris and dead cells. The supernatant was filtered under tangential flow filtration system (TFF) (Formulatrix, USA) with sterile hollow fibre polyether sulfone membranes with 30-50kDa molecular weight cut-off pores to remove huge biomolecules. The filtrate results called HS-MSCs were analysed under ELISA assay including TGF-β (Invitrogen, catalogue #BMS249-4) and IL-10 (Invitrogen, #BMS614) (25–27).

Animals' studies. The sample size was determined using Resource Equation formula: E=N-T (T=number of treatments; N=number of repetition), E=10-20. Twenty male Wistar rats 6 to 8 weeks old were purchased from local breeders (Semarang, Indonesia). They were raised in a controlled environment, with a regular 12-hour light-dark cycle, and randomly assigned to four groups: sham, control T1DM, T1DM with HS-MSCs 0.5 mL intraperitoneal treatment (T1), and TIDM with HS-MSCs 1 mL intraperitoneal treatment (T2). Rats fasted for 12 h and were rendered T1DM by a single intraperitoneal (IP) injection of freshly prepared streptozotocin (STZ) (Sigma-Aldrich, St. Louis, Mo, USA) at a dose of 60 mg/kg of body weight in sodium citrate buffer 0.05 M, pH 4.5. To avoid hypoglycaemia and mortality, rats were permitted to drink 5% glucose solution ad libitum overnight after STZ injection.

Blood samples were taken from the tail vein 72 h after STZ administration, and the fasting blood glucose concentration was determined by means of one touch ultra-glucometer and compa-

tible blood glucose strips. Rats exhibiting FBG ≥250 mg/dl were considered T1DM and were selected for the experiments. Control rats were injected with normal saline solution parallel to the treated groups throughout the course of the study. On day 7, 14, and 21 groups T1 and T2 received a 0.5 mL and 1 mL doses of HS-MSCs injected via intra peritoneal (IP), respectively.

All the mice that were used in this study were handled according to a protocol, which was approved by the Ethical Committee Universitas Islam Sultan Agung Semarang (56/II/2022/Komisi Bioetik).

Collection of sera and tissue samples. After 7 days of HS-MSCs injection (day 28), animals were fasted overnight and afterwards anesthetized under light chloroform anaesthesia. Blood samples were collected by orbital vein and then a glucose level was determined. Pancreatic tissue samples were also taken from rats of all groups for histological and gene expression studies (28).

SOD and IL-6 gene expression by qRT-PCR. Total RNA from rat pancreatic tissue was extracted with TRIzol (Invitrogen, Shanghai, China) according to the manufacturer's protocol. Briefly, first-stranded cDNA was synthesized with 1 g of total RNA using Super-Script II (Invitrogen, Massachusetts, USA). SYBR No ROX Green I dye (SMOBIO Technology Inc, Hsinchu, Taiwan) was used for reverse-transcription in a real-time PCR instrument (PCR max Eco 48), and mRNA levels of the PDGF and GPx genes were measured using the respective primers (Table 1). The following thermocycler conditions were used: initial step at 95 °C for 10 minutes, followed by 50 cycles at 95 °C for 15 seconds, and 60 °C for 1 minute. The gene expression was recorded as the Cycles threshold (Ct). Data were obtained using Eco Software v5.0 (Illumina Inc, San Diego, CA, USA). All reactions were performed in triplicate, and data analysis used the $2^{-\Delta\Delta}$ Ct method (Livak method) (25).

Table 1. Primer sequences for GPx, PDGF, and GAPDH genes

Gene symbol	Primer sequence 5'→ 3'
SOD	Forward SOD 5'- ATGTGTCCATTGAAGATCGTGTGA-3' Reverse SOD 5'- GCTTCCAGCATTTCCAGTCTTTGTA -3'
IL-6	Forward IL-6 5'- CTTGGGACTGATGCTGGTGACA-3' Reverse IL-6 5'- GCCTCCGACTTGTGAAGTGGTA-3'
GAPDH	Forward GAPDH 5'- GTCTCCTCTGACTTCAACAGCG-3' Reverse GAPDH 5'- ACCACCCTGTTGCTGTAGCCAA-3'

Statistical analysis

Data were presented as the mean ± SD. The statistical significance of differences between the groups was examined using ANOVA with post-hoc under the least significant difference (LSD) analysis. The $p < 0.05$ were considered significant.

RESULTS

MSCs were analysed based on their plastic adherent capability under standard culture. After passage four, MSCs showed adhere cells with typical monolayers of spindle-shaped fibroblast-like cells (Figure 1A). To confirm the in vitro differentiation potential of MSCs, osteogenic and adipogenic differentiation media were used to evaluate that these MSCs can differentiate into osteogenic and adipogenic cells. Red colour at osteogenic differentiation assay as calcium deposition was found, indicating that the MSCs differentiate to osteogenic (Figure 1B), and adipogenic differentiation was indicated by accumulation of neutral lipid vacuoles that stained with Oil Red O (Figure 1C). Immunophenotyping UC-MSCs based on flow cytometric surface marker analysis indicated that MSCs were positive for CD90 (98.50%) and CD 29 (95.30%), negative for CD45 (1.60%) and CD31 (0.00%) (Figure 1D).

To induce cytokine and growth factor MSCs, the MSCs was cultured under hypoxic condition with 5% O_2 for 12 h. Under ELISA analysis, we found higher paracrine factors in HS-MSCs compared to normoxic secretome MSCs, including TGF-ß and IL-10 (Figure 2). The IL-10 level increased up to 467±21.30 pg/mL on HS-MSCs compared to

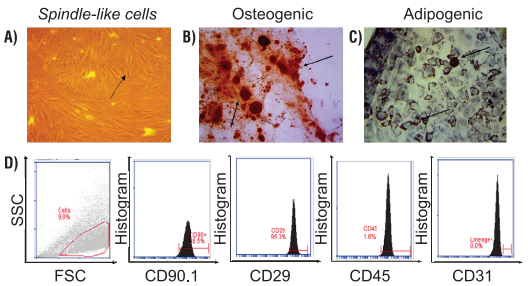

Figure 1. Characterization and validation of mesenchymal stem cells (MSCs). A) Morphological MSCs. The cells appeared as homogeneous spindle-shaped; B) calcium deposition under osteogenic differentiation assay following Alizarin Red staining; C) accumulation of neutral lipid vacuoles stained with Oil Red O; D) the phenotype of MSCs: CD90, CD29, CD45, and CD31

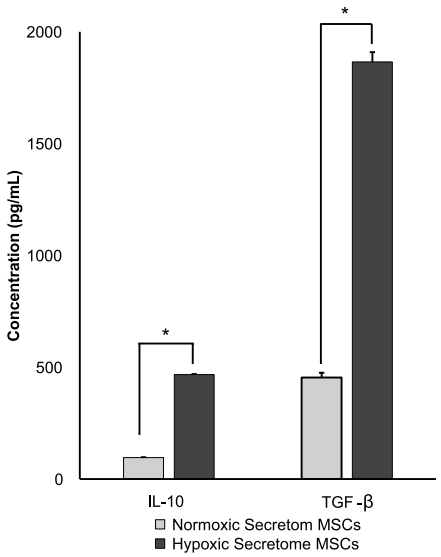

Figure 2. Levels of cytokine and growth factor in hypoxic secretome mesenchymal stem cells (HS-MSCs): all paracrine factors of HS-MSCs were higher than normoxic secretom MSCs
*p< 0.05

normoxic secretome MSCs (96±1.70 pg/ml). Interestingly, TGF-ß level showed the same phenomenon in HS-MSCs, the hypoxic condition strongly induced TGF-ß level (1865±43.50 pg/mL) 3-fold higher than normoxic condition (454±2.89 pg/ml). This clearly indicated that the HS-MSCs significantly contained more of cytokine and growth factor than normoxic secretome MSCs.

To evaluate the effects of administration of HS-MSCs toward inflammation condition on type 1 diabetic mellitus rat model, we determined using qRT-PCR from pancreatic tissue that was applied showing that HS-MSCs significantly decreased IL-6 gene expression in doses-dependent manner (Figure 3A). In the control group it significantly induced IL-6 gene expression 6.78±0.67- fold greater than in the healthy group. Interestingly, the T1 and T2 of HS-MSCs groups significantly suppressing the IL-6 gene expression were 1.88±0.88 and 1.23±0.17- fold, respectively, greater than in the healthy group.

HS-MScs significantly increased SOD gene expression in doses-dependent manner (Figure 3B). In the control group, SOD gene expression was depleted until 0.07±0.03-fold greater than in the healthy group. Interestingly, the T1 and T2 groups significantly increasing the SOD gene expression were 1.49±0.7 and 5.88±1.55- fold, respectively, greater than in the healthy group.

DISCUSSION

Type 1 diabetes mellitus, an autoimmune disease, is characterized by the chronic inflammation of the pancreatic islets of Langerhans (29). T1DM within the ß-Langerhans inflammation produces ROS and proinflammatory cytokines, resulting in a permanent damage to pancreatic β cells (30). In this study, HS-MSCs had significantly increased SOD gene expression in T1DM rats model. This result was in accordance with a previous study suggesting that SOD activity in DM was inhibited (31). Previous studies also reported that

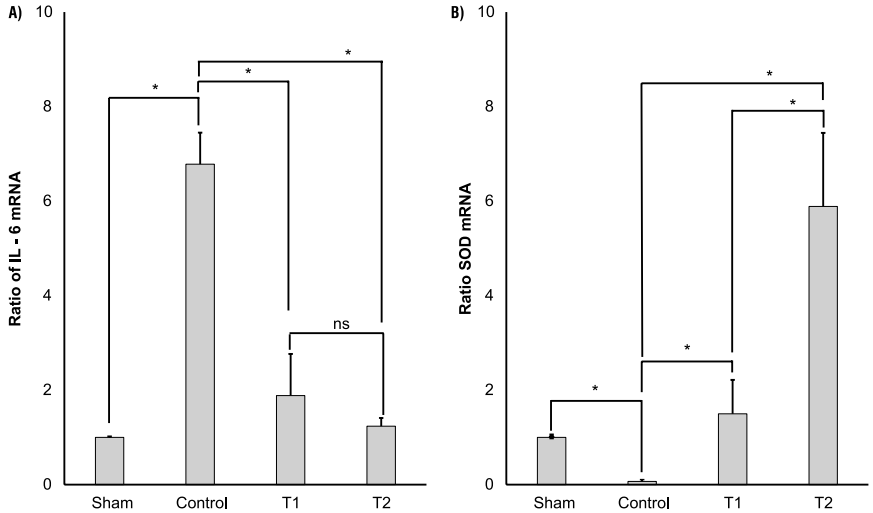

Figure 3. The effect of hypoxic secretome mesenchymal stem cells (HS-MSCs) on the: A) IL-6 and B) superoxide dismutase (SOD) gene expression on type 1 diabetes mellitus (T1DM) rat models. RNA was extracted from the rats pancreatic tissue and analysed for mRNA expression by qRT-PCR (n=5 ±SE). Data are presented as fold change in gene expression relative to UVB unexposed group; *p> 0.05

MSCs could regulate the nuclear-erythroid factor 2-associated factor 2 (Nrf2) dependent on ECH-associated protein 1 such as Kelch (keap-1) under a high level of oxidative stress (32). The Nrf2 dissociates from kaep-1 and translocated to the nucleus leading to dimerizes with Maf and binds to ARE genes, such as HO-1 (33). Furthermore, upregulated HO-1 catalyses the heme to CO, which will activate the NF-kB pathway to promote proinflammatory cytokine expression and prevent the formation of antioxidant enzymes (34). In HS-MSCs, IL-10 can prevent the release of Nrf2 from eep-1, hence increasing SOD expression (35,36). Previous research reported that HS-MSCs enhance glutathione (GSH) and SOD activity leading to the reduction of oxidative stress (37). Some studies elucidate that MSCs suppress oxidative stress leading to inhibiting secretion of proinflammatory cytokine such as IL-6 (38). In addition, exosome of MSCs inhibited of ROS level leading to improvement of pancreatin on DMT1. These phenomena support our study that HS-MSCs significantly induced SOD and inhibit pro-inflammatory cytokine IL-6.

Increased IL-6 cytokine expression can occur in hyperinflammatory environments that persist inflammation and cause pancreatic cell damage (12). A previous study reported that the interaction of IL-6/IL-6R causes dimerization of gp130, which activates JAK family kinases and subsequently induces phosphorylation of STAT1 and STAT3 proteins (39). Phosphorylation of STAT1/3 protein induces the transcription of other proinflammatory genes that can exacerbate pancreatic cell damage. Our study also reported the decreased IL-6 gene expression post HS-MSCs administration. Another study reported that IL-10 secreted by HS-MSCs prevents the development of DMT1 through regulation of innate and adaptive immune cells (40). As anti-inflammatory cytokines and growth factors, IL-10 and TGF-β in H-MSCs suppress the expression of inflammatory cytokines such as IL-6, causing pancreatic cell proliferation and reduced blood glucose levels (6,41,42). Our findings suggest that the HS-MSCs administration reduced IL-6 gene expression by upregulating SOD gene expression.

In conclusion, our findings suggest that HS-MSCs alleviate SOD gene expression and inhibit IL-6 gene expression to induce insulin secretion and regulate proliferation of ß-Langerhans cells. Thus, MSCs become a therapeutic target for T1DM. Furthermore, MSCs might be an attractive therapeutic target to treat inflammation mediated T1DM.

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TRANSPARENCY DECLARATION

Conflict of interest: None to declare.

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