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The Effect of Hypoxic Mesenchymal Cell Secretome Administration on VEGF Levels in Type 1 Diabetes Rats Model

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ABSTRACT

Background: Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by the cessation of insulin production due to pancreatic β-cell damage resulting in an increase in blood glucose. **Objective:** This study aims to analyze the effect of hypoxic secretome MSCs on the angiogenesis process through the observation of VEGF levels in T1DM rat's model. **Methods:** The twenty male Wistar rats were randomly assigned to four groups: control T1DM, T1DM with hypoxic secretome mesenchymal stem cells (HS-MSCs) 0.5 mL intraperitoneal treatment (T1), and TIDM with HS-MSCs 1 mL intraperitoneal treatment (T2). The T1DM rats' model was induced by a single intraperitoneal (IP) injection of freshly prepared streptozotocin (STZ) at a dose of 65 mg/kg of body weight. **Results**: The VEGF levels was analyses under ELISA assay. The results showed that VEGF levels of T1 (68.86±4.78) and T2 (53.83±10.86) groups were significantly upregulated in treatment of HS-MSCs. **Conclusion:** Taken together, HS-MSCs potentially reduce glucose levels on T1DM through VEGF up-regulation.

Keywords: MSCs, Secretome, Hypoxia, VEGF, T1DM.

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by the cessation of insulin production due to pancreatic β-cell damage resulting in an increase in blood glucose of more than 200mg/dL [1]. The pancreas cannot produce insulin; hence patients must receive lifetime insulin therapy [2]. The use of insulin therapy leads to a significant increase in medical costs. The World Health Organization (WHO) (2021) reported that there are 9,000,000 people with T1DM living in high-income countries [3]. This data has increased considerably from the 2020 data where in that period there were only 2,900,000 people with T1DM. T1DM can cause various complications such as coronary heart disease, heart attack, high blood pressure, high cholesterol, high triglycerides, stroke, and heart failure, hypoglycemia, neuropathy, nephropathy, and diabetic ulcers which increase the risk of death [4,5]. On the other hand, the commonly used therapy in T1DM patients is pancreas transplantation, however, this therapy is often rejected and worsens the patient's condition [6,7]. Therefore, a therapy that can regenerate the pancreas to induce natural insulin formation is needed.

Pancreatic organ damage is characterized by decreased angiogenesis, resulting in cell apoptosis[8]. Vascular endothelial growth factor (VEGF) is a major growth factor in the angiogenesis

VEGF plays an important role in the incidence of endothelial dysfunction in DM patients which will lead to microvascular complications [10,11]. Previous studies reported that Mesenchymal Stem Cells (MSCs) can increase the number of pancreatic beta islet cells through suppression of inflammatory damage and immune-mediated antigen rejection [12–15]. Adipose-derived MSCs induced new blood formation in the RSF rat diabetes model by expression of factor-1 through hypoxia-induced VEGF (HIF-1) α. Hypoxia-conditioned MSCs will secrete more growth factor [16–21]. However, the role of secretome hypoxic MSCs on VEGF levels in T1DM condition has not been studied. Therefore, this study aims to analyze the effect of hypoxic secretome MSCs on the angiogenesis process through the observation of VEGF levels in T1DM rat's model.

MATERIAL AND METHODS

Mesenchymal stem cells isolation

Umbilical cord blood was collected from pregnant rats into heparin tube. The buffy coat was isolated by centrifugation (450 × g, 10 min), suspended in 1.5 mL PBS, and used for culture. The separated buffy coat was layered onto equal volume of Ficoll (GE health care, USA) and centrifuged (400 × g, 20 min). Cells at the interface were removed and washed twice in sterile PBS. MSCs were cultured on tissue treated culture plates in DMEM medium supplemented with 10% FBS, penicillin/streptomycin (50U/mL and 50mg/mL, Gibco-Invitrogen, Carlsbad, USA; respectively), and amphotericin B 0.25%. The plates were maintained at 37°C in a humidified atmosphere containing 5% CO2 for 2 days. To exchange the medium, the plates were washed with PBS to remove non-adhered cells and the medium was replaced. The cultures were maintained for an additional week with one medium exchange [22–24].

MSCs characterization by differentiation

To characterize the adherent cells, osteoblastic differentiation was induced by culturing confluent rat MSCs for 3 weeks in osteoblastic differentiation media (all from Sigma) and after three weeks, the cells were stained by Alizarin. To induce adipocyte differentiation, confluent MSCs were cultured 1 to 3 weeks in differentiation medium, and lipid droplet staining was carried out by S Red Oil (Sigma) [25].

Flow cytometric analysis

Flow cytometry was used to assess the immune profile of MSCs, using the standard for MSC as described by the International Society for Cellular Therapy (ISCT). Cells (Passage 4) were harvested, pelleted and resuspended in 1% bovine serum albumin (BSA in PBS), and counted. Each population containing 10^5 cells was used for flow cytometry. Cells were stained with directly PE (phycoerythrin) conjugated antibodies against CD29, CD90, CD31 and CD45 (Ebioscience, Germany). Cells were analyzed on flow cytometry Acurri BD C6plus [26,27].

Hypoxic secretome MSCs isolation

The MSCs already in the wells were put into the chamber. An oxygen meter is installed in the hypoxia chamber. The chamber is closed and secured tightly. CO2 flowed into the chamber through a hose. The oxygen meter was observed until the oxygen in the chamber reached the level of 5% O2 for 24 hours of incubation. Secretome collection was performed using tangential flow filtration (TFF) to obtain MSCs secretome with a size of 10-50 kDa.

DMT1 rat's model

Twenty male Wistar rat, which were 6 to 8 weeks old were purchased from local breeders (Semarang, Indonesia). They were randomly assigned to four groups: 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 have been rendered T1DM by a single intraperitoneal (IP) injection of freshly prepared streptozotocin (STZ) (Sigma-Aldrich, St. Louis, Mo, USA) at a dose of 65 mg/kg of body weight. To avoid hypoglycemia 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 compatible 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.

VEGF analysis under ELISA assay

Blood serum of rats were determined the VEGF levels using an ELISA kit (Thermofisher) according to the manufacturer's protocol. The absorbance at 450 nm was measured using a Bio-Rad Model 3550-UV microplate reader (Bio-Rad Laboratories, Inc.). Experiments were performed in triplicate and repeated 3 times.

Statistical analysis

Data were presented as the mean ± SD. The statistical significance of differences between the groups was examined on SPSS 26.0 (IBM Corp., Armonk, NY, USA) using ANOVA with post-hoc Fisher's LSD analysis. $p < 0.05$ were considered significant.

RESULTS

MSCs isolation and characterization

The MSCs cells had a heterogeneous fibroblastic-like appearance and exhibited distinct colony formation. MSCs have mainly a spindle-shaped appearance with extension in opposite directions from a small cell body (Figure 1A). Alizarin staining clearly showed the formation of calcium oxalates on the differentiated MSCs, which was not observed in the undifferentiated cells (Figure 1B). Intracellular lipid droplets staining using oil red- O proved the adipogenesis of MSCs (Figure 1C). These findings confirmed the characterization of cells as MSCs and show the potential of MSC to differentiate to these lineages, i.e., osteogenic, and adipogenic.

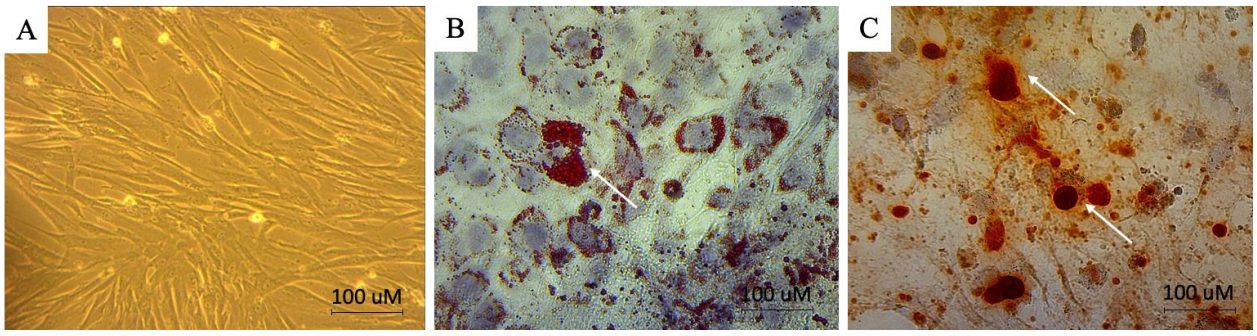

Figure 1. Microscopic images of mesenchymal stem cells isolated from umbilical cord. (a) MSCs spindle-shaped fibroblast- like appearance, extended in opposite directions from a small cell body (passage number 4). (b) adipogenic-induced MSC, intracellular staining using oil red-O. (c) Osteogenic differentiation assay, Alizarin staining specifically shows calcium oxalates in differentiated MSCs (5 days in differentiation medium, number 4).

MSCs surface marker expression

A minimal immune positive criterion for the identification of MSCs cells is the presence of CD90 and CD29 while being negative for CD45 and CD31. Purified MSCs from umbilical cord blood

could be easily characterized by cell markers expressed on their surface. Based on available Abs for MSC, this study elucidated that MSCs were positive for CD90 and CD29 (Figure 2) but were lack of CD45 and CD31 expression.

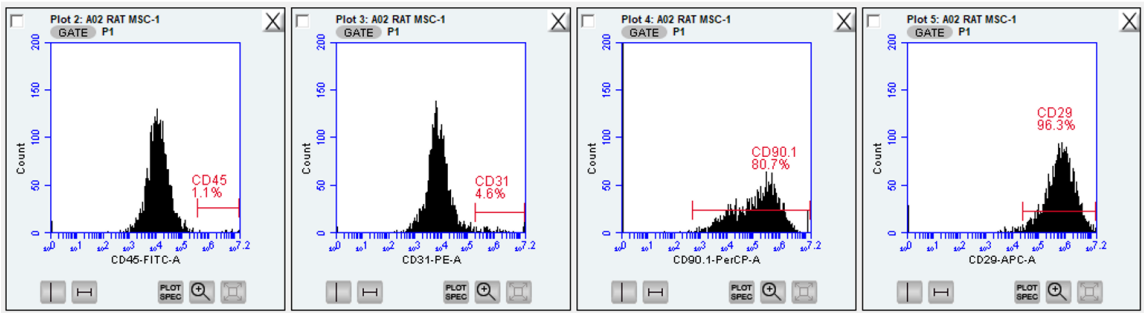

Figure 2. Flow cytometry analysis of cell surface markers present on MSCs derived umbilical cord blood.

T1DM rats model validation

The average fasting blood sugar level in the research rats was observed to be 329 mg/dL, which means that the research rats were confirmed to be DM, in accordance with the provisions of the rats confirmed to be DM if their blood sugar levels were >150 mg/dL. The body weight of Wistar male rats used in this study was 282 grams.

VEGF level on DMT1 under hypoxic secretome MSCs

Based on the findings, the serum from each group was used to measure VEGF levels under ELISA assay. Compared to control groups (10±1.23), the VEGF levels of T1 (68.86±4.78) and T2 (53.83±10.86) groups were significantly upregulated in treatment of HS-MSCs (Figure 3).

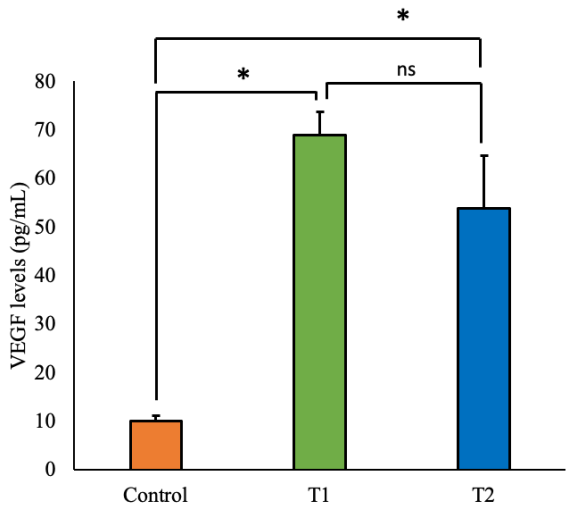

Figure 3. The VEGF levels of blood serum in each group. Data are expressed as the mean ± standard error of the mean. *P<0.05, as indicated. VEGF, vascular endothelial growth factor; n.s., not significant.

DISCUSSION

MSCs cells are considered as multipotent which may differentiate into a variety of cells such as adipocytes, chondrocytes, osteoblasts, and neurons [28,29]. One of greatest aspects of these cells is the

immunomodulatory feature, which makes them a preferable candidate in regenerative medicine [30–32]. MSCs secreted various soluble molecule including cytokine and growth factor that have several functions [33–35]. This study aims to evaluate the secretome hypoxic MSCs (HS-MSCs) on the VEGF level in diabetes mellitus type 1 (T1DM). The various cellular functions of VEGF result from its ability to initiate a diverse, complex, and integrated network of signaling pathways through its main receptor, the kinase insert domain receptor: VEGF can stimulate cell differentiation, proliferation, migration, and survival [9,36,37]. Previous studies have reported that VEGF was critical for the differentiation of endothelial cells, and that nitric oxide was an important effector of the biological actions of VEGF [38,39]. In addition, VEGF has been reported to induce the differentiation of mouse multipotent adult progenitor cells into endothelial cells including in pancreas cells, through a mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 signaling pathway-mediated mechanism [40].

Our findings indicating upregulation of serum VEGF levels reveal that VEGF has important physiological effects on the inhibition mechanisms of angiogenesis as a ligand trap in diabetics and are consistent with the studies evaluating tissue levels in animals [41–46]. Previous research reported that Secretome-MSCs enhance the VEGF levels leading to angiogenesis and cell regeneration [47–49]. Some study elucidates that high level of VEGF induce insulin production [17]. HS-MSCs also contain anti-inflammatory cytokine that causing pancreatic cell proliferation and reduced blood glucose levels [26,50,51]. VEGF induce insulin production through PI3K and PLCy1 pathway leading to decrease glucose levels [12]. Our study concluded that HS-MSCs potentially improve T1DM through VEGF up-regulation.

CONCLUSION

Hypoxic secretome MSCs therapy at doses of 0.5 and 1 cc increased VEGF levels in the T1DM rat model. Hypoxic secretome MSCs can be a candidate therapy to regenerate pancreatic cells in T1DM condition.

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AUTHORS' CONTRIBUTIONS

NDA has made significant contributions to the concept and design, data acquisition, data analysis and supervised the project. FJ has made significant contributions to concept and design, data analysis dan drafting of this manuscript. NDA and ADA strictly revised the important knowledge content of the article and provided technical support. FJ and NDA provided technical support.

COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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