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The Effect of Secretome Hypoxic Mesenchymal Stem Cells on Pro-Inflammatory and Anti-Inflammatory Markers Expression (in Vivo Experimental Study on the Macrophage Cell Polarization of Wistar Male Mice Type 1 DM Model)

A'an Haries Pranowo[1], Danis Pertiwi[2], Agung Putra[2]

[1]Postgraduate student of Biomedical Sciences, Faculty of Medicine, Universitas Islam Sultan Agung, Jl Kaligawe KM 4 Semarang 50012

[2] Stem Cell and Cancer Research, Faculty of Medicine, Universitas Islam Sultan Agung, Jl Kaligawe KM 4 Semarang 50012

ABSTRACT: The autoreactivity of T cells in DMT1 patients causes activation of M1 and M2 macrophages as proinflammatory and anti-inflammatory markers which promote pancreatic cell damage. Secretome Hypoxic Mesenchymal Stem Cells (SH-MSCs) therapy methods play a role in repairing damaged tissue and regenerating pancreatic islet cells. This study aims to examine the effect of SH-MSCs on macrophage cell polarization through the expression of CD68 as a marker of M1 macrophages and CD163 as a marker of M2 macrophages in DMT1 rats. The sample of this study were 5 Wistar rats which were divided into 4 groups; normal group (no treatment), negative control group, and 2 treatment groups with 0.5cc of SH-MSCs (P1 group) and 1cc of SH-MSCs (P2 group). STZ 60 mg/kgBW induced DMT1 in the negative control, P1, and P2 groups. Data were tested parametrically using SPSS 22. The mean of CD68 expression in normal group was 3.06±0.35%, negative control: 21.67±0.93%, P1: 10.98±0.68%, P2: 5.85±0.55%. The mean of CD163 expression in normal group: 22.83±0.51%, negative control group: 3.56±0.50%, P1:15.81±1.80%, P2: 20.80±2.24%. The results of the normality and homogeneity test were $p > 0.05$. Anova test results $p < 0.05$. Post Hoc LSD test results $p < 0.05$. The administration of SH-MSCS was shown to decrease CD68 and increase the CD163 expression significantly in DMT1 rats.

KEYWORDS: CD68, CD163, M1 macrophages, M2 macrophages, DMT1

I. INTRODUCTION

Type 1 diabetes mellitus (DMT1) is an autoimmune disease that causes pancreatic-cell destruction and leads to absolute insulin deficiency. Global data shows that 90% of DM in children and adolescents is T1DM. The prevalence of DMT1 is 5-10% of the total DM patients in the world. Data from the Indonesian Pediatrician Association (IDAI) until the end of 2018 recorded 1220 cases of DMT1 (1.2.3). DMT1 therapy takes a long time and may cause some complications. Continuous treatment may decrease patient compliance, leading to an increase in Diabetic Ketoacidosis (DKA). DKA is an acute complication of T1DM in children with high morbidity and mortality rates (4).

Exogenous insulin therapy in T1DM is not an effective therapy since it may increase the incidence of life-threatening severe hypoglycemia (5). The development of Secretome Hypoxic Mesenchymal Stem Cells (SH-MSCs) therapy has great potential in regenerating islet cells and repairing the function of damaged pancreatic tissue (6). Utilization of SH-MSCs as a therapy for DMT1 has great potential to be developed. IDF 2017 explains that as much as US$850M is spent for DMT1 treatment per year which in turn impacts community productivity (1). SH-MSCs change the pattern of cytokine expression from pro-inflammatory M1 macrophages (such as TNF- and IL-2) to anti-inflammatory M2 macrophages (6.7). Macrophages are components of innate immunity that act as initial defense against pathogens in the body, they are divided into two, classical activation (M1) and alternative activation (M2). The involvement of growth factor PDGF secreted by SH-MSCs acts as an immunomodulator that suppresses autoimmune activity by stopping the production of self-antibodies that attack pancreatic cells. A previous study by Siddiq in 2017 discussed the benefits of the secretome in reducing the expression of IL-17, TNF- and IL-1β which play a role in the pathogenesis of autoimmune diseases, such as Systemic Lupus Erythematosus (8).

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Recent studies have demonstrated that SH-MSCs trigger proliferation and angiogenesis in pancreatic-cell DMT1(9). The discussion regarding SH-MSCs is only limited to self-renewal, further research is needed regarding the effect of SH-MSCs on macrophage polarization as seen from the expression of CD68 and CD163 proteins on pancreatic cell repair. In order to determine the effect of administration of secretome MSCs cell polarization through the expression of CD68 and CD163 proteins in type 1 DM.

II. MATERIAL AND METHOD

This experimental study uses a posttest-only control group design as the design of the study. The sample of this study is 2-3-month-old male white Wistar strain rats. with a body weight of 200 g ± 10 g which was obtained in the Animal House Integrated Biomedical Laboratory, Faculty of Medicine, Sultan Agung University, Semarang. The independent variable in this study was the Secretome Hypoxia Mesenchymal Stem Cell. The sampling technique used in this study is simple random sampling. The sample size used was 6 rats in each group which were randomly taken and divided into 4 groups. There were 24 rats in total that were used in this study.

Grouping and DMT1 Rats Development

Mice that met the inclusion criteria were weighed accurately and put in one cage. On the first day of the experiment, all rats were fasted for 6 to 8 hours before being induced with 15 mL STZ ip according to the dose of Kg/BW and continued with food as usual and 10% sucrose water. On the 2nd experimental day, change the 10% sucrose water to plain water. On experimental day 3, all rats were fasted for 6 to 8 hours. Test blood glucose levels from venous samples were collected from the tail using a digital sugar level check tool. Validation of the experimental animals was carried out to see whether the experimental animals showed the characteristics of DMT1 as seen from the blood glucose level > 200 mg/dL which was stable for 72 hours and the physical characteristics that lead to DMT1 Mice to be treated with SH-MSC were adapted for 5 days.

Isolation of MSCs

The umbilical cord of female white rats at 19 days of gestation was cleaned with PBS solution. The umbilical cord blood vessels were removed then the umbilical cord was cut with a sterile knife into 2-5 mm long and placed evenly in a 66 mm culture dish. The medium used consisted of DMEM culture medium (Sigma-Aldrich, Louis St., MO, MFCD00217342) mixed with 10% FBS, 100IU/ml penicillin/streptomycin (GIBCO, Invitrogen, 15140122) then incubated at 37^0 C with 5% CO_2. Medium replacement is done every 3 days. After 4-5 passes, cells were cultured for 8-10 days until 80% confluent and then harvested by giving 0.25% trypsin-EDTA at 37^0 C. The cells used in this study were cells from the 5th pass.

Characteristics MSCs Phenotype

Expression of isolated MSCs was assessed using immunocytochemistry indicated by the expression of CD73 and CD105 (MSCs markers). After obtaining 60-80% confluent MSCs (3-4 weeks), MSCs were harvested and grown in coverslips with a density of $5x10^3$ -$1x10^4$ cells for 3-4 days then fixed with 4% paraformaldehyde in 90% ethanol for 15 minutes and a temperature of 4^0 C. Cells were incubated with primary antibodies CD73 and CD105 which are markers of character MSCs (1:100) (BD Pharmingan, San Diego. CA USA, no: 561443) for 60 minutes at room temperature. These markers are the minimum criteria that must be used in the MSCs phenotyping test in accordance with the provisions of The International Society for Cellular Therapy (10). CD 105 is a type 1 membrane glycoprotein that functions as an accessory for the TGF-β receptor which is a stemness factor molecule (11). On the other hand, CD 73 has a special role in regulating the inflammatory process of injured tissue (12). Cells were then washed with PBS for 10 minutes added to the second antibody (1:2500 dilution) for 15 minutes at room temperature and then counterstained with 3,3'- Diaminobenzidine (DAB) (Santa Cruz Biotech, SC-24982) and observed with a microscope (13).

The Hypoxic process

MSCs that had reached 80% of confluence were added with a complete medium of up to 10 mL then, the flashed flask containing MSCs was moved into the hypoxic chamber. Nitrogen gas is channeled 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 37^0 C. After 24 hours, the culture medium was taken and filtered using TFT to obtain Secretome Hypoxia-MSCs (SH-MSCs) which were then mixed with a water-based gel according to the dose of the treatment group.

Treatment

The DMT1 rat model was divided into four groups, including 1 group of healthy rats (untreated), 1 control group, and 2 treatment groups with 0.5 cc of SH-MSCs in group P1 and 1 cc in group P2. On the 28th day after treatment, the sample was terminated using a lethal dose of a cocktail. (10 mL cocktail Ketamine 50 mg/kgBW, Xylazine 10 mg/kgBW and Acepromazine mg/kgBW) were

injected intramuscularly before taking the pancreas organ. The expression of M1 and M2 macrophages was seen from the expression of CD68 for the M1 macrophage and CD163 for the M2 macrophage using Immunohistochemistry (IHC). The method to calculate the expression of CD68 and CD163 is by observing the cytoplasm of the epithelial cells and state with percentage.

Data Analysis

Data analysis was carried out by statistical methods including the data normality test with Shapiro-Wilk test. Then continued with the Lavene Homogeneity test. Homogeneous data continued with the One-Way ANOVA parametric test to determine the differences between treatment groups ($p < 0.05$) which was then followed by the Post Hoc LSD test.

III. RESULT

Characteristics of MSCs

The results of the MSC culture were observed microscopically, an image of cells attached to the bottom of the flask with spindle-like cells. After 14 days, red fat disposition was seen using oil red O (Figure 1). This indicates that MSCs can differentiate into adipocytes. The results of MSC isolation were also validated using flow cytometry to determine the ability of MSCs to express several surface markers (15)

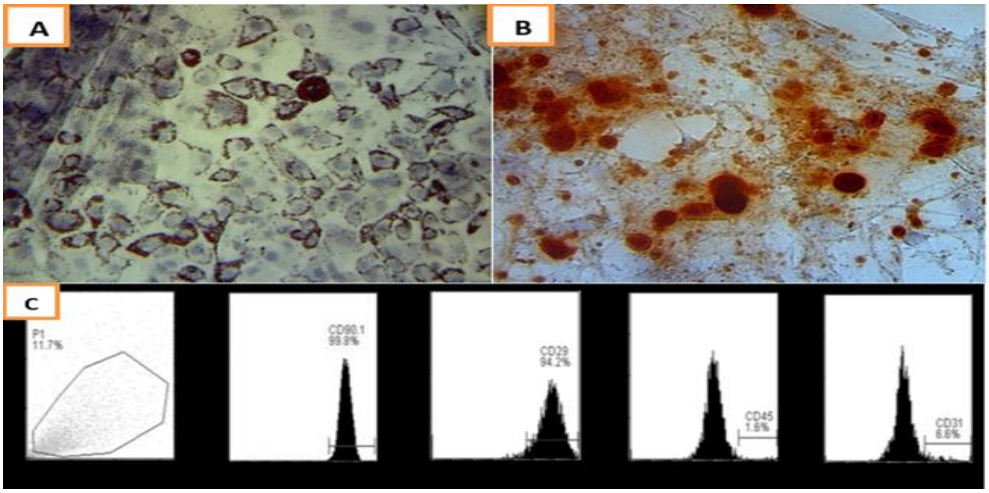


Figure 1. (A) MSCs that differentiate into adipocytes: Microscopic observation at 200x. magnification Alizarin red dye staining makes MSCs appear red which indicates calcium deposits have occurred. This indicates that MSCs can differentiate into osteocytes (B). (C) The results of validated MSCs and examination of the expression of CD90, CD29, CD45, and CD31 using flow cytometry (14).

Validation of Type 1 DM Model Rats

In this study, it was found a decrease in the number and enlargement of rat pancreatic cells and also changes in the structure of the islets of Langerhans (Figure 2). The different condition of the mice in this study was also confirmed by examination of blood glucose level before STZ administration, on days 7, 14, 21, and before termination. There was an increase in the glucose level in the negative control group, the P1 group, and the P2 group on the 7th day of the experiment compared to the glucose level before the STZ injection.

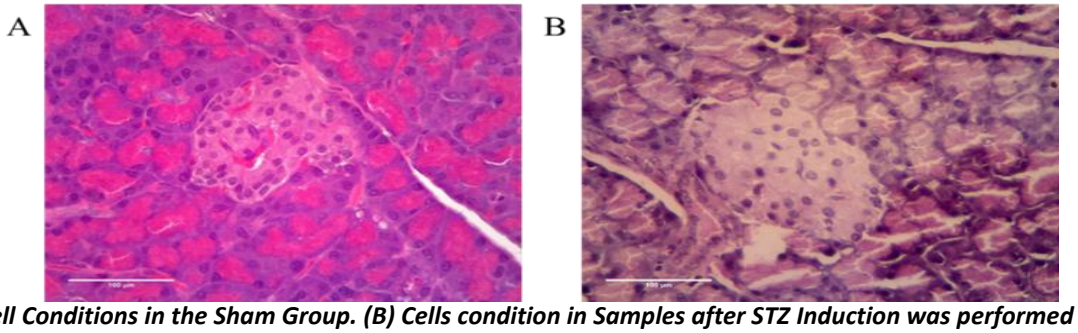


Figure 2. (A) Cell Conditions in the Sham Group. (B) Cells condition in Samples after STZ Induction was performed with a dose of 60mg/kgBW.100x

CD68 expression

In this study, CD68 expression was examined by the IHC method and expressed as a percentage of the total area of pancreatic tissue expressing CD68 (stained in brown) to the total pancreatic tissue area. (Figure 3)

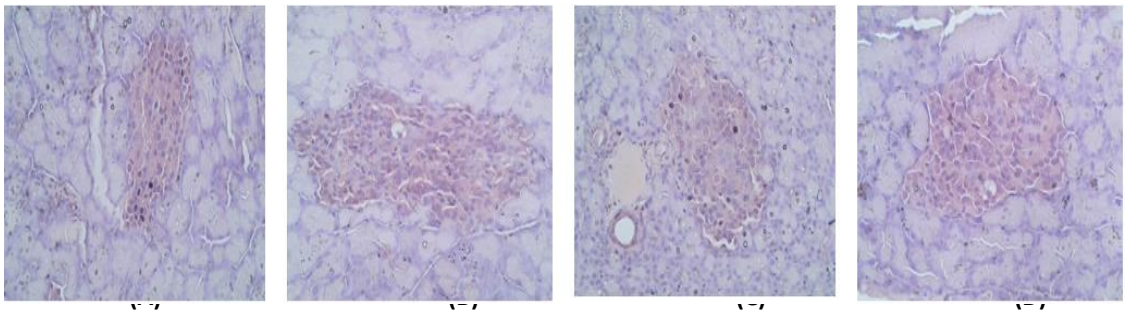


Figure 3. Histology of the pancreas of mice expressing CD68 by HE staining at 40x magnification; (A) healthy rat preparations, (B) negative control preparations (C) P1 preparations, and (D) P2 preparations.

The results of normality and homogeneity tests obtained p>0.05 in all groups, which means that the data are normally distributed and homogeneous. The results of the One-way ANOVA test p = 0.000 (p <0.05) so it can be interpreted that there are significant differences in CD68 expression between groups. Post Hoc test showed that there was a significant difference in CD68 expression between groups (p=0.000). (Figure 4)

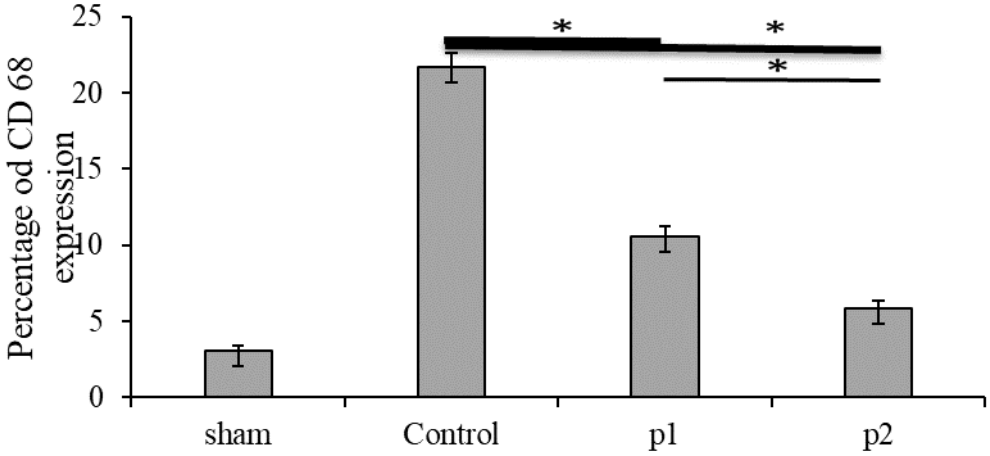
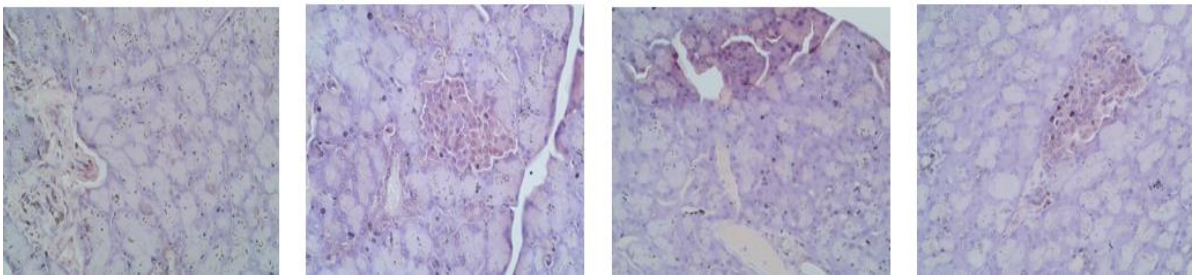


Figure 4. CD68 insulin expression in; healthy rat group: subjects without treatment, Negative control: subjects with diabetes induction without treatment, P1: subjects with diabetes induction given the injection of 0,5 cc of SH-MSC 0, P2: subjects with diabetes induction given IP injection treatment of 1 cc SH-MSC

Expression 163

Expression of CD163 was examined using the IHC method and expressed as a percentage of the total area of pancreatic tissue which expressed CD163 (stained in brown) to the total area of pancreatic tissue. (Figure 5)



(A) (B) (C) (D)

Figure 5. Histology of the pancreas of mice expressing CD163 by HE staining at 40x magnification; (A) healthy rat preparations, (B) negative control preparations, (C) P1 preparations, and (D) P2 preparations

The results of normality and homogeneity tests obtained p>0.05 in all groups, which means that the data are normally distributed and homogeneous. The results One-way ANOVA test got a value of p = 0.000 (p <0.05) so it can be interpreted that there are significant differences in CD163 expression between groups. Post Hoc test results between groups showed that there was a significant difference in CD163 expression between groups (p=0.000). (Figure 6)

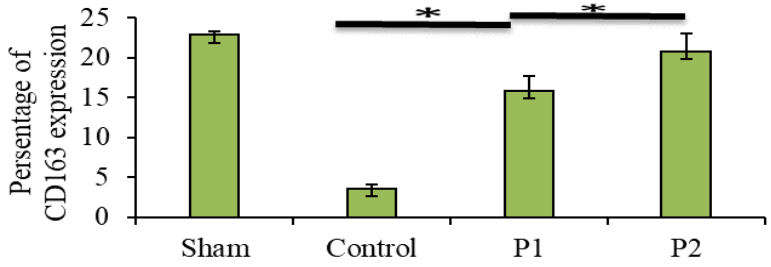


Figure 6. CD163 Insulin expression in healthy rats group, subjects without treatment, Control group: subjects with diabetes induction without treatment, P1: subjects with diabetes induction treated with IP injection 0,5 cc of SH-MSC, P2: subjects with diabetes induction treated with IP injection of 1cc of SH-MSC 1 cc

V. DISCUSSION

DMT1 is an autoimmune metabolic disease that requires lifelong insulin replacement therapy (16). Increased levels of ROS have an impact on the polarization of M1 macrophages which produce proinflammatory cytokines (IL-6 and TNF-) that can interfere with the antigenic response, macrophage function, the healing processes of pancreatic cells, and the emergence of microvascular complications. High levels of proinflammatory cytokines in DM patients initiate the disruption of the inflammatory cascade reaction, hyperinflammation, and insulin resistance (17). SH-MSCs contain growth factors and anti-inflammatory cytokines such as IL-10, IL-12, HGF, TGF-β, VEGF, PDGF, and FGF. which are able to accelerate tissue repair and regeneration (18.19.20,21). Hypoxic MSC conditioning showed a better potential for regeneration of damaged tissue than normoxia MSCs. Hypoxic MSCs are thought to be able to express higher Heat Shock Protein to induce protein folding and optimize intracellular protein function (22). The results of this study showed that 1cc of SH-MSCs had a significant effect followed by 0.5cc on the reduction of CD68 expression in STZ-induced DMT1 rats, which was in accordance with the hypothesis in this study. This finding is in line with a study by Dong. et.al, 2014 which proved that MSCs can suppress M1 macrophages by increasing IL-10 levels and decreasing TNF-α, IL-12, CD68, and HLA class II. Another study explained that the presence of IL-10 caused a decrease in the production of inflammatory cytokines which resulted in a reduced risk of chronic intestinal inflammation in mice (25). The increased expression of CD68+ as an effector cell involves the classic M1 macrophage activity which continues to play a role in releasing pro-inflammatory cytokines such as IL-12, IL-1β and TNF-α. It participates in Th1 induction thereby enhancing the ongoing inflammatory process in pancreatic cells (23). IL-10 contained in SH-MSCs triggers the polarization of M1 macrophages into M2 macrophages which are anti-inflammatory. IL-10 plays an important role in blocking the production of cytokines by activated macrophages. Inhibition of IL-12 production by IL-10 aims to attenuate the development of Th1 cell responses (24).

M2 produces several anti-inflammatory cytokines including IL-10 and IL-1RA. M2 uses arginine as a substrate for arginase 1 (Arg1), then degrades arginine to ornithine, which is a precursor of polyamines, prolines, and collagen required for tissue repair processes. Polyamines are involved in cell growth and cell division, while proline is a major component of collagen (26). The results of this study showed that 1cc dose of SH-MSCs able to increase CD163 expression was better than the group with 0.5 cc of SH-MSCs and the negative control group, respectively by 22% ± 0.5%, 15 %±1.3%, and 3%±0.5%. Administration of a dose of 0.5cc of SH-MSCs increases CD163 expression which explains that a small dose of SH-MSCs can be recommended as initial therapy to repair chronic inflammation in DMT1 patients. Research conducted by Wang is in line with several studies in 2020 which show that the increase of M2 in the inflammatory process accelerates the healing process of diseases in some tissues including cardiovascular, lung, digestive, kidney, and central nervous system diseases(27). Jamiyan et al, (2020) also explained that the accumulation and activation of M2 macrophages can induce local adaptation of immune cells through the production of CCL18, recruitment of naive T cells and dendritic cells that play a role in stopping the ongoing inflammatory process (28).

V. CONCLUSION

The administration of SH-MSC 0.5 cc and 1 cc on macrophage cell polarization can reduce the CD68 protein expression and increase the CD163 expression in DMT1 model rats.

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