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Secretome hypoxia-mesenchymal stem cells decrease tumor necrosis factor-α and interleukin-18 in kidney of type 2 diabetes mellitus model rats

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ABSTRACT

BACKGROUND

Type 2 diabetes mellitus (T2DM) is a chronic disease that affects millions of people worldwide and associated with an increased risk of kidney damage caused by prolonged inflammation. Secretome hypoxia-mesenchymal stem cells (SH-MSCs) have been investigated as a potential therapy for kidney inflammation in T2DM, due to their immunomodulatory properties and ability to promote tissue repair. In this study, we investigated the effects of SH-MSCs on tumor necrosis á (TNF-á) and interleukin-18 (IL-18) in the kidney of the T2DM model rats.

METHODS

A post-test-only control group involving 24 male Wistar rats. The rats were treated with a high-fat diet (HFD) for 4 weeks and streptozotocin-nicotinamide with sucrose solution for 5 days to induce T2DM animal models. Rats were randomly divided into four groups: healthy, control, and groups treated with SH-MSCs T1 and T2, with doses of 250 µL and 500 µL, respectively. TNF-α and IL-18 gene expression was measured by real time polymerase chain reaction (RT-PCR). One Way ANOVA and post-hoc LSD tests were used to determine the significant difference against all groups based on their quantitative measurement.

RESULTS

Administration of the SH-MSCs at a dose of 500µL (T2) was able to significantly reduce TNF-α and IL-18 gene expression when compared to control (T2DM rat without treatment) ($p<0.05$), but not significantly when compared to healthy and SH-MSCs at a dose of 250µL (T1) group ($p>0.05$).

CONCLUSION

This study demonstrated that the SH-MSCs decreased the levels of proinflammatory cytokines TNF-α and IL-18 gene expression in the kidney of T2DM model rats.

Keywords: IL-18, inflammatory, SH-MSCs, T2DM, TNF-α, kidney, rats

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a disease characterized by hyperglycemia and insulin receptor dysfunction due to impaired lipid and carbohydrate metabolism which causes excess glycogen deposition and rejection of insulin resistance.[1,2] Currently, diabetes which is closely related to obesity has become a global epidemic. Adipose tissue is known as a major contributor to the production of pro-inflammatory molecules such as tumor necrosis factor alpha (TNF-α), interleukin-18 (IL-18), interleukin-1 (IL-1), interleukin-6 (IL-6) and C-reactive protein (CRP) trigger the progression of kidney failure.[3,4] In addition, infiltration of type 1 macrophages (M1) in the kidneys of T2DM patients can cause the secretion of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-18 (IL-18).[5,6] Serum IL-18 levels are associated with urinary microglobulin-2 as a marker of tubular dysfunction. Interleukin-18 is able to regulate the synthesis of pro-inflammatory molecules such as IL1, TNF-α and interferon-gamma (IFN-γ) then it will induce chemokine receptors in mesangial cells.[7,8] Tumor necrosis factor-alpha is one of the key pro-inflammatory cytokines that contribute to the development of insulin resistance and T2DM through activating low-grade inflammation by decreasing glucose transporter-4 (GLUT4) and serine phosphorylation of insulin receptor substrate-1 (IRS-1) expression.[2]

Approximately 30% of patients with type 1 DM (T1DM) and 40% of patients with type 2 DM (T2DM) develop a diabetic kidney disease (DKD).[1,6,9] The prevalence is estimated to have reached 537 million people worldwide and is projected to increase to 643 million people in 2030.[10] This phenomenon also leads to the connotation of "diabesity" which indicates the fact that most people with diabetes are overweight or obese.[3] According to WHO, the risk of death for people with diabetes increases by 7 times and makes obesity the biggest chronic health problem in the world that is more serious than malnutrition.[11]

Mesenchymal stem cells (MSCs) that have the potential for self-renewal and multi-directional differentiation play an essential role in regenerative tissue.[12] The paracrine components of MSCs that are commonly called secretome have an important roles in immunomodulatory. However, the secretome are not constant and depends on stimuli that affect the MSCs microenviroment. Hypoxia is a modification technique that aims to change the environment to resemble an inflammatory condition.[13] Hypoxia in MSCs has the advantage of increasing the production of cytokines or extracellular vesicles (EVs) associated with immunomodulation.[14]

Previous studies reported that secretome hypoxia-mesenchymal stem cells (SH-MSCs) therapy was able to reduce serum creatinine (SCr), blood urea nitrogen (BUN), creatinine clearance rate (CCr), urine protein and kidney hypertrophy in DKD patients.[15,16] Secretome hypoxia-MSCs is an active soluble molecule contained lots of cytokines and growth factors that are useful in inhibiting inflammation by suppressing the NF-κB pathway and regenerating wound tissue. Secretome hypoxia-mesenchymal stem cells contains various anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) which are be able to inhibit the inflammatory process.[17] The other study reported that SH-MSCs can reduce podocyte damage and regulate autophagic activity in the kidney of T2DM model rats.[18] Our previous study described that SH-MSC significantly increased anti-inflamatory cytokine by regulate IL-10 on STZ-induced Type 1 diabetes rats.[19] There is potential for SH-MSCs as an alternative agent to prevent kidney failure progression on T2DM. Therefore, in this study, we will investigate the effect of SH-MSCs at concentrations of 250 µL and 500 µL on the expression of TNF-α and IL-18 in T2DM model rats.

METHODS

Research Design

This experimental research was conducted in a completely randomized design with a post-test only control group design to compare the effect of the treatment on the experimental and control groups at the end of the treatment. The research was conducted from January to February 2023 at Stem Cell and Cancer Research (SCCR) Indonesia, Semarang.

MSCs isolation and characterization

The isolation of MSCs from an umbilical cord (UC-MSCs) of 19 days pregnancy of female rat was performed using a previously described method with modification.[20] Briefly, the umbilical cord was mechanically dissected and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) contained 10% FBS (Sigma-Aldrich, St. Louis, MO), 100 IU/mL penicillin/ streptomycin (Sigma-Aldrich, St. Louis, MO) under Normoxia condition. The cultured cells at passages 5 (P5) and under 80% confluence were employed for the next experiment.

The MSC surface markers were determined as previously described.[21] Briefly, the cells at 4th passage were detached and stained with anti-rat monoclonal antibodies including APC-conjugated CD73, FITC- conjugated CD90, PerCP-conjugated CD105, and PE-conjugated hemopoietic stem cell lineage Lin for 30 min at 4°C. The labeled cells were analyzed using flow cytometry BD Accuri C6 Plus (BD Biosciences, San Jose, CA, USA). The MSCs differentiation capacity was determined using osteogenic differentiation assay Briefly, the cells were plated on 4 × 104 cells in 3.5 cm culture dishes under osteogenic medium that composed of DMEM High Glucose supplemented with 10% FBS, 1% Penstrep, 1 × 10"2M sodium β-glycerophosphate, 1× 10"4 M dexamethasone, and 5 × 10"5 M ascorbic acid. The medium was replaced every 3 days for 15 days. The calcium deposition showed a red bright color after Alizarin Red staining (Zigma, Shenzhen, China).

SH-MSCs preparation

MSCs cultured in serum-free complete medium were incubated under hypoxia condition in the hypoxic chamber maintaining a gas mixture composed of 5% O2, 5% CO2, and balanced N2 at 37°C for 24 h. After 24 h incubation, the hypoxia-preconditioned medium was centrifuged at 2000 rpm at 8°C temperature for 20 min and passed through a 0.22 μm filter membrane (Corning, NY, USA) to remove the remaining cells debris. The SH-MSC isolation, especially for 10–50 kDa molecules containing IL-10 (18 kDa) and TGF-β (25 kDa) using tangential flow filtration. The SH-MSCs was keep on 2–8°C temperature until the treatment.

Animals experiments

The sample size was calculated using the Federer formula that obtained 24 rats. The samples used were healthy male Wistar rats with a body weight of around 200-250 g which were fed ad libitum and maintained at a temperature of 28°C and 12 hours of light. Twenty four Wistar strain rat were given HFD treatment for 4 weeks with a special feed composition for obesity animal models. Rats were validated to have obesity by Lee index = "weight (g)/naso-anal length (cm) × 1000, the cube root of body weight (g) divided by the nose-to-anus length (cm) and multiplied by 1000 which approved >300. Rats that have been validated as obese induced by streptozotocin-nicotinamide and treated with sucrose solution for 5 days. Rats were validated to be T2DM by fasted for 6 hours on the fifth day to calculate the HOMA-IR formula = (fasting glucose value (nmol/L) X fasting insulin value (μU/mL)/22.5) (Table 3). After 1 week of acclimatization, the rats were randomly divided into the following four groups: healthy, control, dose 250 μL (T1) and dose 500μL SH-MSCs (T2) group. Each group consisted of six T2DM rats.

TNF-α and IL-18 gene expression

In the 21 day, rat terminated to collect the kidney tissue in RNA later. A total 100mg kidney tissue was extracted with TRI reagent (TRIzol,

Invitrogen, Shanghai, China) according to the manufacturer's protocol. Briefly, the first-strand cDNA was synthesized with 1 µg 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 the max Eco 48 Real Time-Polymerase Chain Reaction (RT-PCR) instrument, and the mRNA levels of TNF-α, IL-18, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured using the respective primers (Table 1). The thermocycler conditions were as follow: Initial step at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s, and 60°C for 1 min. Expression levels were recorded as cycle threshold (Ct). Data were acquired using the Eco Software v5.0 (Illumina Inc, San Diego, CA, USA). All reactions were performed in triplicate, and the data were analyzed using the $2^{-\Delta\Delta}$Ct method.

Statistical analysis

Statistical analysis was performed by SPSS 26.0 for Windows (SPSS Inc., Chicago, IL, USA). All data (from at least three separate experiments) are presented as mean ± standard deviation (SD). Statistical analysis was performed using One-Way ANOVA. Analysis of variance and a least significant difference comparison Post Hoc LSD test were used to analyse the data, and $p<0.05$ indicated statistical significance.

Ethical clearance

This study received Ethical Clearance from the Bioethics Committee of Medical Research/ Medical Faculty of Sultan Agung Islamic University, Semarang (No.64/II/2023/Komisi Bioetik).

RESULTS

MSCs characterization and differentiation

Secretome hypoxia-mesenchymal stem cells (SH-MSCs) were collected from the umbilical-cord MSCs medium of pregnant rats that had reached the 4th passage. The results of the validation of the morphology of the MSCs culture were obtained an image of cells attached to the bottom of the flask with spindle-like cell morphology under microscopic observation, while the results of the validation of osteogenic differentiation showed that MSCs could differentiate into osteocytes as indicated by the red calcium deposits in the MSCs population. Using Alizarin Red staining (Figure 1). 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 CD90 (99.80%), CD29 (94.20%) and did not express CD45 (1.60%) and CD31 (6.60%) (Figure 1).

Characteristics of SH-MSCs

Mesenchymal stem cells incubate under hypoxic conditions for 24 hours then collected the conditional medium. Isolation of cytokines and growth factors using Tangential Flow Filtration (TFF) that based on a combination of molecular weight limit categories regarding previous studies. Molecular isolation using filter cassettes 10-50 kDa, 50-100 kDa, and 100-300 kDa. After filtration, the level of cytokines and growth factors was characterized by ELISA assay. SH-MSCs is known to contain IL-10 of 415.02 ± 7.14 pg/ mL and TGFβ 282.83 ± 6.28 pg/mL (Table 2).

Table 1. Primer sequences

Gene	Sequences
GAPDH	Forward : 5'-TGACAACTTTGGCATCGTGG-3'
	Reverse : 5'-GGGCCATCCACAGTCTTCTG-3'
TNF-α	Forward : 5'-AAATGGGCTCCCTCTCATCAGTTC-3'
	Reverse : 5'-TCTGCTTGGTGGTTTGCTACGAC-3'
IL-18	Forward : 5'- GACAAAAGAAACCCGCCTG-3'
	Reverse : 5'ACATCCTTCCATCCTTCACAG -3'

Note : GAPDH : glyceraldehyde-3-phosphate dehydrogenase; TNF-α :tumor necrosis factor alpha; IL-18 : interleukin-18

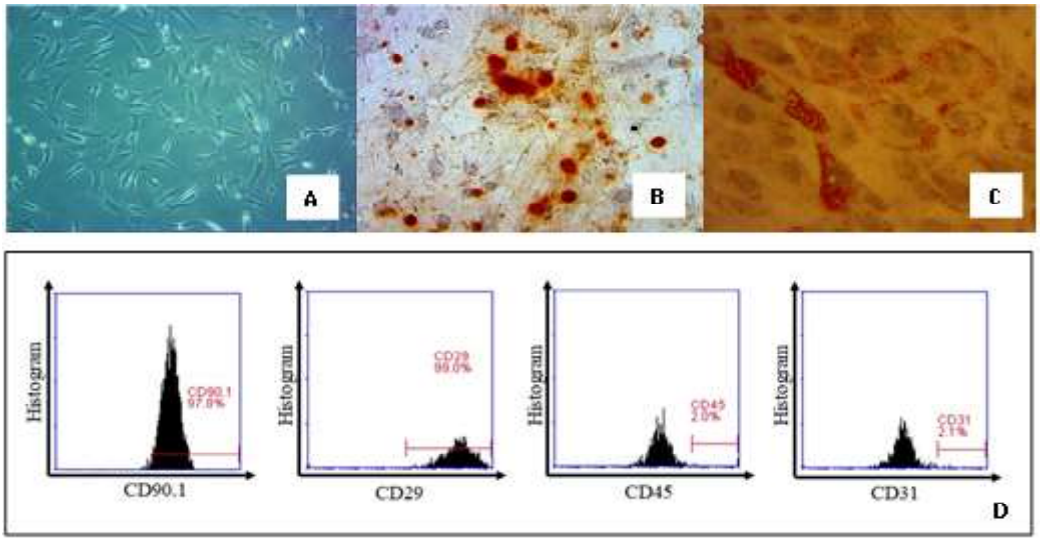

Figure 1. Mesenchymal stem cells (MSCs) validation. (a) Isolated MSCs with 80% confluent showed spindle-like cells (pointed by arrows) at ×100 magnification. (b) Adipogenic differentiation using Oil Red O staining appears in the MSCs population at ×100 magnification. (c) Osteogenic differentiation using Alizarin Red staining appears in the MSCs population at ×100 magnification. (d) Flow cytometry analysis of the expression of CD90, CD29, CD45, and CD31

SH-MSCs downregulated TNF-α and IL-18 expression in the kidney of T2DM model rats

This study compared the gene expression of TNF-α and IL-18 in healthy, control, and treatment groups. The results showed that the gene expression of TNF-α and IL-18 in the control group increased significantly compared to the T1 and T2 groups ($p<0.05$). The analysis also founded that administration of the SH-MSCs at a dose of 500μL (T2) was able to significantly reduce TNF-α and IL-18 gene expression when compared to controls ($p<0.05$), but not significantly when compared to the healthy rat group ($p>0.05$) (Table 4).

One-Way ANOVA test of TNF- α, followed by LSD test showed significant differences between control to T1 and T2 ($p=0.000$) but no significant between healthy to T2 ($p=0.200$) and T1 to T2 ($p=0.074$). And the IL-18 showed significant differences between healthy to control and T1 group ($p=0.000$) but no significant between healthy to T2 ($p=0.906$) (Table 5).

Table 2 . Concentration of IL-10 and TGF-β in SH-MSCs

Cytokine	Concentration
IL-10 (pg/mL)	415.02 ± 7.14
TGF-β (pg/mL)	282.83 ± 6.28

Note :Data presented as mean ± SD;IL-10 : interleukin 10; TGF-β : transformed grow factor- β, SH-MSCs : secretome hypoxia- mesenchymal stem cells

DISCUSSION

In this study, we investigated the effects of SH-MSCs on kidney inflammation in T2DM model rats. Our results demonstrate that the SH-MSCs decreased the gene expression of proinflammatory cytokines TNF-α and IL-18 in the kidneys of these rats. Type 2 diabetes mellitus (T2DM) is a disease characterized by hyperglycemia and insulin receptor dysfunction that caused of proinflammatory cytokine. TNF-α is one of the key pro-inflammatory cytokines that contribute to the development of insulin resistance and T2DM through the activating low-grade inflammation by decreasing glucose transporter-4 (GLUT4). Inflammation in T2DM occurs due to adipocytes that experience hypertrophy due to central obesity, causing tissue death then it triggers the termination of the damage associated molecular pattern (DAMP) and triggers type 1 macrophage to secrete

Table 3 . Parameter validation of T2DM model rats

Parameter	Group	
	Healthy group (n:6)	T2DM model rats (n:18)
Indeks Lee	281.51 ± 4.81	302.60 ± 3.50
Glucose (mmol/L)	5.23 ± 0.30	25.41 ± 0.61
Homa IR	0.68 ± 0.47	1.19 ± 0.16

Note : Data presented as mean ± SD; Homa IR : homeostatic model assessment for insulin resistance

proinflammatory cytokines such as TNF-α and IL-18. The involvement of T cells also secrete IFN-γ which type 1 macrophage will continue to secrete TNF-α and IL-18 that develop local and systemic inflammation which also causes inflammation in the kidney.[20–22]

Various inflammatory parameters predicted of the evolution, initiation, and progression of DKD. Previous study, SH-MSCs showed that the serum levels of TNF-α could be used as an indicator for evaluating the development and progression of diabetes.[23] TNF-α and IL-18 expression were significantly reduced after therapy using the SH-MSCs in T2DM model rats. Several studies also reported that SH-MSCs can decrease the inflammation through the activation of the IL-10 in STAT3 pathways. IL-10 binds to IL-10R activated JAK1 by inducing STAT3 phosphorylation. The STAT3 protein will enter the nucleus and activate the SOSC3 mRNA sequence which will then be expressed intracellularly and can suppress the pro-inflammatory signaling pathway, namely NF-κB. The pressure from the NF-κB pathway will lead to a decrease in the secretion of pro-inflammatory cytokines including TNF-α and IL-18.[24,25] The cytokines IL-10, IF-γ and TGF-β with their cell receptors will program the phosphorylate NADPH which results in the activation of the transcription factor NF-κB pathway through to the mediation of phosphoinositide-3-kinase, MAPK, ERK1/2.[26] Activated NF-κB will migrate into the nucleus, initiate the translation and transcription from various antioxidant gene expressions, including TNF- α and IL-18.

In a T2DM kidney tissue, inflammation is mediated by insulin resistance, high plasma leptin, increased levels of plasma glucose, low levels of adiponectin, and FFAs.[27] The result showed TNF-α expression decreased after SH-MSCs administration. We assume that SH-MSCs contained of IL-10 may reduce the proinflammatory cytokine such as TNF-α and IL-18. Moreover, TNFα and IL-18 also increases ROS levels in kidney cells independently of hemodynamic mechanisms, altering the glomerular capillary wall and, consequently, increasing urinary albumin excretion. Previous studies have shown that TNF-α and IL-18 are involved in the pathogenesis of kidney inflammation in T2DM. TNF-α is known to promote inflammation by inducing the expression of other proinflammatory cytokines, and has been implicated in the development of diabetic nephropathy. IL-18 is also involved in inflammation and has been shown to contribute

Table 4. The results of the RT-PCR test of the gene expression level of TNF- α mRNA in the kidney tissue of each group

Gene expression	Treatment group				p value
	Healthy Rat (n=6)	Control (n=6)	T1 (n=6)	T2 (n=6)	
TNF-α (fold-change)	1.00	6.07 ± 1.45	2.86± 1.25	1.77± 0.59	0.000*
IL-18 (fold-change)	1.00	5.14 ± 1.23	2.12 ± 1.10	0.94± 0.42	0.000*

Notes : Data presented as mean±SD; TNF- α : tumor necrosis factor α; IL-18: interleukin 18; T1: dose 250 µL SH-MSCs; T2: dose 500µL SH-MSCs; *One Way ANOVA test ($p<0.05$ = significant)

Table 5. Results of LSD multiple comparison test

Parameter	Compared	Groups	p-value
TNF-α	Healthy	Control	0.000*
		T1	0.004*
		T2	0.200
	Control	T1	0.000*
		T2	0.000*
	T1	T2	0.074
IL-18	Healthy	Control	0.000*
		T1	0.035*
		T2	0.906
	Control	T1	0.000*
		T2	0.000*
	T1	T2	0.027*

Notes : Healthy : no T2DM, Control = no treatment, T1 = SH-MSCs at a dose of 250µL , T2 = SH-MSCs at a dose of 500µL.
*Significant at $p<0.05$; THF-α : tumor necrosis factor α; IL-18 : interleukin 18

to the development of T2DM. Our findings suggest that the SH-MSCs may be a potential therapeutic option for treating kidney inflammation in T2DM. One possible mechanism for this effect is the ability of the SH-MSC to modulate the immune response and reduce the levels of proinflammatory cytokines in the kidney. Previous studies have shown that MSCs secrete a variety of factors with immunomodulatory properties, including cytokines, chemokines, and growth factors. These factors have been shown to reduce inflammation and promote tissue repair in a variety of diseases.

This study has proven that SH-MSCs have the potential to reduce inflammatory cytokines and promote tissue regeneration. SH-MSCs has the potential to be a future therapy that is effective and low cost for patients with kidney failure or T2DM. Table 5 shows that there are statistically significant differences in the expression of TNF-α and IL-18 genes between the healthy, control, T1, and T2 groups. Based on the results, SH-MSC can be used for clinical trials in patients with kidney disease, especially hemodialysis.

Our study has some limitations. First, we did not investigate the long-term effects of the SH-MSCs on kidney function in T2DM model rats. Additional studies are needed to determine whether the SH-MSCs can improve kidney function in these animals. Second, we did not investigate the mechanisms underlying the effects of the SH-MSCs on TNF-α and IL-18 expression of kidney in serial days. Future studies should investigate these mechanisms to identify potential targets for therapeutic interventions.

CONCLUSION

In conclusion, our study demonstrates that the SH-MSCs has anti-inflammatory effects and reduces the levels of proinflammatory cytokines TNF-α and IL-18 in the kidney of T2DM model rats. These findings suggest that the SH-MSCs may be a potential therapeutic option for treating kidney inflammation in T2DM and support further investigation of the mechanisms underlying the effects of the SH-MSCs on kidney inflammation.

CONFLICT OF INTEREST

The authors declare that there was no conflict of interest.

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AUTHOR CONTRIBUTIONS

RCSI, AP, and ST were responsible for experimental design and developed methodology. RCSI, SSG and NH carried out the experiments. AP and ST interpreted the results, performed data analysis and prepared the figures and tables. RCSI and NH wrote, reviewed, and revised the manuscript. Provided administrative, technical, or material support. AP and ST supervised the study. All authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

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