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Therapeutic Potential of Secretome Hypoxia Mesenchymal Stem Cells: Downregulation of TNF-α and HIF-2α in Metabolic Syndrome-Induced Inflammation in Wistar Rats

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Abstract. Metabolic syndrome (MetS) has become a global health challenge with several associated issues, such as obesity, insulin resistance, dyslipidemia, and hypertension. Important proteins such as Tumor Necrosis Factor-alpha (TNF-α) and Hypoxia Inducible Factor-2 alpha (HIF-2α) regulate the inflammatory process by inducing the expression of pro-inflammatory proteins. This study aims to determine the effect of administering SH-MSCSs on the expression of the TNF-α and Hypoxia Inducible Factor (HIF)-2α genes in the male Wistar rat model with Metabolic Syndrome. This research is an experimental study with a Post-test Only Control Group Design, using a total of 24 male Wistar rats divided into four groups: T1 (Healthy control), T2 (MetS + NaCl), T3 (MetS + administration of SH-MSCs dose 150 uL), and T4 (MetS + administration of SH-MSCs dose 300 uL). SH-MSCSs were administered intraperitoneally four times over 14 days. Adipose tissue TNF-α and HIF-2α gene expression were measured on day 15 using qRT-PCR. TNF-α and HIF-2α gene expression was significantly lower in T3 and T4, compared with the MetS control group (T2). Administration of SH-MSCs was able to reduce the expression of the Tumor Necrosis Factor (TNF-α) and Hypoxia Inducible Factor (HIF)-2α genes in fatty tissue in the male Wistar rat model with Metabolic Syndrome. This study presents a novel approach to treating MetS by demonstrating that the administration of SH-MSCs significantly reduces the expression of pro-inflammatory genes TNF-α and HIF-2α. This finding is beneficial for society as it suggests a potential new therapeutic strategy that could mitigate inflammation and improve health outcomes for individuals suffering from MetS, thereby addressing a critical global health challenge.

Keywords : Metabolic syndrome; Secretome; MSC; TNF-α; HIF-2α.

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INTRODUCTION

Metabolic syndrome (MetS) has emerged as a global health issue characterized by a cluster of interrelated metabolic disorders, including obesity, insulin resistance, dyslipidemia, and hypertension (Li et al., 2023). This complex syndrome significantly elevates the risk of cardiovascular diseases, type 2 diabetes, and other metabolic disorders, imposing a substantial burden on public health systems worldwide. A crucial aspect of MetS pathophysiology involves inflammation and impaired oxygen supply to adipose tissue. Tumor Necrosis Factor-alpha (TNF-α) and hypoxia-inducible factor-2 alpha (HIF-2α) are key proteins in this process (Chen et

al., 2023). TNF-α is a pro-inflammatory cytokine implicated in insulin resistance and adipose tissue dysfunction, while HIF-2α plays a central role in the cellular response to hypoxia, regulating various genes involved in metabolic adaptation and angiogenesis (Castillo-Rodríguez et al., 2022).

Recent research highlights the significance of secretome hypoxia mesenchymal stem cells (SH-MSCSs) due to their active biomolecules, which possess immunomodulatory, anti-inflammatory, and angiogenic properties, in regulating various inflammatory diseases, including MetS (Ferreira et al., 2018; Utami et al., 2023; Vizoso et al., 2017). Data from 2020 indicate that MetS has a prevalence rate of approximately 25% among adults in the United States (Li et al., 2023). Additionally, over the past decade, the prevalence of MetS has increased not only in the United States and Europe but also in Asian countries such as China, India, South Korea, and Indonesia. The rising prevalence of MetS globally affects health quality in every country, including Indonesia (Papaioannu Borjesson et al., 2023). According to the Indonesian Family Life Survey reported in 2019, the current prevalence of MetS in Indonesia is 21.66%, with an estimated incidence rate of around 50% in some provinces (Herningtyas & Ng, 2019). Moreover, the most commonly found MetS components in Indonesia are low high-density lipoprotein (HDL) and hypertension. These MetS conditions can act as triggers or drivers in the development of multiple organ dysfunction (MOD), as several MetS components can negatively impact various organs such as the heart, kidneys, liver, and vascular system. MOD can lead to the dysfunction of two or more organs simultaneously, potentially resulting in prolonged stays in the intensive care unit (ICU) and, in severe cases, death (27%-100%) (Liu et al., 2015).

The bioactive molecules contained in SH-MSCSs play a vital role in intracellular communication, inducing pathological changes and affecting the functions of target cells. This intracellular communication is primarily mediated by growth factors, cytokines, and chemokines, such as Interleukin (IL), Transforming growth factor-β (TGF-β), Platelet-Derived Growth factor (PDGF), and Vascular Endothelial Growth factor (VEGF) (Daryanti et al., 2023; Hartanto et al., 2022; Hidayat et al., 2023). Metabolic syndrome factors can induce a chronic low-grade inflammatory state, characterized by immune cell infiltration, and the production of pro-inflammatory cytokines and chemokines, especially TNF-α, in the adipose tissue environment (Zatterale et al., 2020). Besides inflammation, the pathogenesis of metabolic syndrome is also triggered by hypoxia, hyperplasia, and structural remodeling of blood capillaries through angiogenesis. These processes are regulated by HIF family proteins, such as HIF-1α and HIF-2α. IL-10 and TGF-β are potential anti-inflammatory molecules that control the expression of TNF-α (Masenga et al., 2023).

Previous studies have demonstrated that SH-MSCSs at a dose of 500 µL can reduce IL-6 gene expression and increase SOD in T1DM. Furthermore, in vivo studies have revealed that the secretome of hMSC-EC, or specific proteins enriched in the hMSC-EC secretome, enhances wound healing processes under hyperglycemic conditions (An et al., 2021; Kim et al., 2022). Additionally, hASC and hASC-CM can restore the pro/anti-inflammatory cytokine balance, Th1/Th2 balance, and improve kidney morphology (Darlan et al., 2021, 2022; Kustiyah et al., 2021; Munir et al., 2020). These improvements in MetS conditions can also enhance the repair of MOD. However, the precise molecular mechanisms by which SH-MSCSs regulate and improve MetS remain incompletely studied, particularly at the molecular level. Therefore, this study aims to investigate the effect of SH-MSCSs on the gene expression of TNF-α and HIF-2α in an in vivo experimental design using male Wistar rats as a model for MetS.

METHODS

Study design

This study utilized a post-test-only control group design and was carried out at the Stem Cell and Cancer Research Indonesia in Semarang, Indonesia, from March to May 2024. Approval for the study was obtained from the Ethics Committee of Sultan Agung Islamic University (Approval No. 74/II/2024/Komisi Bioetik).

MSCs culture and isolation.

MSCs were extracted from a pregnant rat at 19 days gestation. The donor rats were anesthetized, and their abdomens were opened. The umbilical cord (UC) was collected under sterile conditions, washed with phosphate-buffered saline (PBS), and the artery and vein were removed. The UC was then cut into pieces measuring 2–5 mm using a sterile scalpel. These sections were placed in T25 flasks with Dulbecco's modified Eagle's medium (DMEM)

(Sigma-Aldrich, St. Louis, MO), supplemented with 10% PBS and 100 IU/ml penicillin/streptomycin (GIBCO, Invitrogen). The cells were incubated at 37°C with 5% CO2, and the medium was changed every three days. Once the cells reached 80% confluence, they were passaged, and MSCs from passages 4 to 6 were used for subsequent experiments (Darlan et al., 2021; Hamra et al., 2021)

Osteogenic and adipogenic differentiation assay of MSCs.

MSCs were cultured in a 24-well plate, with each well containing 1.5 x 10^4 cells in a standard medium composed of DMEM (Sigma-Aldrich, MO), supplemented with 10% FBS (Gibco Invitrogen, NY) and 1% penicillin/streptomycin (Gibco Invitrogen, NY). The culture conditions were maintained at 37°C, 5% CO2, and over 95% humidity. Once the cells reached 80% confluence, differentiation protocols were initiated. For osteogenic differentiation, the medium was replaced with Human MesenCult™ Osteogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore), enhanced with 20% Human MesenCult™ Osteogenic Differentiation 5X Supplement and 1% L-Glutamine (Gibco Invitrogen, NY). The medium was refreshed every three days, and bone matrix formation was visualized using 2% alizarin red staining. For adipogenic differentiation, the cells were switched to Human MesenCult™ Adipogenic Differentiation Basal Medium, with medium changes every other day. On day 35, the cultures were stained with Oil Red O and observed under a microscope.

Characterization of MSCs surface marker.

MSCs were assessed for their expression of specific surface markers using flow cytometry. In brief, the cultured cells were incubated in darkness with primary antibodies, including mouse anti-human CD29, CD90, and a Lin-negative marker (CD45/CD31), followed by a secondary conjugated antibody. The cells were stained with these specific antibodies for 30 minutes at 4°C and then analyzed using a BD Accuri C6 Plus flow cytometer (BD Biosciences, San Jose, CA, USA), with data processed through BD Accuri C6 Plus software (BD Biosciences, San Jose, CA, USA) (Darlan et al., 2022; Hamra et al., 2021).

Hypoxic secretome MSCs (HS-MSCs) Isolation.

Once the MSCs reached 70% confluency in a 75 cm² flask with complete medium, they were rinsed twice with 5 mL of PBS and then incubated in DMEM (Gibco, USA). The cells were placed in a hypoxic chamber (Anaerobic Environment; ThermoForma, Waltham, MA, USA) containing 15 mL of DMEM for 12 hours. This sealed, humidified chamber was kept at 37°C and continuously supplied with a gas mixture of 5% CO2, 10% H2, and 85% N2, maintaining an oxygen level of approximately 0.5%. Following incubation, the hypoxic secretome was collected and centrifuged twice at 1,500 rpm for 3 minutes at 4°C to remove debris and dead cells. The supernatant was then filtered using a tangential flow filtration system (TFF) (Formulatrix, USA) equipped with sterile hollow fiber polyethersulfone membranes with pore sizes allowing for a molecular weight cut-off of 30-50 kDa to eliminate large biomolecules (Putri et al., 2023; Restimulia et al., 2021; Zukhiroh et al., 2022).

SH-MSCs treatment groups

Twenty-four male rats, aged 2-3 months and weighing 200-250 grams, were randomly divided into four groups: K1, K2, K3, and K4, with each group consisting of six rats. T1: Rats receiving standard feed without high-fat diet induction. T2: Rats receiving standard feed and high-fat diet induction with NaCl administration. T3: Rats receiving standard feed and high-fat diet induction with SH-MSCSs administration at 150 µL/200 g body weight, given four times over 14 days. T4: Rats receiving standard feed and high-fat diet induction with SH-MSCSs administration at 300 µL/200 g body weight, given four times over 14 days. On day 15, the rats were euthanized, and abdominal adipose tissue was collected and stored in RNA-later solution for subsequent analysis of TNF-α and HIF-2α gene expression.

High-Fat Diet Induction

The high-fat diet was administered as a high-calorie diet consisting of 50% standard feed, 25% wheat flour, 10% goat fat, 8-9% lard, 5% egg yolk, 1% coconut oil, and 10% NaCl. This diet was provided for 30 days. The nutritional status of the rats was assessed, and metabolic syndrome was confirmed if the Lee index exceeded 300. The high-fat diet was given ad libitum at a rate of 20 g per rat per day for 30 days.

Administration of SH-MSCSs

The SH-MSCSs were administered via intraperitoneal injection to the treatment groups at

doses of 150 µL/200 g body weight and 300 µL/200 g body weight, four times over 14 days (on days 1, 4, 7, and 10). The doses were chosen based on optimization from previous research.

Triglyceride Analysis

The blank, standard, and rat serum samples were prepared. Then, 1000 µL of triglyceride reagent was added to three test tubes. Next, 10 µL of triglyceride standard was added to the tube containing 1000 µL of triglyceride reagent, followed by the addition of 10 µL of serum to the triglyceride reagent. The mixture was incubated for 10 minutes at 37°C. The absorbance was read at 546 nm using a spectrophotometer within 60 minutes.

High-density lipoprotein (HDL) Analysis

A working reagent was prepared by combining 4000 µL of cholesterol reagent with 1000 µL of distilled water in an empty bottle. For the preparation of the supernatant, 500 µL of the working reagent was added to the first tube (standard), and 200 µL of serum was added to the second tube (sample), followed by homogenization. The mixture was incubated for 10 minutes at 20-25°C and then centrifuged at 4000 rpm for 10 minutes. For the HDL cholesterol procedure, three tubes were prepared, with 100 µL of supernatant standard added to the first tube and 100 µL of supernatant sample to the second tube. To all three tubes, 1000 µL of working reagent was added. The mixture was incubated for 10 minutes at 37°C, and the absorbance was read at 546 nm using a spectrophotometer.

Low-density lipoprotein (LDL) Analysis

The blank, standard, and rat serum samples were prepared. Then, 1000 µL of LDL reagent was added to three test tubes. Next, 10 µL of LDL standard was added to the tube containing 1000 µL of LDL reagent, followed by the addition of 10 µL of serum to the LDL reagent. The mixture was incubated for 10 minutes at 37°C, and the absorbance was read at 546 nm using a spectrophotometer.

Total cholesterol Analysis

50 µL of Total Cholesterol Reaction Mix and 50 µL of Free Cholesterol Reaction Mix were prepared for each reaction according to the Cholesterol Assay Kit - HDL and LDL/VLDL (catalog number: ab65390). Then, 20 µL of serum sample was added to each well, followed by the addition of 50 µL of Total Cholesterol Reaction Mix to each well and 50 µL of Free Cholesterol Reaction Mix to each well. The samples were incubated at 37°C for 60 minutes in the dark. The absorbance was read at 570 nm using a spectrophotometer. The total cholesterol concentration was calculated and compared to the standard using the kit's formula.

***TNF-α* and *HIF-2α* gene expression by qRT-PCR**

Total RNA was isolated from rat adipose tissue using TRIzol reagent (Invitrogen, Shanghai, China) following the manufacturer's guidelines. First-strand cDNA synthesis was performed using 1 µg of total RNA with SuperScript II (Invitrogen, Massachusetts, USA). Reverse transcription was conducted on a real-time PCR machine (PCR max Eco 48) utilizing SYBR No ROX Green I dye (SMOBIO Technology Inc, Hsinchu, Taiwan). The mRNA expression levels of TNF-α and HIF-2α genes were quantified with specific primers, and gene expression was measured as the Cycle threshold (Ct). Data analysis was performed using Eco Software v5.0 (Illumina Inc, San Diego, CA, USA), and all reactions were carried out in triplicate. The $2^{-\Delta\Delta}$ Ct method (Livak method) was employed for data analysis (Hartanto et al., 2022; Munir et al., 2023; Paramita et al., 2022).

Table 1. Primer sequence

Primer	Sequences
TNF-α	F: 5'-GCCCTACGGGTCATTGAGAG-3' R: 5'-GAGAGACGACAGACGCAGAC-3'
HIF-2α	F: 5'-GGCAGATCTAACACGCCTTG-3' R: 5'-GAGAGACGACAGACGCAGAC-3'
β-actin	F: 5'-GCCTTCCTTCCTGGGTATG-3' R: 5'-AGGAGCCAGGGCAGTAATC-3'

Statistical analysis

The data are expressed as mean ± standard deviation (SD). To assess the statistical significance of differences between groups, we utilized SPSS version 26.0 (IBM Corp., Armonk, NY, USA) and conducted an ANOVA followed by Fisher's Least Significant Difference (LSD) post-hoc analysis. A p-value of less than 0.05 was considered to indicate statistical significance.

RESULT AND DISCUSSION

The upregulation of TNF-α gene expression in Metabolic Syndrome (MetS) indicates increased transcriptional activity and production of TNF-α molecules in adipose tissue (Masenga et al., 2023). MetS, characterized by various cardiovascular risk factors and metabolic

imbalances resulting from chronic inflammatory conditions, prominently features TNF-α as a key inflammatory mediator. In this research, we utilized mesenchymal stem cells (MSCs), which were evaluated based on their ability to adhere to plastic surfaces under standard culture conditions. By the fourth passage, MSCs displayed adherence with typical monolayers of spindle-shaped, fibroblast-like cells (Figure 1A-B). To verify the differentiation potential of MSCs in vitro, we employed osteogenic and adipogenic differentiation media to assess their ability to transform into osteogenic and adipogenic cells. We observed that MSCs exhibited adipogenic differentiation, as evidenced by the accumulation of neutral lipid vacuoles stained with Oil Red O (Figure 1C). Additionally, a red coloration in the osteogenic differentiation assay indicated calcium deposition, confirming differentiation into osteogenic cells (Figure 1D). Flow cytometric analysis for immunophenotyping showed that MSCs were positive for CD90 (97.80%) and CD29 (98.30%), and negative for CD45 (1.65%) and CD31 (2.30%) (Figure 1E). To stimulate cytokine and growth factor production, MSCs were cultured under hypoxic conditions with 5% oxygen for 12 hours.

SH-MSCSs were isolated from hypoxic MSC culture medium using Tangential Flow Filtration (TFF) with filter chips of 10 kDa and 50 kDa. This fraction contained interleukin-10 (IL-10) and Transforming Growth Factor-β (TGF-β) with molecular weights of approximately 18 kDa and 25 kDa, respectively. The anti-inflammatory cytokines IL-10 and TGF-β present in the SH-MSCs were characterized using the Enzyme-Linked Immunosorbent Assay (ELISA). The concentration of growth factor TGF-B was higher of SH-MSCs compare with IL-10 anti-inflammatory cytokine (Table 2).

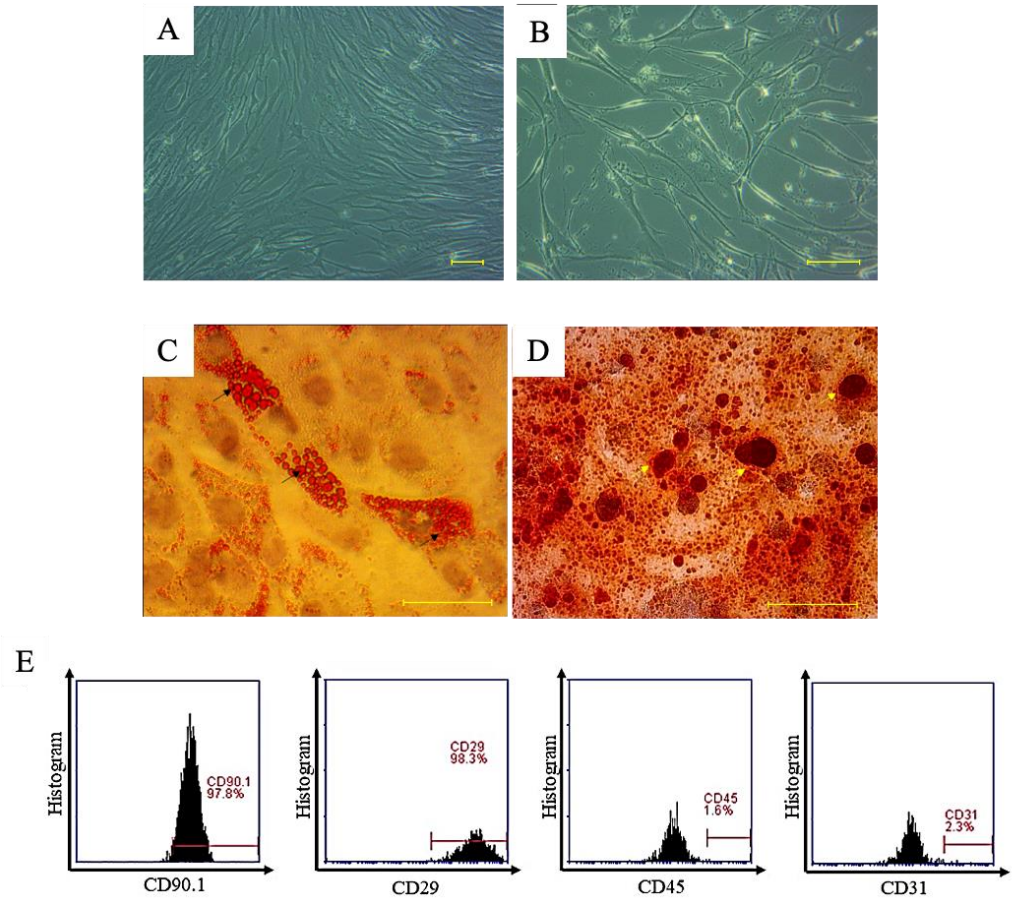

Figure 1. Analysis and confirmation of MSC characteristics. (A) At 10x magnification and (B) 100x magnification, MSCs exhibited a uniform spindle-like morphology. (C) Neutral lipid vacuoles were identified using Oil Red O staining. (D) Calcium deposits were observed in osteogenic differentiation assays, highlighted by Alizarin Red staining. (E) The graphs illustrated the MSC phenotype, showing markers CD90, CD29, CD45, and CD31.

Table 2. Cytokines concentration of SH-MSCs

Protein	Concemtration ± SD (pg/mL)
IL-10	120.03 ± 11.89
TGF-β	733.44 ± 11.47

Metabolic Syndrome (MetS) was induced in the rat model through a high-fat diet with a specific feed composition for 30 days. On the 31st day, the induction of MetS in the rats was validated by measuring serum levels of triglycerides, LDL, HDL, and total cholesterol using spectrophotometry. Rats were confirmed to have MetS if they had a Lee Index >300 and elevated levels of triglycerides, LDL, HDL, and total cholesterol compared to healthy control rats (Table 3). The biochemical validation of serum parameters in MetS rat models demonstrated significant deviations from normal values, confirming the successful induction of MetS.

The expression of TNF-α and HIF-2α genes in adipose tissue samples was measured using qRT-PCR and calculated using the Livak method to obtain Relative quantification (Rq) values. The analysis results indicate that SH-MSCs have a significant ability to reduce the expression of TNF-α and HIF-2α genes in MetS rats, with effects dependent on the administered dose. Figure 2A-B illustrates the data analysis results, where the expression of TNF-α and HIF-2α genes in the T2 group (negative control) showed the highest fold change, while the T4 group exhibited the lowest fold change in gene expression levels. This demonstrates the significant anti-inflammatory effect of SH-MSCs in the MetS model.

Table 3. Biochemical Validation of Serum in Metabolic Syndrome Rat Model

Indicator	Group	
	Healthy group±SD	MetS±SD
Lee index	273.98±4.82	305.06±3.50
Triglycerides (mg/dL)	115.50±19.15	229.33±15.29
LDL (mg/dL)	7.83±1.60	18.00±2.10
HDL (mg/dL)	44.00±2.31	32.67±1.97
Total cholesterol (mg/dL)	56.60±0.57	103.93±1.03

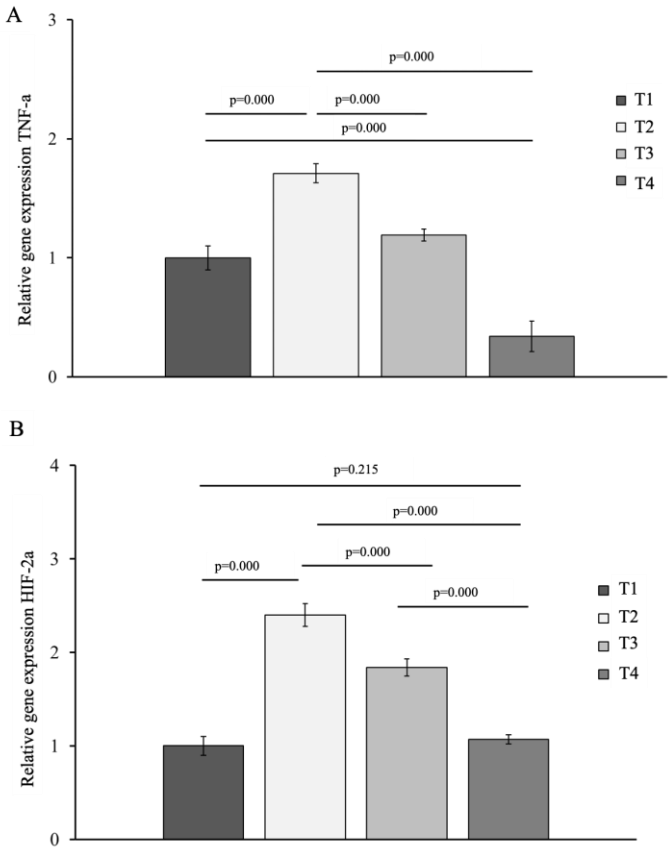

Figure 2. (A) Relative gene expression level of TNF-a and (B) relative gene expression level of HIF-2a. n=6. Values are expressed as mean ± SD. *p < 0.05 indicated significantly different.

TNF-α plays a crucial role in the inflammatory response by activating inflammatory pathways, stimulating the production of other pro-inflammatory molecules, and causing tissue dysfunction, particularly in adipose tissue often associated with metabolic complications (Alicka & Marycz, 2018). This is consistent with data shown in Figure 2, which demonstrates higher TNF-α gene expression in MetS rats induced by a high-calorie diet compared to the healthy group.

The decrease in TNF-α gene expression in groups T3 and T4 is likely due to the anti-inflammatory properties of SH-MSCSs, which can suppress the NF-κB inflammatory pathway. TNF-α gene expression is regulated by a series of molecular events involving various proteins, primarily controlled by the NF-κB pathway (Rahardja et al., 2024). The binding of TNF-α to TNFR1 triggers the formation of different signaling complexes, known as complexes I, IIa, IIb, and IIc, leading to distinct cellular responses (Ebmeyer et al., 2011; Sánchez-Fidalgo et al., 2015). During complex I activation, activated TNFR1 binds to TRADD, followed by the association and interaction of various components, including Receptor-Interacting Serine/Threonine-Protein Kinase 1 (RIPK1), TNFR-associated factor 2 or 5 (TRAF2/5), Cellular Inhibitor of Apoptosis Protein 1 or 2 (cIAP1/2), and the Linear Ubiquitin Chain Assembly Complex (LUBAC) (Holbrook et al., 2019). This signaling pathway activates NF-κB, a transcription factor that translocates to the nucleus to induce the transcription of several pro-inflammatory genes, particularly TNF-α (Jang et al., 2021).

SH-MSCs are believed to suppress TNF-α expression due to their content of anti-inflammatory molecules, especially IL-10 and TGF-β, as shown in Table 5.1. IL-10 binding to IL-10R activates JAK1, inducing STAT3 phosphorylation (Kuntardjo et al., 2019; Liang et al., 2021). The STAT3 protein then enters the nucleus and activates the mRNA sequence of SOSC3 (Suppressor of Cytokine Signaling 3), which is expressed intracellularly and can inhibit the NF-κB pro-inflammatory signaling pathway (Griess et al., 2020; Huang et al., 2020). Suppression of the NF-κB pathway leads to decreased expression and secretion of pro-inflammatory cytokines, including TNF-α and IL-18 (Dong et al., 2015; Giuliani et al., 2018).

The increase in HIF-2α gene expression in MetS conditions indicates enhanced transcriptional activity and production of HIF-2α molecules in adipose tissue (Chan et al., 2019). HIF-2α is a transcription factor involved in the cellular response to hypoxia or oxygen deficiency. Elevated HIF-2α gene expression in MetS may be associated with hypoxia or oxidative stress in the adipose tissue of MetS rats. Increased proliferation and fat accumulation in MetS adipose tissue can elevate oxygen demand (Alicka & Marycz, 2018; Holbrook et al., 2019). Additionally, rapid and excessive adipocyte expansion can cause vascular dysregulation, leading to uneven oxygen distribution within the adipose tissue, triggering micro-hypoxia. This aligns with the data in Figure 2B, which shows a significant increase in HIF-2α gene expression in MetS rats induced by a high-calorie diet compared to the healthy group.

The inhibition of HIF-2α expression by SH-MSCSs is likely due to their anti-inflammatory molecules, particularly IL-10 and TGF-β, through the NF-κB and SMAD pathways. Inactivation of these transcription factors inhibits the transcription of c-Myc (cellular Myelocytomatosis oncogene), a transcription factor for the HIF-2α gene. c-Myc protein can regulate HIF-2α gene expression either by directly binding to the HIF-2α gene promoter sequence or indirectly through the AMP-activated protein kinase (AMPK) pathway (Zhou et al., 2021). Additionally, TGF-β from SH-MSCSs binds to TGFR, activating the SMAD2/3 complex, which subsequently phosphorylates SMAD4 for nuclear translocation as a transcription factor (Ahmadi et al., 2021). SMAD4 directly binds to the c-Myc gene promoter as a repressor. Reduced c-Myc expression correlates with the expression of genes it regulates, particularly HIF-2α. This study has several limitations that need to be addressed for a more comprehensive understanding of the effects of SH-MSCSs on MetS rats (Jang et al., 2021). Firstly, the expression of SOSC3 in the context of NF-κB pathway inhibition after SH-MSCS therapy was not examined. Although this study provided insights into the anti-inflammatory effects at the gene level of TNF-α and HIF-2α, a detailed examination of SOSC3's role in modulating the NF-κB pathway would be essential (He et al., 2014; Sugiyama et al., 2012; Yang et al., 2013). Furthermore, the expression of c-Myc, which may be involved in regulating HIF-2α gene expression after SH-MSCSs administration in MetS rats, was not investigated (Jessika et al., 2024; Mayasari et al., 2023; Restimulia et al., 2022). Understanding c-Myc in this context could offer additional insights into the molecular

mechanisms underlying SH-MSCSs' therapeutic response. Further research is necessary to elucidate the interactions between c-Myc and HIF-2α to better understand their roles in MetS. Thus, additional studies are required to strengthen the understanding of SH-MSCs' therapeutic mechanisms in MetS.

This study presents a novel approach to treating MetS by demonstrating that the administration of SH-MSCs significantly reduces the expression of pro-inflammatory genes TNF-α and HIF-2α. This finding is beneficial for society as it suggests a potential new therapeutic strategy that could mitigate inflammation and improve health outcomes for individuals suffering from MetS, thereby addressing a critical global health challenge.

CONCLUSION

The study highlights the anti-inflammatory effects of SH-MSCSs in Metabolic Syndrome (MetS) rats, evidenced by the reduced expression of TNF-α and HIF-2α genes. The suppression of TNF-α is likely mediated through the NF-κB pathway, aided by anti-inflammatory molecules like IL-10 and TGF-β. Similarly, the reduction in HIF-2α expression is attributed to the inhibition of the NF-κB and SMAD pathways. Despite these findings, further investigation into the roles of SOSC3 and c-Myc in these pathways is necessary to fully elucidate the therapeutic mechanisms of SH-MSCSs in MetS.

REFERENCES

Ahmadi, S. E., Rahimi, S., Zarandi, B., Chegeni, R., & Safa, M. (2021). MYC: a multipurpose oncogene with prognostic and therapeutic implications in blood malignancies. In *Journal of Hematology and Oncology* (Vol. 14, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13045-021-01111-4

Alicka, M., & Marycz, K. (2018). The effect of chronic inflammation and oxidative and endoplasmic reticulum stress in the course of metabolic syndrome and its therapy. In *Stem Cells International* (Vol. 2018). Hindawi Limited. https://doi.org/10.1155/2018/4274361

An, Y. H., Kim, D. H., Lee, E. J., Lee, D., Park, M. J., Ko, J., Kim, D. W., Koh, J., Hong, H. S., Son, Y., Cho, J. Y., Park, J. U., Kim, S. D., & Hwang, N. S. (2021). High-Efficient Production of Adipose-Derived Stem Cell (ADSC) Secretome Through Maturation Process and Its Non-scarring Wound Healing Applications. *Frontiers in Bioengineering and Biotechnology*, *9*. https://doi.org/10.3389/fbioe.2021.681501

Castillo-Rodríguez, R. A., Trejo-Solís, C., Cabrera-Cano, A., Gómez-Manzo, S., & Dávila-Borja, V. M. (2022). Hypoxia as a Modulator of Inflammation and Immune Response in Cancer. In *Cancers* (Vol. 14, Issue 9). MDPI. https://doi.org/10.3390/cancers14092291

Chan, S. M. H., Selemidis, S., Bozinovski, S., & Vlahos, R. (2019). Pathobiological mechanisms underlying metabolic syndrome (MetS)in chronic obstructive pulmonary disease (COPD): clinical significance and therapeutic strategies. *Pharmacology and Therapeutics*, *198*, 160–188. https://doi.org/10.1016/j.pharmthera.2019.02.013

Chen, S., Saeed, A. F. U. H., Liu, Q., Jiang, Q., Xu, H., Xiao, G. G., Rao, L., & Duo, Y. (2023). Macrophages in immunoregulation and therapeutics. In *Signal Transduction and Targeted Therapy* (Vol. 8, Issue 1). Springer Nature. https://doi.org/10.1038/s41392-023-01452-1

Darlan, D. M., Munir, D., Putra, A., Alif, I., Amalina, N. D., Jusuf, N. K., & Putra, I. B. (2022). Revealing the decrease of indoleamine 2,3-dioxygenase as a major constituent for B cells survival post-mesenchymal stem cells co-cultured with peripheral blood mononuclear cell (PBMC) of systemic lupus erythematosus (SLE) patients. *Medicinski Glasnik*, *19*(1). https://doi.org/10.17392/1414-21

Darlan, D. M., Munir, D., Putra, A., & Jusuf, N. K. (2021). MSCs-released TGFβ1 generate CD4+CD25+Foxp3+ in T-reg cells of human SLE PBMC. *Journal of the Formosan Medical Association*, *120*(1), 602–608. https://doi.org/10.1016/j.jfma.2020.06.028

Daryanti, E., Putra, A., Sumarawati, T., Amalina, N. D., Prasetio, A., & Sidiq, H. A. (2023). The Comparison of Normoxic and Hypoxic Mesenchymal Stem Cells in Regulating Platelet-derived Growth Factors and Collagen Serial Levels in Skin Excision Animal Models. *Open Access Macedonian Journal of Medical Sciences*, *11*(A), 181–187. https://doi.org/10.3889/oamjms.2023.10966

Dong, Q. M., Ling, C., Chen, X., & Zhao, L. (2015). Inhibition of tumor necrosis factor-α enhances apoptosis induced by nuclear factor-κB inhibition in leukemia cells. *Oncology Letters*, *10*(6), 3793–3798. https://doi.org/10.3892/ol.2015.3786

Ebmeyer, J., Leichtle, A., Hernandez, M., Ebmeyer, U., Husseman, J., Pak, K., Sudhoff, H., Broide, D., Wasserman, S. I., & Ryan, A. F. (2011). TNFA deletion alters apoptosis as well as caspase 3 and 4 expression during otitis media. *BMC Immunology*, *12*. https://doi.org/10.1186/1471-2172-12-12

Ferreira, J. R., Teixeira, G. Q., Santos, S. G., Barbosa, M. A., Almeida-Porada, G., & Gonçalves, R. M. (2018). Mesenchymal stromal cell secretome: Influencing therapeutic potential by cellular pre-conditioning. *Frontiers in Immunology*, *9*(December), 1–17. https://doi.org/10.3389/fimmu.2018.02837

Giuliani, C., Bucci, I., & Napolitano, G. (2018). The role of the transcription factor Nuclear Factor-kappa B in thyroid autoimmunity and cancer. *Frontiers in Endocrinology*, *9*(AUG), 1–8. https://doi.org/10.3389/fendo.2018.00471

Griess, B., Mir, S., Datta, K., & Teoh-fitzgerald, M. (2020). Free Radical Biology and Medicine Scavenging reactive oxygen species selectively inhibits M2 macrophage polarization and their pro-tumorigenic function in part , via Stat3 suppression. *Free Radical Biology and Medicine*, *147*(December 2019), 48–60. https://doi.org/10.1016/j.freeradbiomed.2019.12.018

Hamra, N. F., Putra, A., Tjipta, A., Amalina, N. D., & Nasihun, T. (2021). Hypoxia mesenchymal stem cells accelerate wound closure improvement by controlling α-smooth muscle actin expression in the full-thickness animal model. *Open Access Macedonian Journal of Medical Sciences*, *9*, 35–41. https://doi.org/10.3889/oamjms.2021.5537

Hartanto, M. M., Prajoko, Y. W., Putra, A., & Amalina, N. D. (2022). The Combination of Mesenchymal Stem Cells and Bovine Colostrum in Reducing α-SMA Expression and NLR Levels in Wistar Rats After 50% Fibrotic Liver Resection. *Open Access Macedonian Journal of Medical Sciences*, *10*(A), 1634–1639. https://doi.org/10.3889/oamjms.2022.10557

He, T., Quan, T., Shao, Y., Voorhees, J. J., & Fisher, G. J. (2014). Oxidative exposure impairs TGF-β pathway via reduction of type II receptor and SMAD3 in human skin fibroblasts. *Age*, *36*(3), 1079–1094. https://doi.org/10.1007/s11357-014-9623-6

Herningtyas, E. H., & Ng, T. S. (2019). Prevalence and distribution of metabolic syndrome and its components among provinces and ethnic groups in Indonesia. *BMC Public Health*, *19*(1). https://doi.org/10.1186/s12889-019-6711-7

Hidayat, M., Kusumo, B., Husain, S. A., Amalina, N. D., & Id, H. A. (2023). Secretome MSCs restore α-Smooth Muscle Actin Protein Tissue Expression in Croton Oil-Induced Hemorrhoid rats. In *International Journal of Cell and Biomedical Science* (Vol. 2, Issue 4).

Holbrook, J., Lara-Reyna, S., Jarosz-Griffiths, H., & McDermott, M. (2019). Tumour necrosis factor signalling in health and disease. In *F1000Research* (Vol. 8). F1000 Research Ltd. https://doi.org/10.12688/f1000research.17023.1

Huang, Y. H., Chen, M. H., Guo, Q. L., Chen, Z. X., Chen, Q. D., & Wang, X. Z. (2020). Interleukin-10 induces senescence of activated hepatic stellate cells via STAT3-p53 pathway to attenuate liver fibrosis. *Cellular Signalling*, *66*(October 2019), 109445. https://doi.org/10.1016/j.cellsig.2019.109445

Jang, D. I., Lee, A. H., Shin, H. Y., Song, H. R., Park, J. H., Kang, T. B., Lee, S. R., & Yang, S. H. (2021). The role of tumor necrosis factor alpha (Tnf-α) in autoimmune disease and current tnf-α inhibitors in therapeutics. In *International Journal of Molecular Sciences* (Vol. 22, Issue 5, pp. 1–16). MDPI AG. https://doi.org/10.3390/ijms22052719

Jessika, C., Putra, A., & Sumarawati, T. (2024). Therapeutic Potential of Secretome from Hypoxic-Mesenchymal Stem Cell (SH-MSC) in Regulating PDGF and IL-1β Gene Expression in Fluconazole-Related Alopecia. *Biosaintifika: Journal of Biology & Biology Education*, *16*(1), 10–22. https://doi.org/10.15294/biosaintifika.v15i1.3499

Kim, J. H., Green, D. S., Ju, Y. M., Harrison, M., Vaughan, J. W., Atala, A., Lee, S. J., Jackson, J. D., Nykiforuk, C., & Yoo, J. J. (2022). Identification and characterization of stem cell secretome-based recombinant

proteins for wound healing applications. *Frontiers in Bioengineering and Biotechnology*, *10*. https://doi.org/10.3389/fbioe.2022.954682

Kuntardjo, N., Dharmana, E., Chodidjah, C., Nasihun, T. R., & Putra, A. (2019). TNF-α-Activated MSC-CM Topical Gel Effective in Increasing PDGF Level, Fibroblast Density, and Wound Healing Process Compared to Subcutaneous Injection Combination. *Majalah Kedokteran Bandung*, *51*(1), 1–6. https://doi.org/10.15395/mkb.v51n1.1479

Kustiyah, A. R., Putra, A., Nasihun, T., & Ramasamy, R. (2021). *The Normal Ratio of Th17 and Th1 Post-mesenchymal Stem Cells Coculture with PBMCs of Systemic Lupus Erythematosus Patients*. *9*, 169–176.

Li, W., Qiu, X., Ma, H., & Geng, Q. (2023). Incidence and long-term specific mortality trends of metabolic syndrome in the United States. *Frontiers in Endocrinology*, *13*. https://doi.org/10.3389/fendo.2022.1029736

Liang, W., Chen, X., Zhang, S., Fang, J., Chen, M., Xu, Y., & Chen, X. (2021). Mesenchymal stem cells as a double-edged sword in tumor growth: focusing on MSC-derived cytokines. In *Cellular and Molecular Biology Letters* (Vol. 26, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s11658-020-00246-5

Liu, X., Tao, L., Cao, K., Wang, Z., Chen, D., Guo, J., Zhu, H., Yang, X., Wang, Y., Wang, J., Wang, C., Liu, L., & Guo, X. (2015). Association of high-density lipoprotein with development of metabolic syndrome components: A five-year follow-up in adults. *BMC Public Health*, *15*(1). https://doi.org/10.1186/s12889-015-1747-9

Masenga, S. K., Kabwe, L. S., Chakulya, M., & Kirabo, A. (2023). Mechanisms of Oxidative Stress in Metabolic Syndrome. In *International Journal of Molecular Sciences* (Vol. 24, Issue 9). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/ijms24097898

Mayasari, Y. I., Subchan, P., Putra, A., Chodijah, C., Hussana, A., Sumarawati, T., Amalina, N. D., & Irawan, R. C. S. (2023). Secretome Hypoxia Mesenchymal Stem Cells Inhibited Ultraviolet Radiation by Inhibiting Interleukin-6 through Nuclear Factor-Kappa Beta Pathway in Hyperpigmentation Animal Models. *Open Access Macedonian Journal of Medical Sciences*, *11*(A), 188–194. https://doi.org/10.3889/oamjms.2023.11222

Munir, D., Lubis, R. R., Darlan, D. M., Putra, A., & Alif, I. (2020). The role of mesenchymal stem cells in decreasing interleukin-12 human systemic lupus erythematosus. *Open Access Macedonian Journal of Medical Sciences*, *8*(A), 787–792. https://doi.org/10.3889/oamjms.2020.5120

Munir, D., Nasution, I. P., Restimulia, L., Putra, A., & Amalina, N. D. (2023). The role of mesenchymal stem cells in allergic rhinitis and its relationship with IL-10, plasma cells and regulatory T cells. *Medicinski Glasnik*, *20*(2), 175–180. https://doi.org/10.17392/1541-23

Papaioannu Borjesson, R., Galli, L., Muccini, C., Poli, A., Clemente, T., Bottanelli, M., Gianotti, N., Nozza, S., Castagna, A., & Spagnuolo, V. (2023). Increasing incidence and prevalence of metabolic syndrome in people living with HIV during the COVID-19 pandemic. *Frontiers in Medicine*, *10*. https://doi.org/10.3389/fmed.2023.1220631

Paramita, D. A., Hermansyah, D., Paramita, D. A., & Amalina, N. D. (2022). Regulation of p53 and survivin by Curcuma longa extract to caspase-3 dependent apoptosis in triple negative breast cancer cells. *Medicinski Glasnik*, *19*(2), 189–196. https://doi.org/10.17392/1453-22

Putri, R. S., Putra, A., Chodidjah, Darlan, D. M., Trisnadi, S., Thomas, S., Amalina, N. D., & Irawan, R. C. (2023). Clitorea ternatea flower extract induces platelet-derived growth factor (Pdgf) and gpx gene overexpression in ultraviolet (uv) b irradiationinduced collagen loss. *Medicinski Glasnik*, *20*(1), 15–21. https://doi.org/10.17392/1530-22

Rahardja, C. K., Mulyani, S. P., & Putra, A. (2024). Secretome of Hypoxic Mesenchymal Stem Cells Improves Fluconazole-Induced Alopecia in Rats via Immunoregulatory Modulation of IL-15 and IFN-γ. *Biosaintifika: Journal of Biology & Biology Education*, *16*(1), 23–36. https://doi.org/10.15294/biosaintifika.v15i1.3500

Restimulia, L., Ilyas, S., Munir, D., Putra, A., Madiadipoera, T., Farhat, F., Sembiring, R. J., Ichwan, M., Amalina, D., & Alif, I. (2021). *The CD4+CD25+FoxP3+ Regulatory T Cells Regulated by MSCs Suppress Plasma Cells in a Mouse Model of Allergic Rhinitis 256 The CD4+CD25+FoxP3+ Regulatory T Cells Regulated by MSCs Suppress Plasma Cells*

in a Mouse Model of Allergic Rhinitis. https://doi.org/10.5455/medarh.2021.75

Restimulia, L., Ilyas, S., Munir, D., Putra, A., Madiadipoera, T., Farhat, F., Sembiring, R. J., Ichwan, M., & Amalina, N. D. (2022). Rats' umbilical-cord mesenchymal stem cells ameliorate mast cells and Hsp70 on ovalbumin-induced allergic rhinitis rats. *Medicinski Glasnik*, *19*(1). https://doi.org/10.17392/1421-21

Sánchez-Fidalgo, S., Villegas, I., Rosillo, M. Á., Aparicio-Soto, M., & de la Lastra, C. A. (2015). Dietary squalene supplementation improves DSS-induced acute colitis by downregulating p38 MAPK and NFkB signaling pathways. *Molecular Nutrition and Food Research*, *59*(2), 284–292. https://doi.org/10.1002/mnfr.201400518

Sugiyama, Y., Kakoi, K., Kimura, A., Takada, I., Kashiwagi, I., Wakabayashi, Y., Morita, R., Nomura, M., & Yoshimura, A. (2012). Smad2 and smad3 are redundantly essential for the suppression of inos synthesis in macrophages by regulating IRF3 and STAT1 pathways. *International Immunology*, *24*(4), 253–265. https://doi.org/10.1093/intimm/dxr126

Utami, A., Putra, A., Wibowo, J. W., Amalina, N. D., & Satria Irawan, R. C. (2023). Hypoxic secretome mesenchymal stem cells inhibiting interleukin-6 expression prevent oxidative stress in type 1 diabetes mellitus. *Medicinski Glasnik : Official Publication of the Medical Association of Zenica-Doboj Canton, Bosnia and Herzegovina*, *20*(2). https://doi.org/10.17392/1538-23

Vizoso, F. J., Eiro, N., Cid, S., Schneider, J., & Perez-Fernandez, R. (2017). Mesenchymal stem cell secretome: Toward cell-free therapeutic strategies in regenerative medicine. *International Journal of Molecular Sciences*, *18*(9). https://doi.org/10.3390/ijms18091852

Yang, H., Li, G., Wu, J. J., Wang, L., Uhler, M., & Simeone, D. M. (2013). Protein kinase a modulates transforming growth factor-β signaling through a direct interaction with Smad4 protein. *Journal of Biological Chemistry*, *288*(12), 8737–8749. https://doi.org/10.1074/jbc.M113.455675

Zatterale, F., Longo, M., Naderi, J., Raciti, G. A., Desiderio, A., Miele, C., & Beguinot, F. (2020). Chronic Adipose Tissue Inflammation Linking Obesity to Insulin Resistance and Type 2 Diabetes. In *Frontiers in Physiology* (Vol. 10). Frontiers Media S.A. https://doi.org/10.3389/fphys.2019.01607

Zhou, Y., Gao, X., Yuan, M., Yang, B., He, Q., & Cao, J. (2021). Targeting Myc Interacting Proteins as a Winding Path in Cancer Therapy. In *Frontiers in Pharmacology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fphar.2021.748852

Zukhiroh, Z., Putra, A., Chodidjah, C., Sumarawati, T., Subchan, P., Trisnadi, S., Hidayah, N., & Amalina, N. D. (2022). Effect of Secretome-Hypoxia Mesenchymal Stem Cells on Regulating SOD and MMP-1 mRNA Expressions in Skin Hyperpigmentation Rats. *Open Access Macedonian Journal of Medical Sciences*, *10*(A), 1–7. https://doi.org/10.3889/oamjms.2022.10348