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ORIGINAL ARTICLE

Effect of MSCs on vascular endothelial growth factor (VEGF), C-reactive protein (CRP), tumour necrosis factor-alpha (TNF-α), triglyceride, pancreatic beta cells and insulin resistance in obese type 2 diabetic rat model

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ABSTRACT

Aim To investigate the potential of SG followed by injection of MSCs in type 2 diabetic rats with obesity in improving IR.

Methods This study used a pre and post-control group design with 24 rats divided into three groups: control (C), SG, and SG + MSCs (SG+M). On day 10 the levels of vascular endothelial growth factor (VEGF), tumour necrosis factor-alpha (TNF-α), C-reactive protein (CRP), triglyceride (TG), pancreatic beta cells, and homeostasis model assessment of IR (HOMA-IR) were evaluated using qRT-PCR, ELISA, and immunohistochemistry.

Results A significant decrease of TNF-α (1.179 pg/mL), CRP (209 pg/mL), and TG levels (82,8315,02 pg/mL) in all treatment groups on day 10, in which SG + M group showed optimum inhibition, was found ($p<0.05$). This result was in line with the optimum increase of VEGF (8.1500±2.47397) and pancreatic beta cell (10.1783±0.47) in SG + M group ($p<0.05$). Moreover, our study also revealed the optimum decrease of HOMA-IR in SG+M group on day 10 (49.8233±1.07303).

Conclusion A combination of SG and MSCs can optimally improve insulin resistance by inhibiting TNF-α, CRP, and TG level and upregulating VEGF and pancreatic beta cell in an obese T2DM rat model.

Keywords: adipose tissue, bariatric surgery, cytokines, progenitor cells, sleeve gastrectomy

INTRODUCTION

The accumulation of body fat in obesity can cause adipose cell hypertrophy and cell hypoxia. This can increase the production of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF)-α and IL-6 that make the body undergo low-grade chronic inflammation, which can be identified through blood C-reactive protein (CRP) level (1). The cells hypertrophy can also be associated with reducing adipose tissue capacity as a storage area for energy in the form of triglycerides (TG) when the body is in the process of eating and releasing free fatty acids (FFA) in a fasting state (2). High level of pro-inflammatory cytokines in the body are among the causes of impaired insulin signalling through several pathways, resulting in insulin resistance or T2DM (3).

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Surgery is currently an alternative for weight loss; one example is bariatric surgery sleeve gastrectomy. The principle of bariatric surgery itself is to slow down gastric emptying thereby reducing food intake (2). Mesenchymal Stem Cells (MSCs) are an adjuvant therapy for bariatric surgery; the formation of new blood vessels is influenced by the ability of MSCs to differentiate into endothelial cells, secrete soluble factors including angiogenic factors, and the formation of vascular smooth muscle that plays a role in bringing together endothelial vessel walls (4). MSCs can also increase endothelial cell proliferation and vascular permeability (5). Vascular endothelial growth factor (VEGF) is a glycoprotein that plays an important role in vasculogenesis during embryogenesis and the formation of new blood vessels (angiogenesis), the presence of VEGF is a parameter to assess vascular growth in the pancreas (5).

The chronic inflammation that occurs in the obese body will cause insulin resistance. It is a condition where normal level of insulin does not have the same effect on muscle and adipose cells. It makes glucose levels remain higher. To compensate for this

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phenomenon, the pancreas in insulin-resistant individuals is stimulated to release more insulin to decrease blood glucose (6). In some people with T2DM, the initial damage appears in pancreatic beta cells and is manifested as impaired insulin secretion (7).

One therapy option to resolve obesity is using a surgical method called sleeve gastrectomy (SG) (8). Previous studies have stated that SG could reduce BMI and improve glucose tolerance, but it is not followed by a decrease in inflammatory markers in adipose tissue in over 12 months (9). Therefore, adjuvant therapy is needed to suppress the inflammation in the patient's body. Potential biologic therapy that can be used is the injection of MSCs (10).

MSCs are multipotent adult progenitor cells that can regenerate an immune response (11). Stem cells can degenerate, and they can repair or replace damaged tissue along with the formation of nerve and muscle tissue. These stem cells differentiate into certain cells and induce growth factors that can potentially affect physiological processes locally and systemically (12). MSCs home to the site of inflammation in the body and release anti-inflammatory mediators so the inflammation and insulin resistance that occurs can be reduced (13). According to Qi (2019) after the injection of adipose-derived MSCs (AD-MSCs) in rats that suffer T2DM, there was an improvement in insulin sensitivity, also a significant decrease in TNF-α and TG (14). Those results are caused by the cells ability to differentiate MSCs into insulin-producing cells (IPCs), which restore insulin levels and glucose uptake (14). The reduction of the inflammatory state drag down the blood CRP level (15). Some research led to the conclusion that mesenchymal stem cells act as a pro-angiogenic agent and immunomodulator in the regeneration of beta cells pancreas, and MSCs produce several cytokines and growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1, hepatocyte growth factor (HGF), and others (10). When interacting with their site of implantation in the tissue, the resulting factors have anti-apoptotic, mitogenic, and angiogenic effects (5).

Previous research by our group demonstrated that combining sleeve gastrectomy with mesenchymal stem cells could modulate the pro-inflammatory environment in obese diabetic rats (7). **This study extends our previous work by analysing additional molecular markers and observing enhanced effects of SG+MSCs in the early phase.**

The aim of this study was to determine the effect of MSCs on VEGF, CRP, TNF-α, triglyceride pancreatic beta cell and insulin resistance in obese type 2 diabetic rat model.

MATERIALS AND METHOD

Materials and study design

Twenty-four male Wistar rats weighing 170-200g were maintained from the Integrated Research and Development Laboratory, Gadjah Mada University, Yogyakarta, Indonesia in the period between November and December 2021. Rodents were confined at 24±2 °C and 60% relative humidity, with a 12:12 hour light-dull cycle (research facility standard). Every one of the animals was empathetically treated as per the rules for the creature care and the conventions supported by the Ethics Committee of Health Studies, Faculty of Medicine, Sultan Agung Islamic University, Semarang, Indonesia (permit number: No. 238/VIII/2021/Komisi Bioetik).

The rats were randomly divided into three groups after a week of adaptation with each group having eight rats (n=8): control group (C), SG only (SG), and SG followed with 1cc site intraperitoneal injection containing 1×10^6 of MSCs (SG+M). Sample size was determined based on previous studies investigating similar inflammatory and metabolic endpoints in rat's models of obesity and T2DM (16).

Rats were randomly assigned to the treatment groups to minimize bias. Rats were fed with high caloric and fatty diet consisting of 60% Comfeed pars, 27.8% starch, 2% cholesterol, 0.2% cholic acid, 10% lard, and 2cc fructose/day. On days between 26 and 28, the animal model was induced by injecting the rats with 230 mg/kg nicotinamide (NA) intraperitoneally 15 min before a single dose of intraperitoneal administration of 65 mg/kg streptozotocin (STZ)(17). After four weeks of inducing obesity and diabetes, the rats were validated to assess obesity using the Lee index (18) and IR using blood samples taken from the tail vein or lateral vein, and the homeostasis model assessment of insulin resistance (HOMA-IR) (19) was calculated using insulin and glucose from blood samples. Rats were considered obese if the lee index was >300 and considered T2DM if the HOMA-IR was >2.

Methods

Sleeve gastrectomy. Three days after STZ induction, the SG and SG+M group were getting an SG procedure with the method previously described by Ekasaputra et al (2022) with slight modification (7). The abdominal cavity rats on SG and SG+ M groups were opened with an oblique left subcostal incision under general anaesthesia using 20 mg/kg body weight of ketamine hydrochloride. Stomach was dissected above the major curvature line until it remained 50% of its volume. The residual stomach was then closed using a continuous suture of 5–0 PDS. The abdominal wall was closed with a whole layer simple suture 3-0 Vicryl (7).

Isolation and culture of MSCs. Mesenchymal stem cells (MSCs) were extracted from the Wharton's jelly of rat umbilical cords. The transport medium used phosphate buffer solution (PBS) (Gibco TM Invitrogen, NY, USA) with 5% Penstrep antibiotic. Mesenchymal stem cells (MSCs) were extracted from the Wharton's jelly of rats' umbilical cords. The tissue was finely minced and transferred into 75 cm² culture flasks pre-filled with ulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Louis St., MO) supplemented with antibiotics 100 IU/cc penicillin/streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS) (Gibco) TM Invitrogen, NY, USA). The cultured Wharton's Jelly was incubated at 37 °C and 5% O_2. The MSCs typically appeared within 7 to 10 days; the medium was changed in 3-day intervals. After reaching 80% confluence, the MSCs were passaged by trypsin then the fourth passage was used for the experiment (20).

Characterization of MSCs. To confirm the MSC-like surface antigens, plastic adherent stromal cells at the fourth passage were characterized by flow cytometry assays. After trypsinized and pelleted, the cells were subsequently incubated using fluorescein allophycocyanin (APC)-, isothiocyanate (FITC)-, phycoerythrin (PE), and peridinin-chlorophyll-protein (perCP)-Cy5.5.1- conjugated anti-rat CD90.1, CD29, CD31, and CD45 antibodies (BD Bioscience, San Jose, CA, USA) for 30 min at room temperature in the dark. An isotype-specific conjugated anti-IgG (BD Bioscience, San Jose, CA, USA) was used as the negative control. The cells were then washed twice using PBS. The analyses were performed using a BD Accuri C6 Plus flow cytometer (BD Bioscience, San Jose, CA, USA). The post-acquisition

analysis was performed using the BD Accuri C6 Plus software (BD Bioscience, San Jose, CA, USA)(21).

Osteogenic and adipogenic differentiation of MSCs. We further performed the osteogenic differentiation assay in the fifth passage. The MSC-like cells were cultured in a standard medium containing DMEM with 10% FBS, 1% penicillin (100 U/mL)/ streptomycin (100 μg/mL), and 0.25% amphotericin B at 37°C and 5% CO2 until reaching 95% confluency. Then, the standard medium was replaced using an osteogenic differentiation medium containing Rat MesenCult Osteogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore) with 20% Rat MesenCult Osteogenic Differentiation 5X Supplement (Stem Cell Technologies, Singapore), 1% L-glutamine (Gibco Invitrogen, NY, USA), 1% penicillin (100 U/mL)/streptomycin (100 μg/mL), and 0.25% amphotericin B. For inducing adipogenic differentiation, the UC-MSC-like with 95% confluency were cultured using adipogenic differentiation medium made of Rat MesenCult MSC Basal Medium (Stem Cell Technologies), Rat MesenCult Adipogenic Differentiation Supplement (Stem Cell Technologies), 1% L-Glutamine (GibcoInvitrogen), 1% penicillin/ streptomycin (100 U/mL; respectively) (Gibco Invitrogen), and 0.25% amphotericin B (62.5 μg/ mL) (Gibco Invitrogen). The medium was changed every 3 days. Calcium and lipid deposition was shown by alizarin red staining and oil red O staining, respectively (Sigma-Aldrich, Louis St, MO), followed by 21 days of induction.

MSCs administration. The SG+M group was treated after the SG procedure by MSCs intraperitoneally at doses of 1×10^6. On the other hand, the control and the SG groups intraperitoneally received sodium chloride (NaCl).

Blood glucose monitoring. Seven days after the induction of STZ and ten days after SG and MSCs injection, the blood sample was taken from infraorbital venous rats. The measurement was performed on day 10 post-treatment to allow adequate time for both surgical recovery and therapeutic response to MSCs. This time point was chosen based on previous studies indicating that the acute post-operative inflammatory phase typically subsides within 5–7 days, allowing for clearer differentiation of the treatment effects from those attributable to transient surgical intervention (7,22). Fasting plasma glucose levels were determined by blood samples using glucose strips and devices from the Accu-Chek (Roche, Basel, Switzerland).

TNF-α, CRP, insulin, and HOMA-IR level analysis. The serum from blood samples on day 10 was separated and analysed by ELISA kit. The assay was performed at room temperature according to the manufacturer's instructions (Sigma-Aldrich). The results were analysed at a wavelength of 450 Å using a microplate reader (Bio-Rad, CA, USA). Specifically, HOMA-IR was used to evaluate IR before and after the treatment using the following formula HOMA-IR= (fasting insulin μU/mL×-fasting glucose mg/dL/)/22.5 × 18.

Pancreatic beta cell analysis. The slide of pancreas organ samples on day 10 was analysed by immunohistochemistry (IHC).

TNF-α and VEGF level analysis. The RNA from pancreas organ samples at day 10 was separated and analysed by qRT-PCR. The assay was performed at room temperature according to the manufacturer's instructions (Sigma-Aldrich). The results were analysed using Kappa SYBR Fast Master Mix 2x (KAPA Biosystem, KK4600, Massachusets USA).

Statistical analysis

The data were shown in mean±standard deviation (SD). For intergroup comparison, data were first assessed for normality using the Shapiro–Wilk test. For normally distributed data, one-way ANOVA followed by LSD post-hoc was used. For non-parametric data, Kruskal–Wallis test followed by Mann-Whitney test was applied. All results are expressed as mean ± SD and $p<0.05$ was considered statistically significant.

RESULTS

Characterization of Human MSCs (H-MSCs)

MSCs derived from rat umbilical cord were cultured through five passages under standard conditions, exhibiting a typical spin-

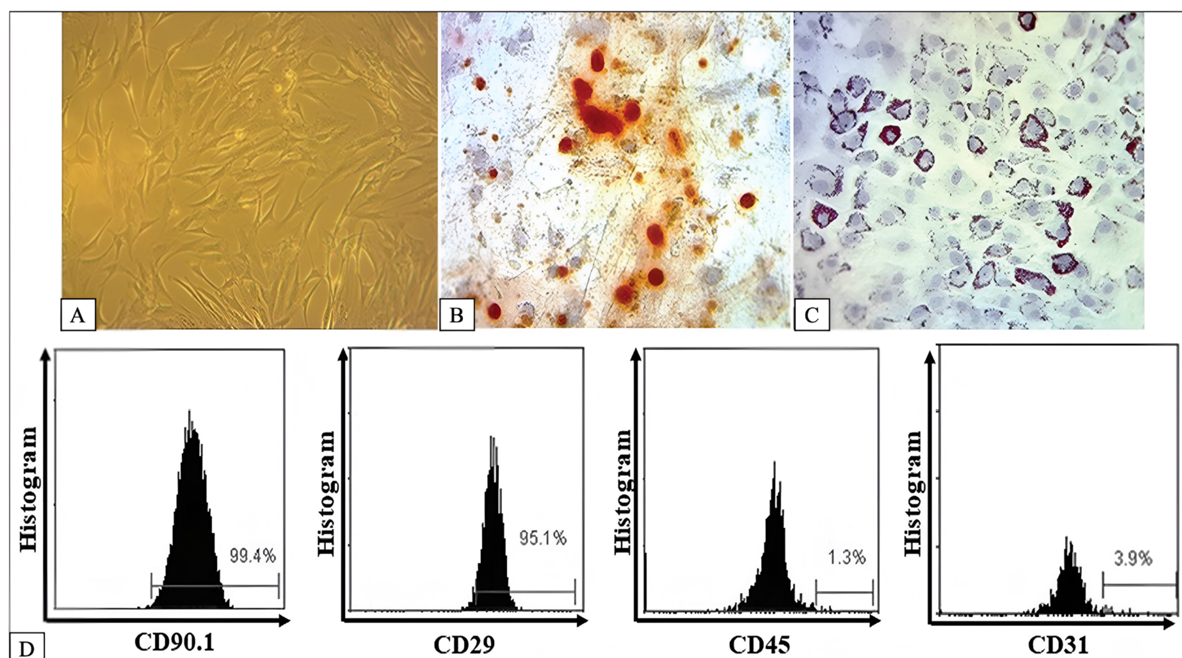

Figure 1. A) In vitro culture of human MSCs (H-MSC) showed spindle-like shaped characteristic (magnification 100x); B) osteogenic and C) adipogenic differentiation of H-MSCs was evidenced by calcium and adipose deposition appearance as a matrix visualized by red colour using Alizarin red and oil red staining, respectively (scale bar 50 μm) (magnification 100x); D) Immunophenotyping characterization of H-MSCs showed a high level of CD90.1, CD29, and low expression of CD45, CD31

dle-shaped fibroblast morphology (Figure 1A). Differentiation into osteogenic and adipogenic lineages was confirmed through calcium and lipid staining using Alizarin Red and Oil Red O, respectively, demonstrating their multipotency (Figure 1B and 1C). Flow cytometry revealed strong surface expression of CD90.1 (99.4%) and CD29 (96.9%), with minimal detection of hematopoietic markers CD45 (1.9%) and CD31 (3.7%) (Figure 1 D).

Reduction in TNF-α levels with combined SG and MSCs treatment

To investigate the capacity of SG and its combination with MSCs in suppressing proinflammatory cytokine, the level of TNF-α was measured with ELISA and qRT-PCR 10 days after the treatments. As the data were normally distributed and showed homogenous variances, one-way ANOVA followed by LSD post hoc test was applied. According to ELISA results, all treated groups exhibited a statistically significant reduction in TNF-α levels when compared to the control group ($p<0.05$). The greatest reduction in TNF-α was observed in the SG+M group, reaching a concentration of 1.179 pg/mL (Figure 2A). PCR test showed that there was a significant decrease of TNF-α level in all treatment groups, compared to the control group ($p<0.05$). The optimum decrease of TNF-α was shown in SG + M group with a mean 0.13±0.07 (Figure 2B).

Combination of SG and MSCs optimally downregulates CRP and TG levels

To investigate the capacity of SG and its combination with MSCs in suppressing CRP and TG, the level of CRP and TG was measured with ELISA 10 days after the treatments. ELISA assay showed that there was a significant decrease of CRP level in all treatment groups, compared to the control group ($p<0.05$). The optimum decrease of CRP was shown in SG+M group with 209 pg/mL (Figure 3A). In the TG levels the data did not meet normality assumptions, so the Kruskal–Wallis was test followed by Mann–Whitney U test. ELISA assay also showed a significant decrease of the TG level in all treatment groups compared to the control group ($p<0.05$). The optimum decrease of TG was shown in SG+M group with 82.8315.02 pg/mL (Figure 3B).

Increased VEGF level and pancreatic beta cell restoration with the combination of SG and MSCs

Quantitative RT-PCR analysis was demonstrated in 10 days and showed significant upregulation of VEGF gene expression in all treatment arms, with the highest increase recorded in the SG+MSC group (8.15 ± 2.47; $p < 0.05$) (Figure 4A). Immunohistochemical evaluation revealed a parallel increase in pan-

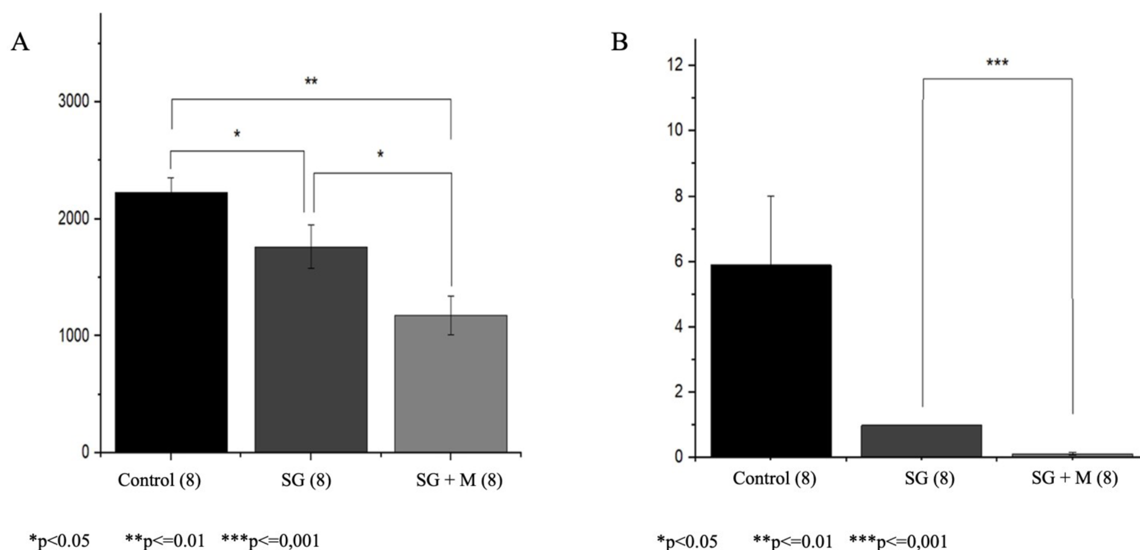

Figure 2. Sleeve gastrectomy and its combination of mesenchymal stem cells decrease the level of tumour necrosis factor alpha (TNF -α) in type 2 diabetes mellitus (T2DM) rats. A) Data are expressed as mean±SD and measured using ELISA; B) Data are expressed as means±SD and measured using qRT-PCR

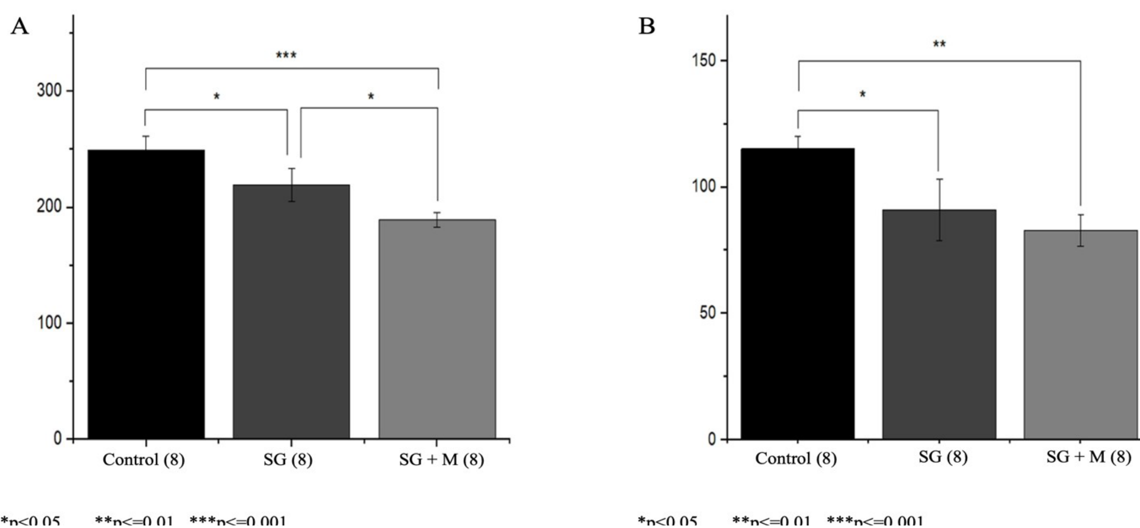

Figure 3. Sleeve gastrectomy and its combination of mesenchymal stem cells decrease the level of C-reactive protein (CRP) in type 2 diabetes mellitus (T2DM) rats. A) Data are expressed as mean±SD and measured using ELISA; B) Data are expressed as mean±SD and measured using qRT-PCR

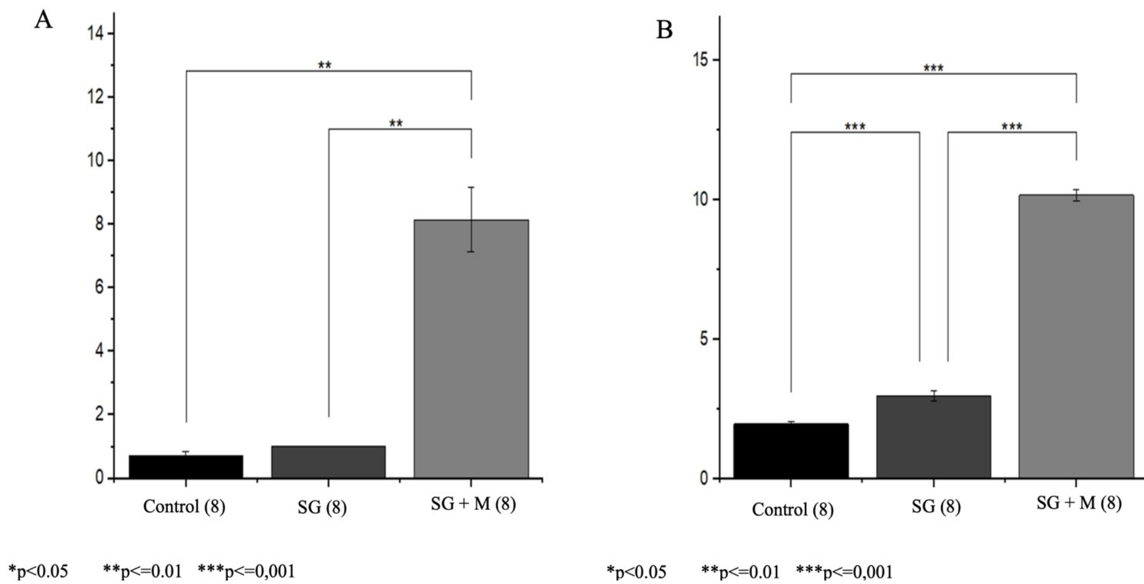

Figure 4. Sleeve gastrectomy and its combination of mesenchymal stem cells, (A) to enhance the level of vascular endothelial growth factor (VEGF) in type 2 diabetes mellitus (T2DM) rats and B) to enhance Pancreatic Beta Cells. Combination of SG and MSCs optimally improves IR in obese diabetic rats

creatic beta cell density after 10 days, with the SG+MSC group displaying the highest mean count (10.1783 ± 0.47; $p < 0.05$) (Figure 4B) indicating enhanced regenerative activity.

Improvement in insulin sensitivity assessed by HOMA-IR

To examine the capacity of SG and its combination in improving IR, the level of HOMA-IR was measured using ELISA. HOMA-IR was analysed after the treatment using the following formula HOMA-IR = (fasting insulin µU/mL × fasting glucose mg/dL/)/22.5 × 18. Ten days after the treatments, the level of HOMA-IR was measured and revealed significant improvement in all treated groups compared to the diabetic control ($p<0.05$). The SG+MSC group showed the greatest reduction, with a final value of 49.82±1.07, reflecting improved glycaemic control and insulin responsiveness (Figure 5).

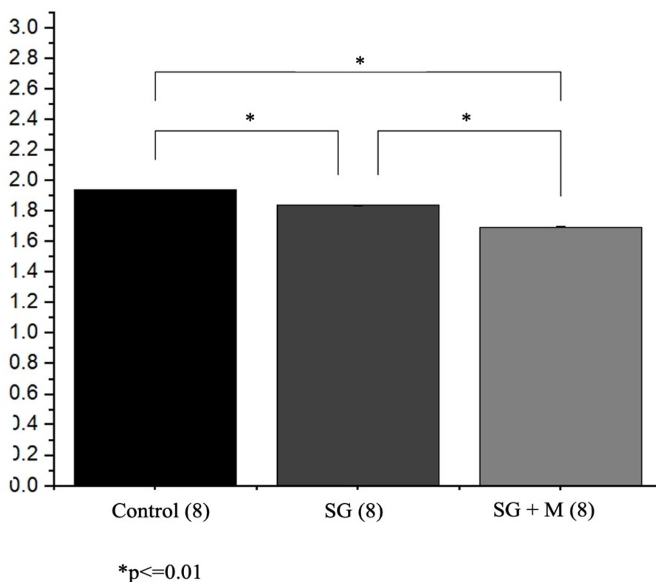

Figure 5. The capacity of sleeve gastrectomy and its combination with mesenchymal stem cells to suppress homeostasis model assessment of insulin resistance (HOMA-IR) level in type 2 diabetes mellitus (T2DM) rats. Data are expressed as means ± SD

DISCUSSION

This study showed a reduced level in the mean data of TNF-α, CRP, TG, and insulin resistance index, as well as an increased level of VEGF and pancreatic beta cells in the treatment group compared to the control group. This improvement is ascribed to changes in gastric metabolic effects after bariatric surgery. The previous studies have shown that systemic inflammation was reduced, insulin sensitivity increased, and glucose tolerance increased within 12 months after SG, but this systemic improvement is not suspected to be a direct benefit of bariatric surgery (9). Adjuvant therapy is needed to reduce the levels of TNF-α, CRP, triglycerides, and insulin resistance index, as well as increase levels of VEGF and pancreatic beta cells swiftly.

The result of our study showed that TNF-α and CRP levels significantly decrease after the injection of MSCs. This situation follows the theory that MSCs can suppress inflammation that occurs in an obese body, which is characterized by a decrease in inflammatory mediators such as TNF-α and IL-6 so that CRP values can also be decreased (14). MSCs can inhibit dendritic cell maturation so that it will decrease TNF-α expression. In addition, MSCs will polarize pro-inflammatory macrophages (M1) into anti-inflammatory macrophages (M2), which will release anti-inflammatory cytokines such as IL-10 in high concentrations and help relieve inflammation (13).

Previous research showed a decrease in TNF-α expression in mice with T2DM treated with adipose tissue-derived stem cells (ASCs) (22). MSCs showed good improvement due to their homing and paracrine abilities. Homing is the ability of MSCs to migrate to areas of inflammation due to stimulation of inflammatory cell molecules such as TNF-α. In addition, MSCs have paracrinization abilities by releasing communicator molecules such as anti-inflammatory and pro-regenerating (13).

TG levels were also found to be decreased in the treatment group. This is because SG could reduce the capacity of adipose tissue as a TG storage site (2). In addition, SG can also improve metabolic complications because adipose tissue secretes more adiponectin by increasing energy expenditure so TG that is stored in adipose tissue will be used as energy (23). SG works by reducing the appetite-stimulating hormone, namely

ghrelin, so that the need for food will still be suppressed even though calorie intake and body weight have decreased. The effect of this mechanism leads to the accumulation of TG outside adipose tissue such as in the liver, pancreatic cells, and skeletal muscle which store excessive sources of fatty acids so that they experience lipoapoptosis (24).

Performing SG reduces TG and fatty acid levels in the pancreas as well as the liver and muscles (23). The presence of adjuvant therapy in the form of MSCs can differentiate into IPCs to restore insulin levels and glucose uptake by increasing insulin sensitivity by reducing triglyceride accumulation (14). This is supported by previous studies which showed that there was a partial loss of total fat mass from subcutaneous adipose tissue (SAT) and intramuscular adipose was also greatly reduced after surgery (24). This is also associated with a decrease in the capacity of adipose tissue as a fat storage site in the form of TG and FFA (2).

The increase in pancreatic VEGF levels in MSCs group was due to its ability to secrete various bioactive molecules such as VEGF through paracrine signaling. This is in accordance with previous studies (10) that MSCs produce some cytokines and growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1, hepatocyte growth factor (HGF), and others. When MSC interacts with their implantation site in the tissue, the resulting factors will produce anti-apoptotic, mitogenic, and angiogenic effects (5). VEGF plays a very important role in increasing vascular regeneration, angiogenesis, and reducing the number of apoptotic cells (5). So, if there is cell repair, MSCs will release several growth factors which are generally used as an indicator of islet cell growth in pancreas (5).

This study results also came across an increase in the number of pancreatic beta cells in the group given MSCs. This result is in line with previous studies which stated that an increase in the number of pancreatic beta cells could occur due to MSCs ability to do self-renewal and differentiate into more specific cells, including pancreatic beta cells (14). MSCs act as pro-angiogenic agents and immunomodulators in the regeneration of pancreatic cells (14).

The mean insulin resistance index in the treatment group was also lessened compared to the control group. This climbdown of mean insulin resistance index is a sign of an improvement in the body's sensitivity to insulin (9). In conditions where the body experiences insulin resistance, there is a disturbance in insulin signaling, which results in decreased insulin sensitivity (3). This disruption can occur through the IKK/NF-kB pathway and the TNF-α-activated JNK pathway. The activation of this pathway leads to an increase in IKK-mediated serine phosphorylation at IRS-1 or IR, suppresses the expression of the GLUT4 molecule, and causes serine and threonine phosphorylation of IRS-1(25).

Following the theory, MSCs will increase the expression of IR and GLUT4 in the body so that there is an improvement in insulin signaling and sensitivity (14). Previous research by Kim et al. (26) showed an improvement in GLUT4 expression and a decrease in IRS-1 phosphorylation in the administration of umbilical cord MSCs to mouse muscle cells leading to an improvement in insulin resistance (26,27). Another study showed an improvement in pancreatic cells and a decrease in TNF-α expression resulting in the improvement in insulin resistance in rats with type 2 diabetes who were treated with adipose tissue-derived stem cells (ASCs) (22).

Even though this study has a decent result, its limitation was that the levels of other inflammatory mediators such as IL-6 and IL-10 were not assessed after the ingestion of MSCs. This study also did not assess the effect of MSCs on the expression or amount of IR which has a direct effect on insulin sensitivity and MSCs without undergoing sleeve gastrectomy. As a result, it was not possible to fully differentiate whether the observed effects of MSCs in the SG+MSCs group were purely adjunctive to the surgical intervention or if MSCs have a therapeutic effect independently. In addition, there was no regular observation in the long term on the decrease in the level of TNF-α, CRP, and TG and the enhanced level of VEGF and pancreatic beta cells and the improvement in the insulin resistance index of VEGF and pancreatic beta cell and the improvement in the insulin resistance index. Another limitation of this study is that there was no assessment of pro-inflammatory markers to see the improvement of the inflammatory process which would later affect insulin sensitivity and triglycerides. In addition, there was no assessment of IRS-1 level that could affect insulin sensitivity. While this study demonstrates significant findings, future research with larger sample sizes and longer observation periods is warranted to further validate the statistical power and reproducibility of the observed effects.

In conclusion, this study winds up that in obese diabetic rats, the combination of SG and MSCs can optimally improve IR. This improvement is followed by the decreased level of TNF-α, CRP, and TG and the enhanced level of VEGF, pancreatic beta cell simultaneously. Our findings are important for understanding the potency of SG and its combination with MSCs as a potent immunomodulatory mediator in obese T2DM.

AUTHOR CONTRIBUTION

Data curation, IA.; Formal Analysis, V.M.E.; Methodology, V.M.E., FRM; Conceptualization, V.M.E., S.I., T.S., G.H.A., N.L.S.., R.M., and W.L.; Supervision, V.M.E., A.P., T.S, S.I., M.N.M, A.D.A. and F.R.M.; Validation, A.P., I.A.; Project administration, A.D.A, G.H.A., N.L.S.., R.M., and W.L..; Writing – review & editing, V.M.E., F.R.M.. S.I. and T.S.; Visualization, F.R.M., M.N.M., G.H.A., N.L.S.., R.M., and W.L.; Writing – original draft, V.M.E., F.R.M.; All authors have read and agreed to the published version of the manuscript.

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TRANSPARENCY DECLARATION

Competing interests: None to declare.

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