Anti-inflammatory effect of UC-MSC secretome on diabetic ulcer model rats: Study of Ccl22 and Cxcl12 mRNA expression

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ABSTRACT

Introduction: Diabetes mellitus is a metabolic disorder which is characterized by chronic hyperglycemia condition and this condition can cause some complications such as diabetic ulcers. Secretome from UC-MSC is an alternative treatment that being developed for accelerating wound healing of diabetic ulcers. The aim of this research is to study the effect of UC-MSC secretome for wound healing of diabetic ulcers in Nicotinamide-Streptozotocin (NA-STZ) induced Wistar rats and the mechanism through mRNA CCL22 and CXCL12 expression study.

Materials and Methods: Fifteen male Wistar rats were divided into the diabetes group and the normal group. The diabetes group consists of the diabetes control group given by 0.02 ml MEM-α, the secretome group 0.004 ml, the secretome group 0.02 ml, and the secretome group 0.4 ml, and the normal group. Wounds are created on the back of the hyperglycemia rats and given the treatment intradermally. Wounded skin was collected for RNA extraction. RNA total yield used for cDNA synthesis. cDNA used as a template for Polymerase Chain Reaction (PCR) and using Agarose electrophoresis to view mRNA CCL22 and CXCL12 expression. Gene expression was normalized using mRNA GAPDH. Online software Targetscan and miRTarbase are used for selection of miRNA which have potentially targeted CCL22 and CXCL12 mRNA.

Results: NA-STZ affect increasing blood glucose. Injection of 0.02 ml UC-MSC secretome showed a better impact on wound healing than the diabetes control group. Lower expression of mRNA CCL22 and CXCL12 showed in male Wistar rats induced NA-STZ with UC-MSC secretome treatments.

Conclusion: UC-MSC secretomes have potential therapy as anti-ulcer on hyperglycemia male Wistar rats induced NA-STZ with probability via hsa-23a-3p in nuclear factor-kappa β (NF-κβ) pathway via inflammation mechanism.

KEYWORDS:
Diabetic ulcer, UC-MSC secretome, Nicotinamide-Streptozotocin (NA-STZ), mRNA CCL22, mRNA CXCL12

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder caused by insufficient production of insulin or insulin resistance at the insulin receptors on the cell membrane. Based on the International Diabetes Federation (IDF), there are 536.6 million cases of diabetes worldwide. The prevalence of diabetes in persons between the ages of 20 and 79 was estimated to be 10.5 % in 2021 and is expected to rise to 12.5 % in 2045. Type 2 diabetes (DMT-2) is a subtype of diabetes mellitus characterized by hyperglycemia, which is can be caused by either impaired or insulin secretion failure in the body. About 80-90 % of diabetes mellitus patients have DMT-2.[1] Hyperglycemia is a dysregulation of blood glucose causes microvascular and macrovascular disease such as peripheral neuropathy and peripheral arterial disease which is involved in pathogenesis of diabetic foot ulcers (DFU).[2] The wound dressing treatment for diabetic ulcers can promotes wound healing through the angiogenesis process. However, if wound dressing not routinely monitored and replaced by an expert it will cause an infection. Thus, adequate inhibition of peripheral neuropathy and angiopathy is crucial for the diabetic ulcers wound healing.

Mesenchymal stem cells (MSC) are multipotent non-hematopoietic cells with spindle shape, highly replicative in

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vitro, that derive from the mesoderm germ layer from different tissue. Based on their origin, MSC can be classified into neonatal tissues, such as placenta, fetal blood, umbilical cord tissue, and adult tissues consists bone marrow, thymus, brain, liver, and adipose tissue. The different sources of MSC lead to diverge of proliferation and growth rates, differentiation and immunomodulatory potentials and regenerative properties.[3]

Thus, MSC have great potential for tissue repair and recently used for medical treatment. Nevertheless, there are controversial issues such as autoimmune effect and ethical clearance on using of MSC.

Secretome is the MSC secrete that consist of many crucial biological molecules such as cytokine, chemokines, enzymes, growth factors and extracellular vesicle that can modified by conditioned media.[4] The CM represents the complete culture medium together with the secretome and biological factors secreted into the extracellular space by the cells.[5] The cytokine responsible for immunomodulation such as interleukin 1 receptor antagonist (IL-Ra), competitive inhibitor of interleukin such as IL-13, IL-10, IL-17, and monocyte chemoattractant protein such as CXC motif ligand 12 (CXCL12) and C-C motif chemokine ligand 22 (CCL22). The presence of multiple growth factors consists vascular endothelial growth factor (VEGF), hepatocyte growth factor 1 (HGF-1), and tumor growth factor B (TGF-B). Extracellular vesicle (EV) derived from MSC considered as "cellular waste", recently get recognized for important players in intercellular communication. EV have ability to transport key molecules including functional RNA (mRNA and miRNA).[6] Therefore, secretome have potential as therapeutic agent. In vivo study showed that hWMSC-EC can repair ischemic tissue by promoting neovascularization and re-endothelialization through the secretion of pro-angiogenic factors.[7] Many studies shows that wound repair involves many growth factors such as PDGF and VEGF from BM-MSC for enhancing the wound healing in mice via recruitment, survival, and proliferation of keratinocyte, fibroblast, and endothelial cell to the wound and subsequent neovascularization.[8]

Umbilical Cord Mesenchymal Stem Cells (UC-MSC) have been shown in dozens of studies to be a prospective and optimal therapeutic approach in all kinds of MSC, owing to their advantageous characteristics, including easy extraction, low cost, noninvasive collection procedure, plentiful content, and low immunogenicity.[9-10] UC-MSC can promote tissue regeneration via the secretion of bioactive factors known as UC-MSC secretome. Compared to cell-based therapy with stem cell transplantation, which have a risk such as infection and pro-tumorigenicity, cell-free therapy with secretome injection is less immunogenic, safer, less expensive, and more practical application.[11] Due to the bunch of benefits, this study using In vivo model for evaluate the secretome efficiency in wound healing.

In vivo model are chosen for this wound healing pre-clinical study due to highly medical relevant if the wound is generated to mimic the wound seen in clinical practice. Compared with In vitro studies which is have limitation cannot reproduce biological conitions such as enzymes, cells, and tissue interactions.[12] Wistar rats are frequently used in pre-clinical studies to evaluate the novelty of medicine. A popular DMT-2 mimic model is the NA-STZ (Nicotinamide-Streptozotocin) rat model.

NA have a function on protecting β cells pancreas from the β-cytotoxic effects of STZ. This compound combination produces a model of insulin-deficient, but not insulin-resistant. It is characterized by moderate hyperglycemia associated with an approximately 60 % loss of β-cell function.[13] The mechanism NA-STZ in DMT-2 rat model starts with STZ entering cells by the glucose-2 transporter (GLUT-2) because it have structural similarity to glucose.[14] STZ in the cell, damaged the DNA and activate poly-ADP-ribose polymerase-1 (PARP-1), a polymerase that uses NAD^+ as a substrate to repair damaged DNA. As a result, NAD+ potentially depleted by the activated PARP-1, thereby leading to cell death. Therefore, the NA injection before STZ injection in mouse can decrease the effects of STZ in two ways. One is that NA is a direct inhibitor of PARP-1, the other way NA is precursor of NAD^+.[15]

Hyperglycemia in DMT-2 causes wounds to exhibit excessive oxidative stress which is break the balance between oxidative and antioxidative activities. The escalation of oxidative activity in the cells inhibit the cell function normally and delayed wound healing. In this condition, important regulators nuclear factor kappa B (NF-κβ) pathways play the key role oxidative stress in diabetic wounds.16 The vascular endothelial cell membrane contains the AGE receptor (RAGE), which can activate the transcription factor nuclear factor-kappa β (NF-κβ) and resulting oxidative stress, inflammation, and complications related to diabetes.[17] This activation modify the responses of switch control signals to pro-inflammatory gene transcription.[18]

C-C Motif Chemokine Ligand (CCL22) and C-X-C Motif Chemokine Ligand (CXCL12) are pro-inflammatory that involve in control the immune system. Innate immune cells such as macrophages, dendritic cells, and endothelial cells release CCL22 or macrophage-derived chemokine (MDC) and has CC chemokine receptor 4 (CCR4). CCL22 were able to induce keratinocyte migration and/or proliferation in vitro. CCL22 also has a role to Treg cell recruitment which can cause dysregulation of wound healing.[19]

The other pro-inflammatory, CXCL12 can promote the function of CD57+ CD8+ T cells via Erk 1/2 signaling which is activate the pro-inflammatory cytokine such as IL-15.[20] Higher expression of CXCR4/CXCL12 can recruit M1 macrophages, produce inflammatory cytokines, and cause insulin resistance.[21] CXCR4/CXCL12 is widely expressed in various tissues and organs. It is involved in physiology pathological processes such as growth and development, immune-inflammation, tumor invasion, wound healing, fibrosis through various pathogenic mechanism. Hyperglycemic condition in DMT-2 can lead into CXCR4/CXCL12 dysregulation which is significantly hampers the wound healing process.[22]

The aim of this study is to observe the effect of UC-MSC secretome injection on wound healing of diabetic ulcers and CCL22 and CXCL12 mRNA expression in a wistar rat induced NA-STZ model. In this study, secretome are collected from PT

Tristem Medika Indonesia who collaborated with the Faculty of Medicine UGM team for UC-MSC secretome production for diabetic ulcer therapy. Secretome products consist miRNA which have an advantage as cell-free therapy and targeted gene therapy has been profiled and normalized using β-actin and obtain top 10 highest miRNA expression (hsa-miR-23a-3p, hsa-miR-4445 + miR-7975, hsa-miR-1915-3p, hsa-miR-4488, has-miR-125b-5p, hsa-miR-21-5p, hsa-miR-130a-3p, hsa-miR-320e, hsa-miR-494-3p, and hsa-miR-4516).[23] The highest miRNA expression is miR-23a-3p which also potentially targets mRNA CCL22 and CXCL12.

MATERIALS AND METHODS

Animals Handling

The fifteen male Wistar rats, weighing between 180-230 g were purchased from the Pharmacology and Therapy Department, Gadjah Mada University. The rats are used in this study because it is sensitive to Streptozotocin, unlike rabbit which is resistant even with higher dose.24 Rats were kept in broad cable-based polypropylene cages and the rats were given a week to adjust the room temperature (24 – 25 oC), humidity (ranging from 55 – 60 %), and light/dark (12/12 hours). Animals had unrestricted access to rodent laboratory pellets and water. The handling of experimental animals was conducted according to BPOM No.10/2022 guidelines for the care and use of laboratory animals. This study protocol was approved by the Medical and Health Research Ethics Committee Faculty of Medicine, Public Health and Nursing University Gadjah Mada (Reference KE/FK/1116/EC/2023).

NA-STZ Wistar Rats Model Induction

A minimum sample size of animals involved was calculated according to the following equation: E = total number of animals – total number of groups. The "E" value should be between 10 and 20 to be considered as adequate. Higher animals should be added if the E value is less than 10, and the sample size should be reduced if it is higher than 20.[25] The final number of subjects in this study is 15. Rats were chosen randomly and divided into two groups: diabetes group and normal group. The diabetes group consist rats injected by Nicotinamide which is dissolved 0.9 % sodium chloride solution and obtained 230 mg/kg in concentration. NA administration using 1 ml syringe and inject it intraperitoneally into the rat. Fill a 1.5 ml microcentrifuge tube with 32.5 mg of STZ, cover it with aluminum foil, and prepare for the citrate buffer. Dissolve STZ to a concentration of 65 mg/kg in citrate buffer (pH 4,5). STZ injection intraperitoneally is conducted after a 15-minute NA injection.[26]

Diabetic Ulcer Model Induction

Fasting blood glucose of the diabetic rat group in 14 days after NA-STZ is measured using a glucose meter. Rats that had a fasting blood glucose level of more than 150 mg/dl were confirmed as hyperglycemia conditions. The normal group and the diabetes group injected 0.2 ml of ketamine intramuscularly, then returned the rats to their cage, and waited until the rats no longer reacted to pain. The wound is conducted on rat backs after removing the hair on the back clearly. The excision wound model size is 64 cm^2 square-shaped and made with sterile surgical scissors. Povidone-iodine was applied to the wound, and then covered with clear tape and sterile gauze. The rats return to their cage and are placed in a sterile area.

Administration of Secretome and Wound Closure Measurement

The diabetes group was separated into four subgroups which are given the following treatments: The normal group (A) which is not giving any treatments, diabetes control (B) which is injected by MEM-α 0.02 ml, secretome 0.4 ml (C), secretome 0.02 ml (D), and secretome 0.004 ml (E). At each point of the wound one injection in five days. A mobile phone camera is used to take a photo and a millimeter block is used to measure the closure of the wound. Next, the wound measured with ImageJ (Ver 1.54) and the data analyzed using One Way ANNOVA. Analysis of wound closure percentage using the following formula:

Symbol:
A_0 = wound area in 0 day (mm^2)
A_n = wound area in n-day (mm^2)

$$\textit{Wound healing rate } (\%) = \frac{A_0 - A_n}{A_0} \times 100\ \%$$

Skin Sample Collection

The skin with scar after wound conduction is collected on day twenty-two. Rats are anesthetized with 0.2 ml ketamine hair on the back was removed clearly before skin collection. The scar and ± 5 cm from the scar area are collected and preserved in 0.2 ml RNAlater and stored at -20 °C.

RNA Extraction and cDNA Synthesis

The Quick-RNA Miniprep Plus Kit (Zymo) protocol is used for RNA extraction. 150 grams of collected skin were weighed. RNA concentration was determined with Nanodrop spectrophotometry, yielding results with good purity and concentration. The concentration of RNA determines the RNA dilution in NFW. RNA is used as a template in cDNA synthesis using The Excel RTTM Reverse Transcriptase (SMOBIO) protocol. A total of 10 µl volume in the PCR tube contains 1 µl OligoDT and 9 µl RNA template diluted in NFW. Then, incubate it for 5 minutes at 70o C and left in an ice flake for 1 minute. Add 4 µl 5x RT buffer, 5 µl DEPC-Treated H2O, and 1 µl RTase mix into the mixture and get the 20 µl in volume total. Homogenize the mixture, and incubate for 5 minutes, then incubate in a thermocycler on first strand synthesis condition with temperature setting: 25 °C for 10 minutes; 42 °C for 50 minutes, 85 °C for 5 minutes, 4 °C for 10 minutes. cDNA then stores at -20 °C.

CCL22 and CXCL2 mRNA Expression

CCL22 and CXCL2 mRNA were amplified with specific primers using the Polymerase Chain Reaction (PCR) method. Add 1 µl cDNA from RNA template with 5 µl Powerpol 2X PCR with dye (ABclonal), 0.4 µl specific primer of CCL22 (F: 5'ACTTCAGACCTCCGATGCAG 3' ; R : 5 'GGGTGACGGATGTAGTCCTG 3') , CXCL12 (F: 5' GCCTTAAACAAGAGGCTCAAG 3'; R:5' CTTCAGACCTAGGCTCCTTCT 3') , GAPDH (F : 5' TGAGAAGCTGGTCATCAAC 3' ; R:5' GCATCACCCCATTTGATGTT 3') , and 3.2 µl NFW and mix it.

The mixture conducted for PCR method using a thermocycler with temperature setting: Pre-Denaturation: 98 oC for 45 seconds; Denaturation: 98 °C for 10 seconds; Annealing with gradation temperature 52 °C (CCL22), 58 °C (CXCL12, GAPDH) for 30 seconds; Extension: 72 °C for 20 seconds: post-Extension: 72 °C for 5 minutes; Hold: 4 °C for 10 minutes.

In this study, PCR method followed by electrophoresis are used to ensure the PCR amplicon primer binds to the target gene and to validate the mRNA expression. 0.5 grams of Agarose are weighed, then add it to 50 ml of 1X TBE buffer and heat it in the microwave for 1 minute. Add 1 µl loading dye and mix it with pipetting. Run the electrophoresis chamber at 50 volts for 20 minutes. Agarose was then visualized under UV light and the result was taken by camera. The density of the specific band result was then measured using ImageJ software.

miRNA Targeting CCL22 and CXCL12 mRNA Selection

Selection of miRNA which have potentially targeted CCL22 and CXCL12 are using online software TargetScan (https://www.targetscan.org) and miRTarbase (https://mirtarbase.cuhk.edu.cn). The acquired mRNA gene target position on conserved miRNA was compared to miRNA profiled from the UC-MSC secretome by the PT Tristem Indonesia team and the Faculty of Medicine, Public Health, and Nursing UGM.

Data Analysis

Body weight, fasting blood glucose, wound closure, and densitometry of electrophoresis band data were analyzed using computerized statistical program, GraphPad Software. The statistical analysis that used on this study is the ANOVA with normality, homogeneity, and independence assumption are checked. Each group have same sample size (n=5 rat) and rat are placed in the different cage, so this study meets the ANOVA assumption requirements. If the ANOVA test result is p-value <0.05, the Tukey post-hoc test is conducted and using p-value <0,001 for significant threshold.

The Tukey post-hoc test is used due to there are equal numbers of subjects contained in each group for which comparisons of the data are being made. It is used to determine whether any group or set of treatment conditions significantly differs from one or more others.

RESULTS

Body Weight and Fasting Blood Sugar

Based on the ANOVA test followed by Tukey Post Hoc test (Figure 1), the body weight of diabetes group is decreased in week 1 (Figure 1B) and week 2 (Figure 1C) after the NA-STZ injection and the body weight on week 3 (Figure 1D) shows the body weight is increasing.

Macroscopic wound observation on day 7 (Figure 2), shows that all groups are on inflammation phase which is characterized by heat, read color, swelling, and pain. The purposes of inflammation phase to remove necrosis tissue and prevent microbe infection. The diabetes control group (B) compared with the diabetes group that received 0.02 ml secretome (D) showed a significant result (p<0.01) (Figure 3A) which indicate diabetes group that received 0.02 ml secretome (D) had better effect on wound healing than normal group.

Macroscopic wound observation (Figure 2) and One Way ANOVA followed Tukey Post Hoc Test on day 14 (Figure 3B) showed that the diabetes group received 0.02 ml secretome (D) perfectly closed (100%). Normal group (A), diabetes control group (B), and the diabetes group received 0.004 ml (E) still not closed perfectly (± 90%). Significant results (p<0.01) in the normal group and diabetes control group compared with the diabetes group received 0.4 ml secretome (C) (Figure 3B).

CCL22 and CXCL12 mRNA Expression

The PCR method was used to observe the CCL22 and CXCL12 mRNA expression in the diabetes group and normal group. ImageJ software was used to measure the densitometry of the CCL22 and CXCL12 mRNA. Gene target amplification showed that all three genes-CCL22 (78 bp), CXCL12 (96 bp), and GAPDH (78 bp) were amplified on a specific target. Target gene amplification with various densities was shown by agarose electrophoresis. A thin band visualized the CCL22 gene on the diabetes group received 0.4 mL secretome (C) and diabetes group received 0.02 mL secretome (D). The CXCL12 gene band in the diabetes group received 0.4 mL secretome (C) also looks thinner than in another group. This result may secretome have a paracrine effect in decreasing CCL22 and CXCL12 mRNA expression.

One Way ANOVA followed Tukey Post Hoc Test also showed a lower expression of CCL22 and CXCL12 mRNA on day 21 (Figure 4A and Figure 4B) in the the diabetes group received 0.4 mL secretome (C), diabetes group received 0.02 mL secretome (D), and diabetes group received 0.004 mL secretome (E).

Gene target prediction analysis using Targetscan (https://www.targetscan.org) and miRTarbase (https://mirtarbase.cuhk.edu.cn), showed hsa-miR-23a-3p targeting CCL22 3' UTR gene on 842-849 bp position with strong binding affinity on duplex structure (MFE value) is -17,7 (kcal/mol), -15,80 (kcal/mol), -10,50 (kcal/mol) (Figure 5) and targeting CXCL12 3' UTR on 1452-1458 bp position with strong binding affinity on duplex structure (MFE value) is 13,10 (kcal/mol), -10,00 (kcal/mol), -12,00 (kcal/mol) (Figure 5).

DISCUSSION

Body Weight and Fasting Blood Sugar

Body weight is one of the developed DMT-2 indicators. Polyuria, polydipsia, polyphagia, and body weight loss are signs of diabetes mellitus (DM). Body weight loss may be caused by decreasing insulin production or insulin resistance. The inability of glucose to enter the cells was due to insulin resistance. Lipolysis and the breakdown of fat into energy are conducted to restore the energy. Subsequently, a decrease in body weight is caused by the breakdown of structural muscle proteins due to insufficient energy.

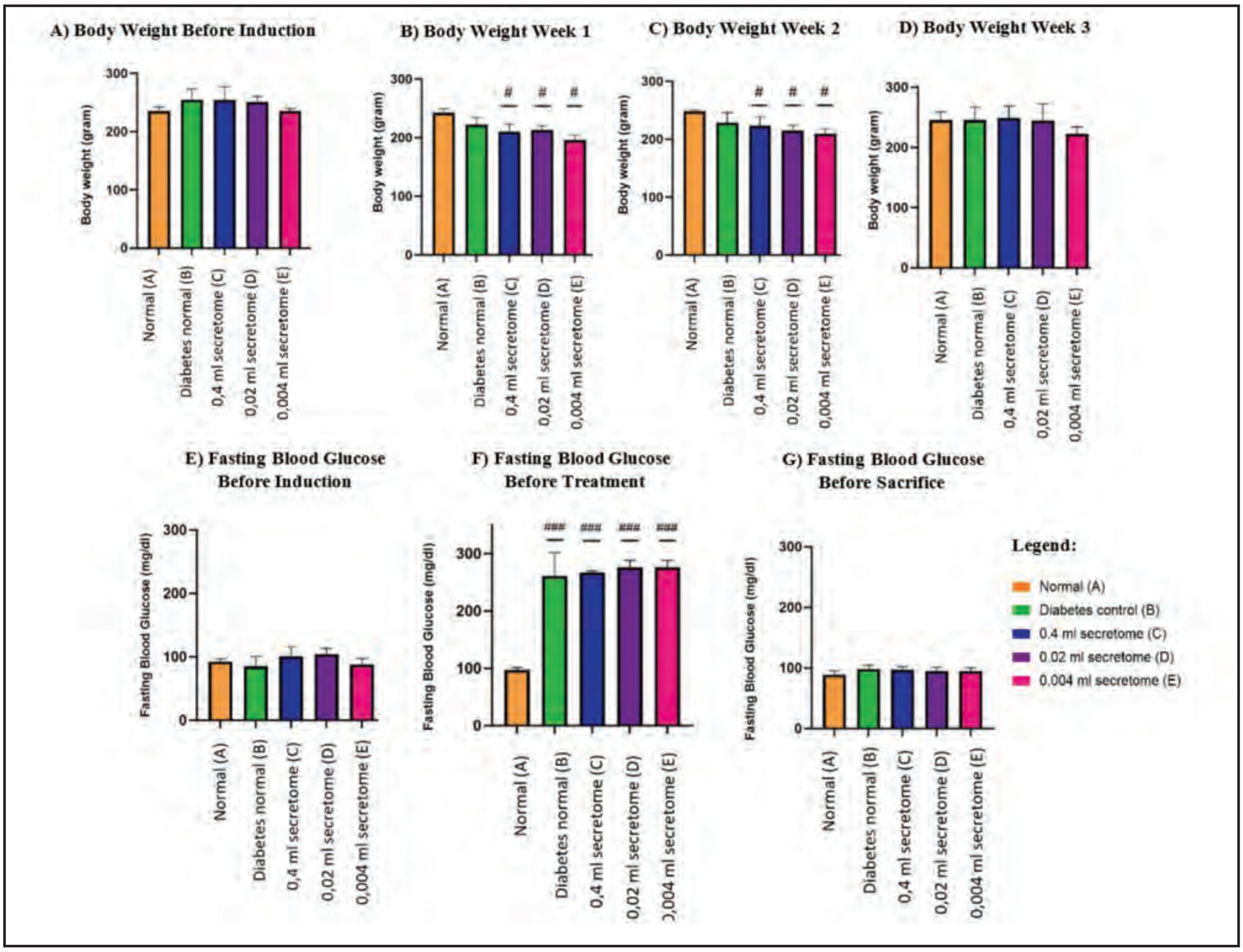


Fig. 1: Body weight and fasting blood glucose of 5 sample NA-STZ induced male Wistar. ANOVA followed by Tukey Post Hoc Test to evaluate the significance. The p-value ($p < 0.05$ vs normal) was statistically significant

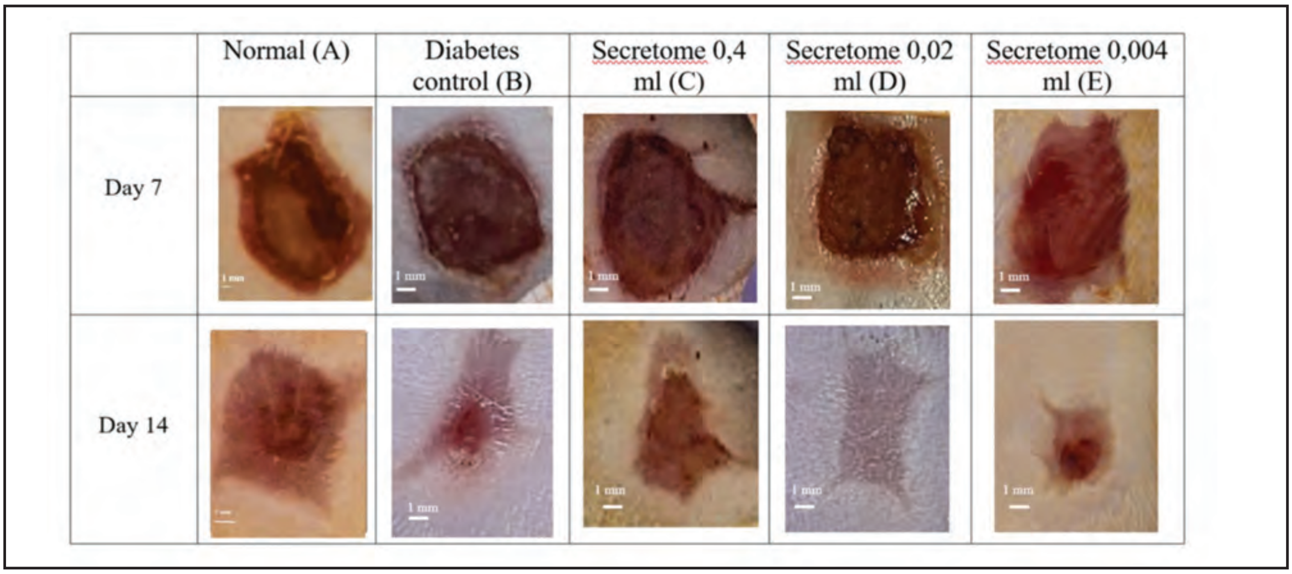


Fig. 2: Macroscopic wound healing observation on day 7 and day 14. Image scale = 1 mm/px

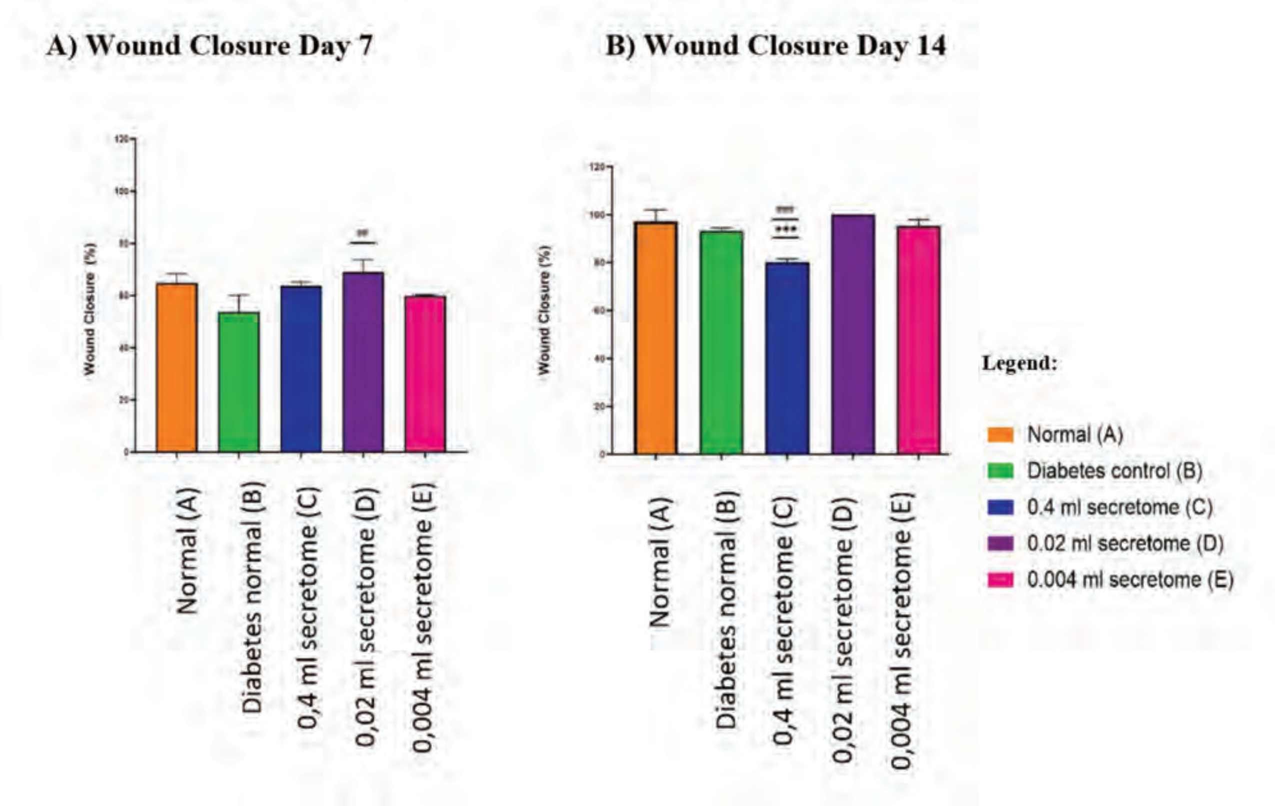


Fig. 3: Wound closure of 5 sample NA-STZ induced male Wistar. ANOVA followed by Tukey Post Hoc Test to evaluate the significance. The p-value (##p < 0,01, ###p<0,001 vs normal; ***p < 0,001 vs diabetes control) was statistically significant

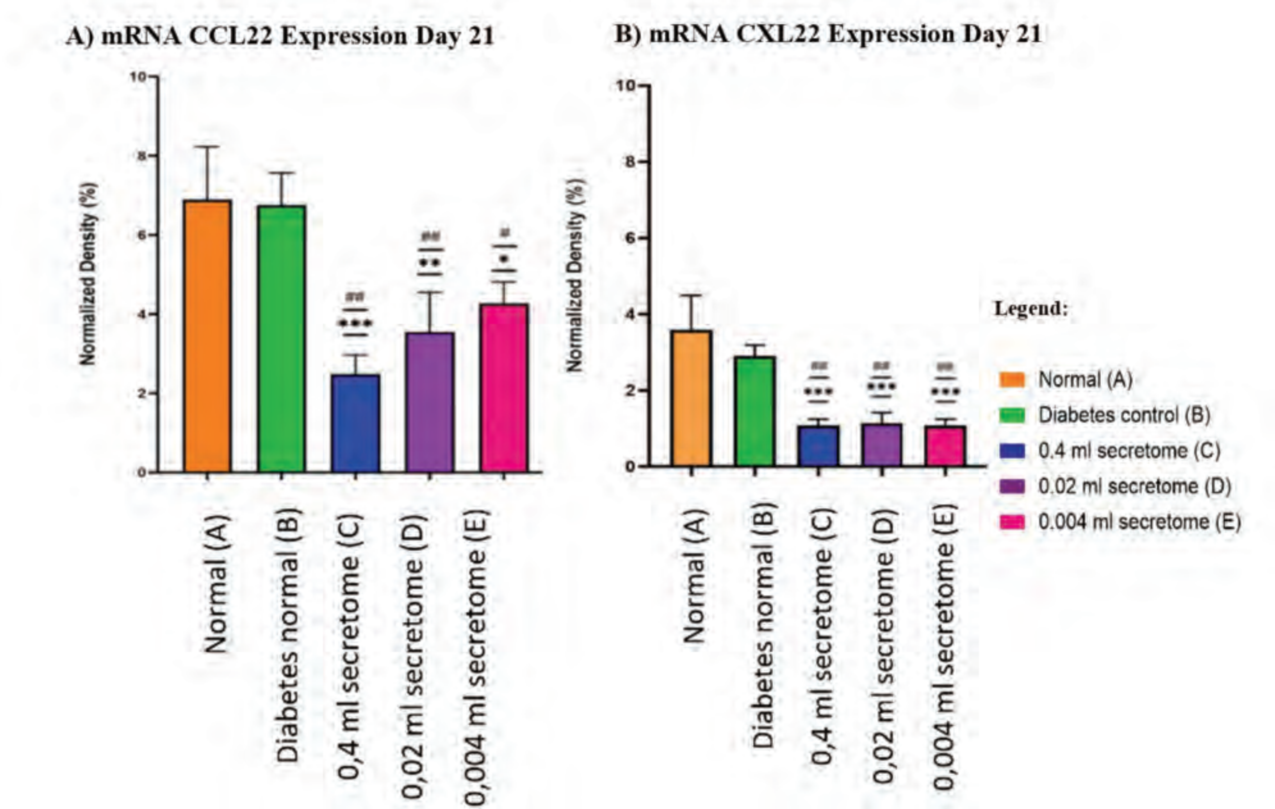
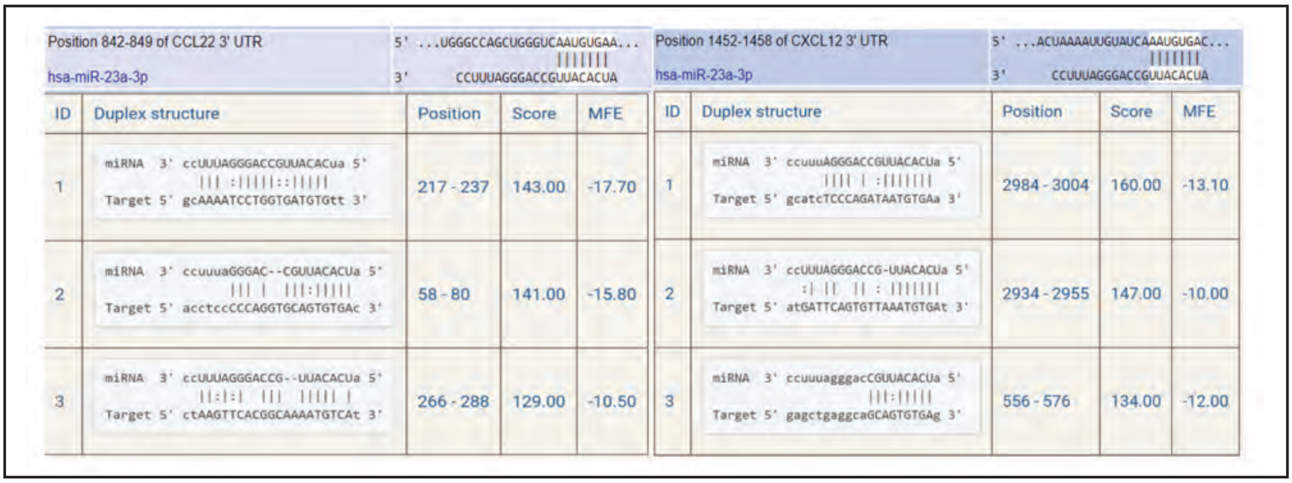


Fig. 4: mRNA CCL22 and mRNA CXCL12 expression of 5 sample NA-STZ induced male Wistar. ANOVA followed by Tukey Post Hoc Test to evaluate the significance. The p-value (*p < 0,05, **p< 0,01, ***p< 0,001 vs kontrol diabetes; #p< 0,05, ##p < 0,01 vs normal) was statistically significant



Position 842-849 of CCL22 3' UTR
hsa-miR-23a-3p

```
5' ...UGGGCCAGCUGGGUCAAUGUGAA...
                      |||||||
3'      CCUUUAGGGACCGUUACACUA
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ID	Duplex structure	Position	Score	MFE
1	miRNA 3' ccUUUAGGGACCGUUACACua 5' ||| :|||||::||||| Target 5' gcAAAATCCTGGTGATGTGtt 3'	217 - 237	143.00	-17.70
2	miRNA 3' ccuuuaGGGAC--CGUUACACUa 5' ||| | |||:||||| Target 5' acctccCCCAGGTGCAGTGTGAc 3'	58 - 80	141.00	-15.80
3	miRNA 3' ccUUUAGGGACCG--UUACACUa 5' ||:|:| ||| ||||| | Target 5' ctAAGTTCACGGCAAAATGTCAt 3'	266 - 288	129.00	-10.50

Position 1452-1458 of CXCL12 3' UTR
hsa-miR-23a-3p

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5' ...ACUAAAAUUGUAUCAAAUGUGAC...
                      |||||||
3'      CCUUUAGGGACCGUUACACUA
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ID	Duplex structure	Position	Score	MFE
1	miRNA 3' ccuuuAGGGACCGUUACACUa 5' |||| | :||||||| Target 5' gcatcTCCCAGATAATGTGAa 3'	2984 - 3004	160.00	-13.10
2	miRNA 3' ccUUUAGGGACCG-UUACACUa 5' :| || || : ||||||| Target 5' atGATTCAGTGTTAAATGTGAt 3'	2934 - 2955	147.00	-10.00
3	miRNA 3' ccuuuagggacCGUUACACUa 5' |||:||||| Target 5' gagctgaggcaGCAGTGTGAg 3'	556 - 576	134.00	-12.00

Fig. 4: Position and duplex structure has-miR-23a-3p tergeting mRNA CCL22 and CXCL12 sequence

The injection of secretome can improve the repairment and regeneration of β-cells. Consequently, normal glucose regulation including weight growth is caused by the regeneration of β-cells. Based on the other researcher, in a rat model of mild hyperglycemia, STZ can trigger the proliferation which can lead to β-cells regeneration. Fasting blood glucose is checked before NA-STZ injection to ensure normal glucose level. NA-STZ injection was conducted after the adaptation period, and then fasting blood glucose was checked 14 days post-injection and before sacrifice. Fasting blood glucose before treatment of NA-STZ analyzed by ANOVA and followed by Tukey Post Hoc Test, showed significant results between the diabetes group and normal groups (p<0,001) (Figure 1.F). This result caused by the toxicity of STZ on the β-cells pancreas which is can effect on increase of blood glucose or hyperglycemia. Fasting blood glucose measurement before sacrifice (Figure 1.G) showed blood glucose returned to a normal condition which is caused by β-cells regeneration after injection of NA-STZ on a mild dose.[27]

Wound Healing after UC-MSC Secretome Treatment

In the diabetes group received 0.02 mL of secretome (D) shows better performance on wound healing than the normal group (A) and other diabetes group (B, C, E). This result caused by secretome injection which is contains soluble factors (growth factors, cytokines, chemokines, and enzymes) and extracellular vesicles that have a paracrine effect on tissue regeneration and reduce the pro-inflammatory production such as TNF-α, IL-6, and IL-10.[28] The use of secretome as cell-free therapy is to overcome the limitation of the stem cell therapy which have insufficient integration of the cells at the site of the implantation which can significantly reduce the efficacy of the treatment. The cost of cell therapy, adequately qualified staff and suitable facility core are other limitations on its everyday clinical use.

Based on wound healing image (Figure 2) and ANOVA Test followed Tukey Post Hoc test (Figure 3) can be concluded that wound healing (A) and diabetes group received 0.02 mL of secretome (D) has a better performance than the diabetes group received 0.4 ml secretome (C). This effect known as by paradoxical drug reaction, which is constitute an outcome that is opposite from the outcome that would be expected from the drug's known actions. Apparent clinical paradoxical or bidirectional effects and reactions ensue when conflicts arise at different levels in self-regulating biological systems, as complexity increases from subcellular components, such as receptors, to cells, tissues, organs, and the whole individual.[29] This effect case might related to the aggregation of the high dose secretome components in wound area which is also increases the immunogenic response.[30] Exosome-derived bone marrow-mesenchymal stem cell (BM-MSC) lower doses also found to be neuroprotective (anti-apoptotic, anti-necrotic, and anti-oxidant), but higher doses harm the neuron.[31]

CCL22 and CXCL12 mRNA Expression

The result on CCL22 and CXCL12 mRNA gene expression based on Figure.3 shows lower expression in diabetes group received secretome (C, D, E). This effect might cause by secretome which is contains molecular bioactive including soluble factors such as cytokines, chemokines, enzymes and extracellular vesicles. Extracellular vesicles contain exosomes which have an important role in cellular communication involving miRNA and protein delivery.[32] miRNA is a short nucleotide with 19-28 nucleotides including a short noncoding single-stranded RNA class that regulate cellular processes like cell death, and differentiation miRNA regulates gene target expression via incomplete pairing on the 3'-untranslated region (3'-UTR) from the mRNA target.[33]

Secretome UC-MSC which has been profiled showed the 10 highest miRNA expression and hsa-miR-23a-3p is the top highest miRNA expression which was normalized using β-actin.23 Gene target prediction analysis using Targetscan (https://www.targetscan.org) and miRTarbase (https://mirtarbase.cuhk.edu.cn), showed hsa-miR-23a-3p targeting CCL22 3' UTR gene on 842-849 bp position with strong binding affinity on duplex structure (MFE value) is -17,7 (kcal/mol), -15,80 (kcal/mol), -10,50 (kcal/mol) and targeting CXCL12 3' UTR on 1452-1458 bp position with strong binding affinity on duplex structure (MFE value) is 13,10 (kcal/mol), -10,00 (kcal/mol), -12,00 (kcal/mol). MFE value showed the lowest energy from the secondary structure for mRNA binding whereas more negative MFE value, the binding of the target gene was stronger.[34]

Thus, indicating hsa-miR-23a-3p affected on lowering CCL22 and CXCL12 mRNA expression in the nuclear factor-kappa β (NF-κβ) pathway via inflammation mechanism. The availability of hsa-miR-23a-3p in UC-MSC secretomes may have a potential targeting therapy on diabetic ulcer therapy. The potential can be used for the development of the secretomes by standardizing the composition of biomaterials, especially miRNA in sccretome that shows quality, stability, security, and efficacy as important factors for clinical trials. The limitation in our study is no validation of miRNA expression on gene target. Future studies should investigate whether miR-23a-3p actually target the CCL22 and CXCL12 gene.

CONCLUSIONS

UC-MSC secretome injection at 0.02 ml gives wound healing effect on NA-STZ- induced male Wistar rats. Giving UC-MSC secretome to NA-STZ-induced Wistar rats showed lower CCL22 and CXCL12 compared to NA-STZ induced Wistar rats that were not given UC-MSC secretome.

CONFLICT OF INTEREST

The authors have no conflicts of interest associated with the material presented in this paper.

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