RESEARCH ARTICLE

Combination of Insulin and UC-MSCs Secretome Therapy Demonstrates A Trend of Liver Injury Improvement in Diabetic Metabolic Dysfunction-associated Steatotic Liver Disease (MASLD) Rat Model

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Abstract

BACKGROUND: Metabolic dysfunction-associated steatotic liver disease (MASLD) coexists with diabetes mellitus and presents a significant therapeutic challenge due to its complex metabolic and hepatic pathology. Insulin therapy remains the cornerstone for glycemic management, but its effects on hepatic steatosis is inconsistent. Complementary therapies are needed to address this limitation. Umbilical cord-derived mesenchymal stem cells (UC-MSCs) secretome has emerged as therapeutic approach for metabolic disease due to its anti-inflammatory, immunomodulatory, and antioxidant properties. This study was conducted to evaluate whether adjunctive administration of UC-MSCs secretome could enhance therapeutic outcomes, particularly in alleviating hepatic steatosis in a diabetic MASLD rat model.

METHODS: Male rats were grouped into normal and diabetic MASLD rat model, and treated with either growth medium, insulin, UC-MSCs secretome, or combination of insulin and UC-MSCs secretome for 4 weeks. Blood samples were collected and liver enzymatic and lipid profile were analyzed using chemistry analyzer. Liver tissue was also obtained for assessment of liver fibrosis using Hematoxylin-Eosin staining, interleukin (IL)-6 measurement using enzyme-linked immunosorbent assay (ELISA), and superoxide dismutase (SOD) activity measurement using colorimetry.

RESULTS: Combined insulin and UC-MSCs secretome therapy showed a trend toward better blood glucose management and lipid parameters, although not statistically significant. Administration of insulin, secretome, and their combination was associated with higher hepatic SOD levels and lower IL-6 protein levels, although not statistically significant. Combined therapy also reduced hepatomegaly compared to untreated diabetic MASLD rat model, and improved hepatic steatosis while significantly alleviating fibrosis severity.

CONCLUSION: Combination of insulin and UC-MSCs secretome therapy in diabetic MASLD was able to improve several parameters related to hepatic steatosis compared with the untreated diabetic MASLD rat model. These findings suggest that UC-MSCs secretome might serve as a potential adjunct to insulin therapy.

KEYWORDS: metabolic dysfunction-associated steatotic liver disease, type 2 diabetes, cell free therapy, umbilical cord mesenchymal stem cells, secretome

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Introduction

By 2024, diabetes mellitus affects more than 588 million people worldwide, accounting for 9.3% of deaths. As diabetes prevalence rises, related complications, including metabolic dysfunction-associated steatotic liver disease (MASLD), are expected to increase.(1) MASLD is characterized by excessive accumulation of lipids in hepatocytes (hepatic steatosis), in combination with cardiometabolic risk factors, such as diabetes. When hepatic steatosis is accompanied by lobular inflammation and hepatocellular injury, the condition progresses to steatohepatitis (MASH). MASLD occurs in 51-65% among patients with type 2 diabetes mellitus (T2DM).(2–5) The coexistence of diabetes and MASLD leaves a significant burden for effective clinical management. Hyperglycemia and systemic insulin resistance (IR) in diabetic condition exacerbates hepatic IR, leading to excessive lipid accumulation and accelerating disease progression toward advanced stages.(6) Persistent hyperglycemia also promotes oxidative stress and stimulates the secretion of pro-inflammatory cytokines, resulting in a chronic, low-grade inflammatory milieu that drives progressive liver injury.(7) Consequently, patients with diabetic MASLD face substantially higher risks of systemic complications compared with non-diabetic individuals, including 1.6-fold increase in mortality.(8)

To date, insulin therapy remains the cornerstone for glycemic management, but its effects on hepatic steatosis are inconsistent since it does not directly target the chronic inflammation, lipid accumulation, and hepatocellular injury that drive the progression of MASLD. Healthcare management of diabetic MASLD primarily focuses on early diagnosis and lifestyle correction, as no disease-specific pharmacological therapy has yet been approved.(9,10) In advanced stages of chronic liver disease, liver transplantation remains the definitive curative option, which is severely limited by donor scarcity, high procedural costs, risk of graft rejection, and post-transplant complications.(11) These limitations highlight an urgent need for safe, effective, and scalable regenerative strategies for the treatment of diabetic MASLD.

Stem cell therapy has emerged as a promising alternative to liver organ transplantation, offering advantages such as potent regenerative capacity, relative cost-effectiveness, and wider availability. Mesenchymal stem cell (MSC)-based interventions have also shown therapeutic promise in T2DM, with several studies demonstrating improved glycemic control and reduced Hemoglobin A1c (HbA1c) levels following treatment.(12–14) Nevertheless, clinical application of cell-based therapy is constrained by safety concerns, including portal hypertension and cell embolism, which limit the number of cells that can be safely infused. Challenges related to efficient cell engraftment, targeted homing, and maintenance of cell viability in hostile pathological microenvironments remain substantial as well.(15) In addition, variability in MSC source, donor characteristics, and manufacturing protocols may compromise therapeutic consistency and hinder the standardization of production methods, thereby motivating the development of cell-free therapeutic approaches, such as MSCs secretome.(16,17)

In this context, cell-free secretome therapy offers a safer and more practical approach, knowing that the therapeutic efficacy of MSCs is largely mediated by their paracrine activity rather than direct cell replacement. The myriad bioactive factors secreted by MSCs, which usually referred to as the secretome, hold a pivotal role in modulating cell signaling, regulating inflammation, and promoting tissue repair.(11) Previously, we demonstrated that secretome derived from human umbilical cord MSCs (UC-MSCs) exerts multiple therapeutic effects *in vivo*, including improved glycemic control (18) and attenuation of liver cirrhosis.(19) However, the potential role of UC-MSCs' secretome in restoring hepatic function under diabetic MASLD conditions has not yet been elucidated. In the present study, rat model of diabetic MASLD was established, and the therapeutic effects of UC-MSCs secretome to this rat model, either alone or in combination with insulin, was investigated. The finding of this study might propose the potential of UC-MSCs secretome as an adjuvant to insulin therapy for improving hepatic histopathological features and selected biochemical parameters in diabetic MASLD.

Methods

Production of UC-MSCs Secretome

Secretome was produced from human UC-MSCs obtained from previous study.(20) In brief, MSCs were initially expanded in Minimum Essential Medium Alpha (MEMα) (Gibco, Life Technologies, Grand Island, NY, USA) containing 20% fetal bovine serum (FBS) (Gibco) and 1% antibiotic-antimycotic (Ab/Am) (Sigma Aldrich, St. Louis, MO, USA) until passage 1. The cells were subsequently cultured in MEMα containing 10% FBS and 1% Ab/Am and expanded to passage 6 at a seeding density of 9,000 cells/cm^2. Upon reaching approximately 80–90% confluency,

the cells were exposed to FBS starvation and a hypoxic environment (5% O_2) for 72 hours. The culture medium was then collected and centrifuged at 1,500 rpm for 10 minutes to remove cellular debris. The resulting supernatant was sterile-filtered through a 0.22 µm membrane filter, yielding approximately 200 mL of secretome per production batch with a total protein concentration of 1.88 mg/mL, as determined using a bicinchoninic acid (BCA) assay. The biological characterization of the UC-MSCs and its secretome has been reported previously using the same manufacturing protocol.(20) The final secretome was tested negative for bacterial and fungal culture tests, as well as endotoxin and mycoplasma tests. For all experiments, the fresh secretome was used and stored at -80°C for no longer than 1 month without undergoing any freeze-thaw cycles prior to administration.

Establishment of Diabetic MASLD Model

Male Sprague Dawley rats aged 8–10 weeks (n=25) were obtained from the Indonesian Food and Drug Authority, Jakarta, Indonesia. The number of animals in this study was calculated through the ANOVA Research Equation formula, accounting the 3Rs principle (Replacement, Reduction, and Refinement).(21,22) Animals were housed within controlled environmental conditions: relative humidity of 65–75 % and a 12:12 h light-dark cycle. Rats were given *ad libitum* access to food and water during a one-week of acclimatization period, using standard chow diet (PT Surya Sains Indonesia, Tangerang, Indonesia). Following acclimatization, rats were divided into normal (n=5) and diabetes mellitus with MASLD (DM/MASLD; n=20) groups.

DM/MASLD model was established through high-fat diet (HFD) feeding and streptozotocin (STZ) injection. (23,24) A high-fat diet (33% fat, 37% carbohydrate, 13% protein content; PT Surya Sains Indonesia) was given continuously throughout the study, starting 4 weeks before diabetes induction until endpoint. Subsequently, 30 mg/kg BW STZ (Cat. No. S0130; Sigma Aldrich) was injected intraperitoneally (i.p.) to better mimic T2DM phenotype, which was characterized by partial β-cell dysfunction. (25) At day 9 post-STZ injection, fasting blood glucose (FBG) was measured through a tail prick method using a glucometer (Accu-Chek® Active, Roche Diagnostics, Basel, Switzerland); rats were confirmed as diabetic with FBG >250 mg/dL.(11)

Animal Grouping and Treatment

After diabetes confirmation, rats were grouped randomly into five experimental groups (n=5): Normal, Diabetic, Insulin, Secretome, and Co-treatment; in which the investigators were blinded to the group allocation. Rats in the Normal group were continuously fed a standard chow diet and received i.p. injections of 0.5 mL serum-free MEMα twice weekly for 4 weeks. For Diabetic group, each rat was injected i.p. with 0.5 mL serum-free MEMα. Rats in Insulin group was administered with 1 unit of insulin per rat (NovoRapid®; Novo Nordisk, Copenhagen, Denmark) subcutaneously (s.c.); Rats in Secretome group was injected with 0.5 mL secretome i.p. (containing 0.94 mg of total protein); while rats in Co-treatment group was treated with both 1 unit of insulin via s.c. and 0.5 mL secretome via i.p. Injections were given twice a week for four weeks, resulting in a total of 8 injections (Figure 1). Rats were subsequently observed for an additional 2 weeks until endpoint. The animal experiment was performed at Tarumanagara Human Cell Technology Laboratory (Jakarta, Indonesia), following the ethically approved protocol.

Euthanasia and Sample Collection

At endpoint, rats were anesthetized using 10% Ketamine (40–80 mg/kg BW; Kepro, Woerden, Netherlands) and 2% Xylazine (5–10 mg/kg BW; Xyla, Interchemie, Hapert,

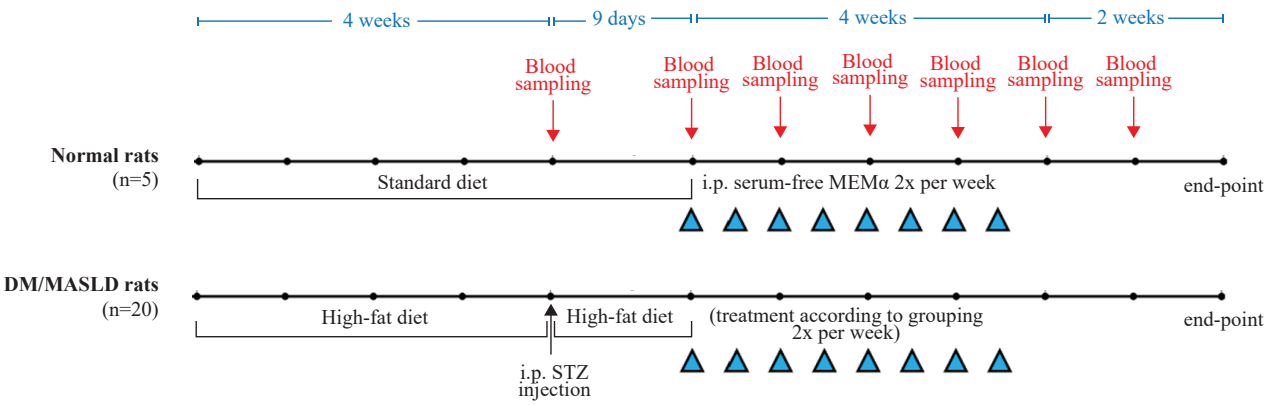

Figure 1. Study design and experimental timeline.

Netherlands) via i.p. injection. Under deep anesthesia, euthanasia was performed through cardiac puncture-induced exsanguination. The whole blood samples were then collected to obtain blood serum for further biochemical analysis, while whole liver tissue was also obtained for determining the liver-to-body weight ratio and liver histopathology.

Liver Enzymes and Lipid Profile Analysis

The collected serum was used for biochemical analysis of liver enzymes and lipid profile. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured as markers of hepatocellular injury using commercially available enzymatic assay kits as follow: ALT (Code No. 1-203-0060; Biomaxima, Lublin, Poland) and AST (Code No. 1-208-0060; Biomaxima) according to the manufacturer's instructions. While the lipid profiles, including serum total cholesterol, triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels were measured using commercially available enzymatic assay kits, as follow: total cholesterol (Code No. 1-023-0200; Biomaxima), TG (Code. No. 87-2; Labtest Diagnóstica, São Paulo, Brazil), LDL (Code. No. 30185; Labkit, Chemelex, Canovelles, Spain), and HDL (Code. No. 1-029; Biomaxima) according to the manufacturers' instruction. These liver enzymes and lipid concentrations were determined spectrophotometrically using an automated clinical chemistry analyzer, and the absorbance was measured using Photometer 5010 (Riele, Berlin, Germany). All biochemical testing were performed by the Pathology and Anatomy Laboratory, Primate Research Center, Bogor Agricultural University (Bogor, Indonesia).

Liver-to-Body Weight Ratio and Hepatic Protein Analysis

At the endpoint, whole liver tissue was collected and weighed to determine the liver-to-body weight ratio (%). (26) Subsequently, a liver section of 0.1 g (caudate lobe), was dissected and stored in –80°C for total protein isolation. Protein isolation was performed through liquid nitrogen pulverization using a cocktail of radioimmunoprecipitation assay lysis buffer (RIPA) (Cat. #E-BC-R327; Elabsicence, Houston, TX, USA) and 1% protease inhibitor cocktail (Cat. #HY-K0010; MedChemExpress, South Brunswick, NJ, USA). Quantification of total protein was performed using Bradford assay (Cat. #E-BC-K168-M; Elabscience).

The total protein concentration of each sample was normalized prior to assay, and equal amounts of protein were subjected to enzyme-linked immunosorbent assay (ELISA) to determine the content of pro-inflammatory factor interleukin-6 (IL-6) using Rat IL-6 DuoSet ELISA kit (Cat. #DY506; R&D Systems, Minneapolis, MN, USA). Samples were added to ELISA plates coated with capture antibody, followed by incubation with detection antibody and streptavidin–horseradish peroxidase conjugate. After substrate reaction, absorbance was measured using a microplate reader, and IL-6 concentrations were calculated from the standard curve and normalized to tissue protein concentration. Antioxidant activity was also analyzed using Superoxide Dismutase (SOD) Colorimetric Activity Kit (Cat. #EIASODC; Invitrogen, Waltham, MA USA) according to the manufacturer's instruction. Briefly, liver tissues were homogenized in an appropriate assay buffer, and the homogenates were centrifuged to obtain the supernatant for analysis. SOD activity was determined based on the inhibition of superoxide-dependent colorimetric reaction, and absorbance was measured using a microplate reader. SOD activity was calculated from the standard curve.

Histopathological Evaluation

The remaining liver section of the median lobe, was preserved in 4% paraformaldehyde before undergoing dehydration with graded alcohol and xylene. The tissues were subsequently paraffin-embedded, sectioned into thin slices, and mounted on glass slides for histological staining. All histopathological processing, staining and evaluation were performed at the Pathology and Anatomy Laboratory, Primate Research Center, Bogor Agricultural University. Histological scoring was conducted by a board-certified veterinary pathologist who was blinded to the treatment groups.

To confirm and evaluate the MASLD severity, tissue slides were stained with Hematoxylin-Eosin (HE) staining. Briefly, tissue slides were stained with hematoxylin for 10 minutes (ScyTek Laboratories, West Logan, WV, USA), followed by eosin staining (Thermo Fisher Scientific, Waltham, MA, USA) for 1 minute. MASLD was confirmed and graded using activity scoring system, assessing two key histopathological features: steatosis and inflammation. (27) Macrovesicular steatosis, microvesicular steatosis, and hepatocellular hypertrophy were assessed under 400× magnification and individually scored (0 to 3) based on the percentage of affected area (Supplementary Table 1). The unweighted sum of these three parameters was calculated as the steatosis cumulative score, yielding a total score ranging from 0 to 9. Inflammation was assessed under 100× magnification based on the number of inflammatory foci per field of view, resulting in an inflammation score ranging

from 0 to 3. Based on the cumulative score, liver pathology was categorized as follows: Normal (no MASLD): steatosis score 0 with inflammation score 0 – 3; MASLD: steatosis score 1 – 9 with inflammation score 0; and MASH: steatosis score 1 – 9 with inflammation score 1 -3.

Sirius Red staining (Picro-Sirius Red ab246832; Abcam, Cambridge, UK) was also employed to evaluate hepatic fibrosis based on the Ishak scoring system, which classifies fibrosis into seven stages ranging from no fibrosis (normal) to cirrhosis.(28) Tissue sections were incubated in Picro-Sirius Red solution for 60 minutes and subsequently rinsed with 0.5% acetic acid solution and absolute alcohol. Slides were observed under the light microscope in 400× magnification.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics (IBM Corp., Armonk, NY, USA). The Shapiro-Wilk normality test was performed to determine the distribution of the data. Body weight and blood glucose analysis over designated timepoints was assessed using repeated measures Analysis of Variance (ANOVA). For other normally distributed data, one-way ANOVA followed by Tukey's multiple comparison test was used, whereas non-normally distributed data were analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test, where applicable. Histopathological diagnosis frequencies were compared using Fisher's exact test. Differences between groups were considered statistically significant at $p<0.05$ and highly significant at $p<0.01$.

Results

Diabetic MASLD Rat Model was Successfully Established

A diabetic MASLD model was successfully established in rats through combined HFD feeding and STZ administration. All groups exhibited progressive body weight gain during the HFD induction period (Table 1). Nine days after STZ injection, FBG levels increased to >300 mg/dL, while the body weight slightly declined in all diabetic MASLD rats, thereby confirming their diabetic state. During the treatment period, all diabetic groups showed gradual increase in their body weight, although the rate of weight gain remained lower than that of the Normal group. Statistical analysis demonstrated a significant effect of time ($p<0.001$) and a significant time×treatment group interaction ($p<0.001$). However, the overall treatment group effect was not statistically significant ($p=0.324$), indicating that body weight trajectories differed over time despite comparable overall body weights among the diabetic groups.

Combination of Insulin and UC-MSCs Secretome Therapy Slightly Lowered FBG Levels

Diabetic group exhibited persistently elevated FBG levels throughout all observation timepoints. Both Insulin and Secretome groups demonstrated minor changes in FBG levels over the treatment period, with values remaining within a comparable range during the treatment period. Notably, co-treatment with insulin and secretome resulted in a decreasing trend for up to three weeks after treatment initiation (239.40±109.95 mg/dL at week 3 *vs*. 323.20±16.54 mg/dL at baseline), although no further decrease thereafter (Figure 2A). Repeated measures ANOVA revealed significant effects of time ($p<0.001$), treatment group ($p<0.001$), and the time×treatment group interaction ($p<0.001$) on FBG levels. At the final observation, FBG levels remained higher in all untreated and treated diabetic groups than in the Normal group; however, only the Diabetic group differed significantly from the Normal group ($p=0.007$).

Insulin and/or UC-MSCs Secretome Therapy Improved Lipid Profiles

By the study end-point, all diabetic MASLD rats displayed worsened lipid profiles, characterized by elevated total

Table 1. Body weight progression during the experimental period.

Group	Time of Sampling							
	Before HFD	Before STZ Injection	9 Days After STZ Injection	1 Week After Intervention	2 Weeks After Intervention	3 Weeks After Intervention	4 Weeks After Intervention	5 Weeks After Intervention
Normal	195±23	259±10	273±8	281±7	287±9	296±10	308±10	324±9
Control	216±12	270±15	252 ±20	256±24	256±29	258±30	259±33	266±33
Insulin	215±18	279±31	258±32	262±32	264±33	268±31	274±33	281±39
Secretome	221±8	275±19	259±21	256±22	257±23	255±24	258±20	264±16
Co-treatment	212±21	260±25	250±25	250±24	252±21	256±21	259±22	267±23

Data were presented as means±SD. Statistical analysis was performed using repeated measures ANOVA, with $p<0.05$ is considered significant.

cholesterol, TG, and LDL levels (Figure 2B-2D). Significant total cholesterol and TG levels were elevated in the Diabetic group compared with the Normal group (p<0.001 for total cholesterol and p=0.004 for TG level). Treatment with insulin, secretome, or their combination resulted in lower total cholesterol levels than those observed in the Diabetic group, although only the Secretome group demonstrated a significant reduction (p=0.038). Reduced TG and LDL levels were also seen in all treatment groups, although these differences did not reach statistical significance. Interestingly, HDL levels in the Diabetic and Co-treatment groups were comparable to those of the Normal group, while monotherapy of Insulin or Secretome showed higher HDL levels compared with Normal group (p=0.013 and p=0.002, respectively) (Figure 2E).

Combination of Insulin and UC-MSCs Secretome Therapy Lowered Live Enzymes Parameters

Analysis of liver enzyme levels revealed no consistent trend among groups (Figure 3A-3B). At study end-point, AST and ALT levels were slightly higher in Diabetic group than in Normal group, although these differences were not significant (AST: 148.06±104.62 U/L *vs.* 141.8±14.70 U/L; ALT: 83.72±38.63 U/L *vs.* 58.2±15.01 U/L). All treatment groups exhibited lower AST and ALT levels compared to Diabetic group, with lowest values observed in the Co-treatment group (AST: 53±39.99 U/L; ALT: 18.80±21.14 U/L). AST levels in the Co-treatment group were significantly lower than the Normal group (p=0.004), whereas ALT levels in the Co-treatment group were significantly lower than those in the Diabetic group (p=0.002).

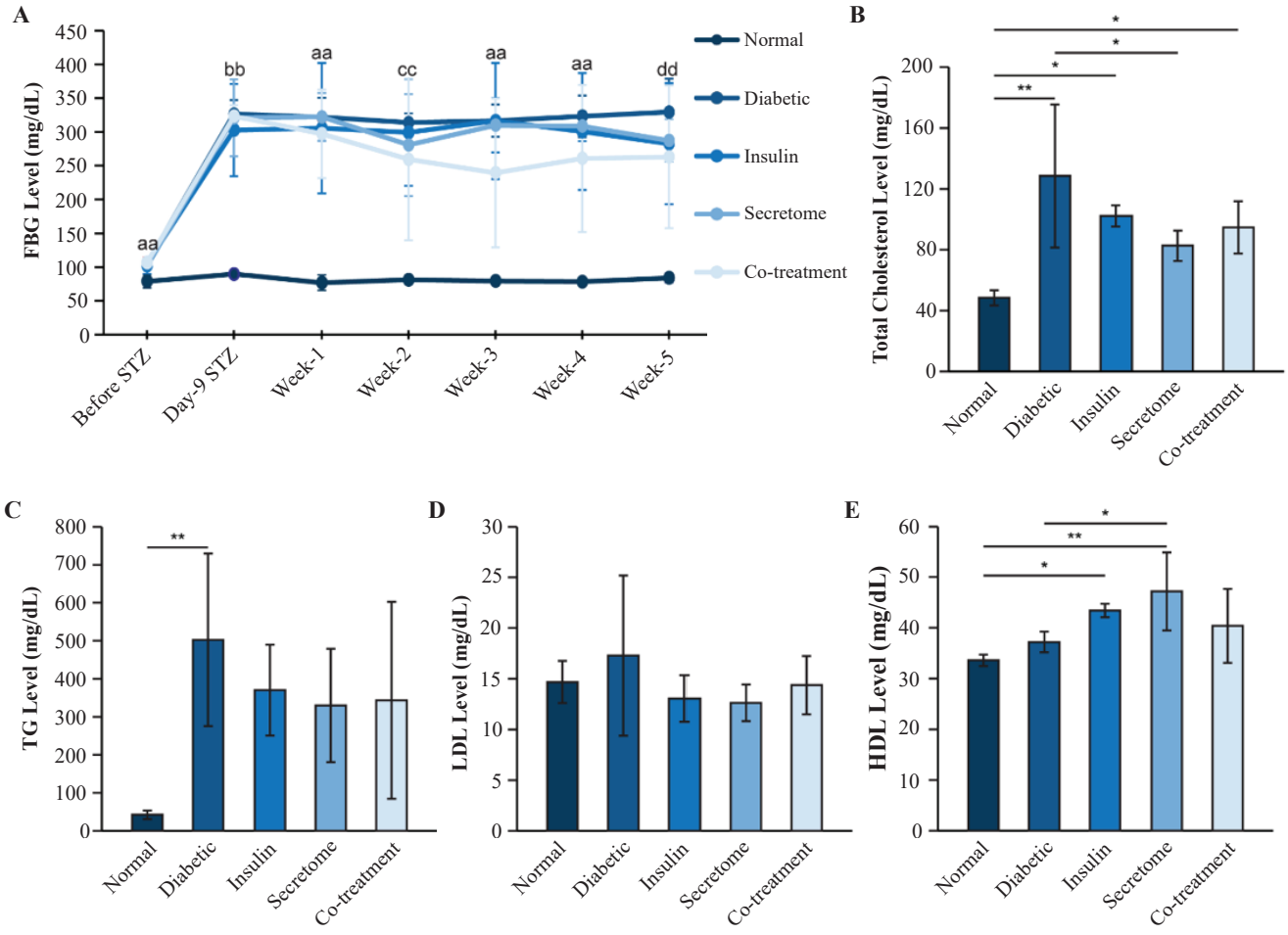

Figure 2. Secretome effects on blood glucose and lipid profile in diabetic MASLD rat. A: FBG levels across designated timepoints, as detected through a tail-prick method. Before STZ: before STZ injection; Day-9 STZ: 9 days after STZ injection; Week-1 to -5: week(s) after intervention. B: Total cholesterol level at end-point. C: TG level at end-point. D: LDL level at end-point. E: HDL level at end-point. Data were shown as means±SD. Panel A was analyzed using repeated measures ANOVA; ^{aa}p<0.01 all groups *vs.* Normal group; ^{bb}p<0.01 Diabetic and Secretome *vs.* Normal group; ^{cc}p<0.01 Insulin *vs.* Normal group; ^{dd}p<0.01 Diabetic *vs.* Normal group. Panel B, C, and E were analyzed using one-way ANOVA followed by Tukey's multiple comparison test; Panel D was analyzed using Kruskal Wallis followed by Dunn's post hoc test; *p< 0.05, **p<0.01.

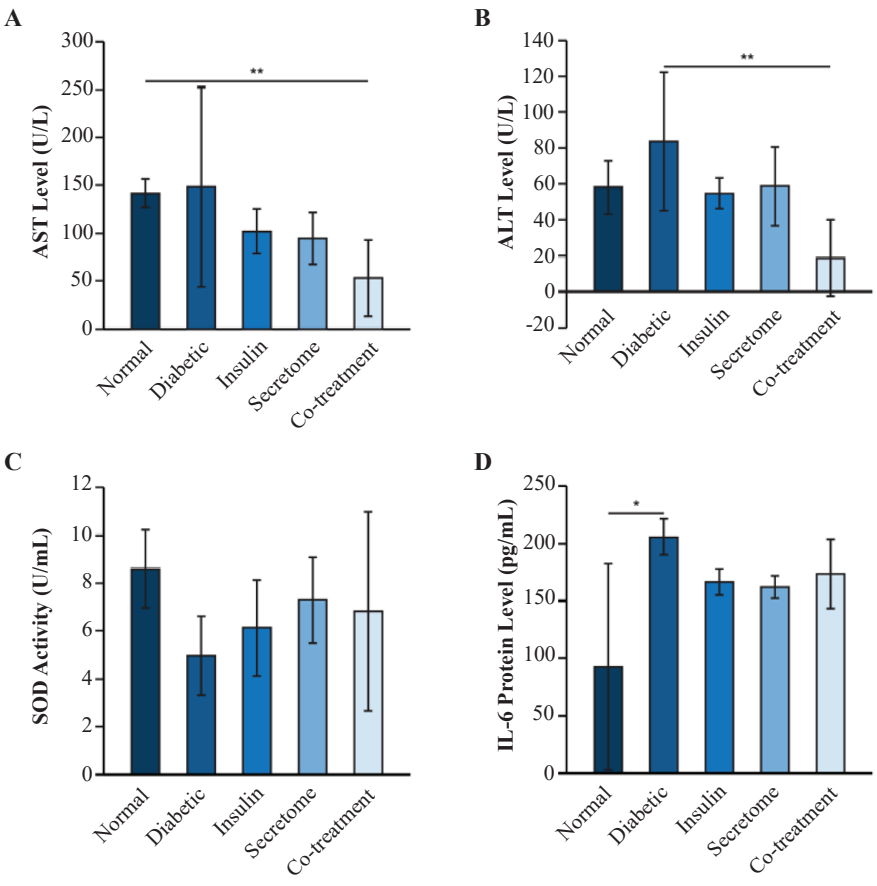

Figure 3. Effect of secretome treatment on liver function and hepatic inflammatory and oxidative stress markers in diabetic MASLD rats. A: AST level at end-point. B: ALT level at end-point. C: SOD activity at end-point. D: Serum IL-6 at end-point. Data were shown as means±SD. Panels A, C, and D were analyzed using Kruskal-Wallis followed by Dunn's post hoc test; Panel B was analyzed using one-way ANOVA followed by Tukey's multiple comparison test; *p<0.05, **p<0.01.

Insulin and/or UC-MSCs Secretome Therapy Showed a Trend of Improved SOD Activity and Reduces IL-6 Level

Rats in Diabetic group exhibited lower hepatic SOD activity than the Normal group (4.97±1.66 U/mL vs. 8.60±1.62 U/mL) (Figure 3C). Although the differences did not reach statistical significance, the Insulin, Secretome, and Co-treatment groups showed higher hepatic SOD activity than the Diabetic group (6.13±2.01 U/mL, 7.29±1.78 U/mL, and 6.84±4.15 U/mL, respectively). In line with this, hepatic IL-6 protein levels were significantly higher in Diabetic group than in the Normal group (205.80±15.70 pg/mL *vs.* 92.23±89.96 pg/mL, p=0.02) (Figure 3D). Although lower hepatic IL-6 levels were observed in the Insulin, Secretome, and Co-treatment groups (166.57±11.52 pg/mL, 162.09±10.02 pg/mL, and 173.70±30.16 pg/mL, respectively), these differences were not significant.

Insulin and/or UC-MSCs Secretome Therapy Showed a Trend of Restoration of Hepatomegaly and Hepatic Steatosis Condition

By the study end-point, rats in Diabetic group showed contrast discoloration and developed hepatomegaly, as evidenced by a significantly increased liver-to-body weight ratio compared with Normal rats (4.62±0.35% *vs.* 3.32±0.21%, p<0.001; Figure 4). Treatment with insulin or secretome alone resulted in reduced liver-to-body weight ratio (4.16±0.20% and 4.19±0.22%, respectively), although not statistically significant. Notably, combined insulin and secretome treatment showed macroscopic liver appearance comparable to the Normal group, with preservation of the normal reddish coloration, as well as significantly lower hepatomegaly, with the liver-to-body weight ratio approaching that of the Normal group (3.85±0.25%, p<0.001 against Diabetic).

Further histological examination of liver tissue from the Normal group revealed preserved lobular architecture with hepatocytes displaying well-defined cell borders and centrally-located nuclei (Figure 5A). In contrast, liver sections from the Diabetic group showed architectural disruption, characterized by extensive hepatocellular steatosis with lipid droplets occupying large areas of hepatocytes' cytoplasm, displacing the nucleus from its central position toward the cell periphery. Inflammatory foci were also prominent in this group, corresponding to a moderate degree of inflammation (Table 2). According to the activity scoring criteria, steatosis without inflammation is classified as MASLD, whereas steatosis accompanied by

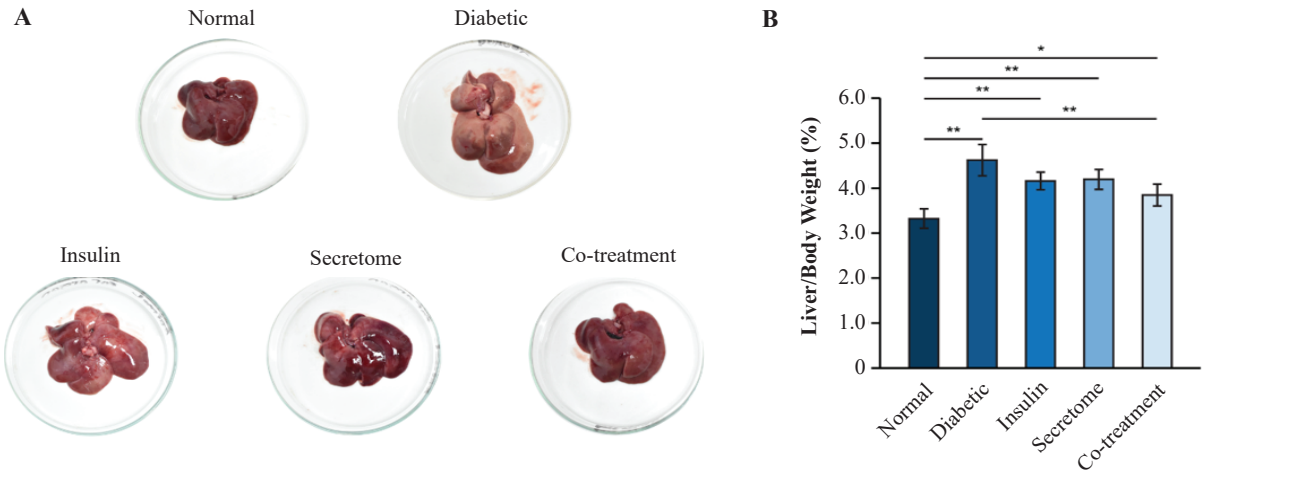

Figure 4. Effects of combined insulin and secretome therapy on hepatomegaly in diabetic MASLD rats. A: Representative images of whole liver specimens collected at the end-point. B: Quantification of liver-to-body weight ratio across experimental groups. Data were presented as means±SD (n=5). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test; **p*<0.05, ***p*<0.01.

inflammatory activity is diagnosed as MASH. Accordingly, the presence of inflammation led to 60% of rats in Diabetic group being classified as MASH at the study end-point (Figure 5B).

Treatment with insulin alone was associated with an improved histopathological profile, with 20% of rats developing MASH and 40% exhibiting MASLD features. Administration of secretome alone displayed a largely preserved hepatic histology, with 80% of rats classified as normal and only 20% developing MASH. Most notably, co-administration of insulin and secretome led to substantial restoration of hepatic morphology, marked by reduced lipid accumulation and fewer degeneration changes compared with Diabetic group. In this group, 80% of rats were classified as normal, while the remaining 20% exhibited MASLD features. Compared with the Normal group, the Co-treatment group demonstrated lower cumulative steatosis and inflammatory scores (cumulative steatosis score: 0.2±0.4 *vs.* 2.2±2.0, p=0.133; inflammatory score: 0 *vs.* 2, p=0.124), although these differences did not reach statistical significance.

Insulin and/or UC-MSCs Secretome Therapy Improved Hepatic Fibrosis

Consistent with MASLD pathology, liver sections from Diabetic group exhibited significantly increased collagen deposition compared to Normal rats, as indicated by a higher hepatic collagen percentage fraction (8.20±3.39% *vs.* 3.98±0.65%, p=0.02) (Figure 5C). Co-treatment resulted in a reduction of hepatic collagen accumulation (6.43±1.29%), although not statistically significant when compared to the Diabetic group. Assessment of fibrosis severity using the Ishak scoring system, which evaluates fibrosis based on portal expansion, architectural distortion, and nodularity, revealed that rats in Diabetic group predominantly exhibited fibrous expansion of most portal areas, corresponding to an Ishak score of 2 (2.20±0.45) (Figure 5D). Co-administration of insulin and secretome significantly alleviated fibrosis severity, restoring the fibrosis stage to an Ishak score of 1.00±0.00 (p=0.03 *vs.* Diabetic group), indicative of fibrous expansion limited to some portal areas.

Discussion

In this current study, the potential of UC-MSCs secretome as an adjuvant to insulin in ameliorating key pathological features of diabetic MASLD rat model was observed. The pathophysiology of diabetic MASLD involves a complex interplay between IR, dysregulated lipid metabolism, oxidative stress, and chronic inflammation. As a result, in addition to persistent hyperglycemia, liver biopsies from affected individuals commonly show excessive glycogen accumulation, hepatic steatosis with hepatocyte ballooning, inflammatory cell infiltration, and interstitial fibrotic proliferation.(11,26,29,30) In line with this paradigm, the untreated diabetic MASLD rats in the present study exhibited sustained hyperglycemia, dyslipidemia, hepatomegaly, elevated hepatic inflammatory markers, hepatic steatosis, and fibrosis. These findings confirm the successful disease model establishment which recapitulates key aspects of human diabetic MASLD.

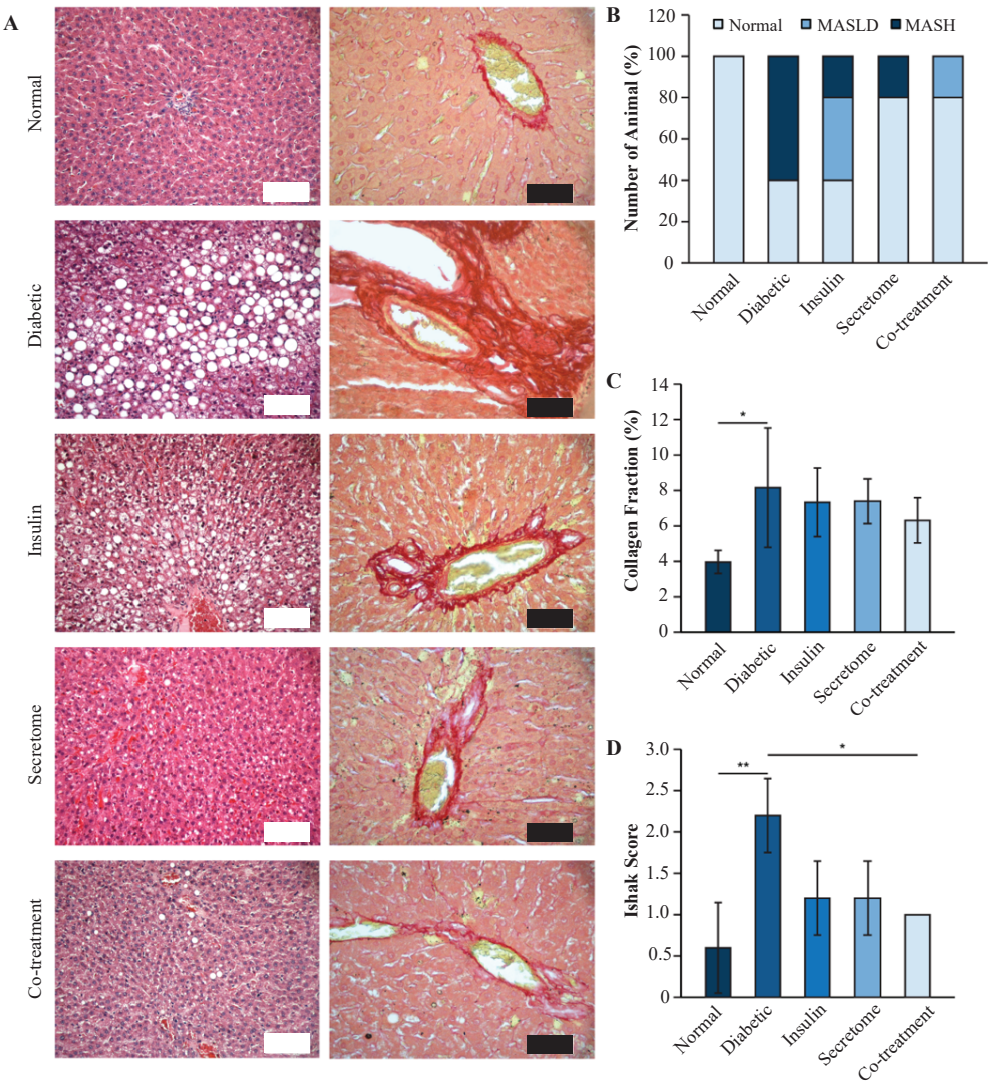

Figure 5. Effects of insulin and secretome administration on histological liver restoration and hepatic fibrosis in diabetic MASLD rats. A: Representative images of liver histology stained with Hematoxylin-Eosin (left panels) and Sirius Red (right panels). White bar: 100: µm; Black bar: 50 µm. B: Percentage number of animals developing MASLD, as graded using MASLD activity scoring system. C: Quantification of hepatic collagen percentage fraction. D: Quantification of Ishak fibrosis score. Data were presented as means±SD. Panel B was analyzed using Fisher's exact test; Panel C was analyzed using one-way ANOVA followed by Tukey's multiple comparison; Panel D was analyzed using Kruskal Wallis followed by Dunn's post hoc test; *p<0.05, **p<0.01.

Current therapeutic strategies for diabetic MASLD should concurrently target metabolic dysregulation and progressive hepatic injury, particularly fibrosis, with several pharmacological agents currently under investigated. However, robust evidence supporting their long-term safety, efficacy, and regenerative capacity in MASLD remains limited.(31) Consequently, insulin therapy remains the preferred treatment for glycemic control in patients with coincident T2DM and decompensated liver disease.(32)

Although UC-MSCs cell infusion has been shown to improve glucose and lipid metabolism *in vivo* (9), its clinical translation in diabetic MASLD remains limited and

Table 2. Histological scoring of hepatic steatosis and inflammation in diabetic MASLD rats.

Group	Mean Steatosis Score			Steatosis Cumulative Score	Mean Inflammatory Score	Overall Diagnosis
	Macrovesicular Steatosis	Microvesicular Steatosis	Hypertrophy			
Normal	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0 (Normal)	Normal
Diabetic	0.4±0.5	1.0±1.0	0.8±0.8	2.2±2.0	2 (Moderate)	MASH
Insulin	0.0±0.0	0.8±0.8	0.2±0.4	1.0±1.0	0 (Normal)	MASLD
Secretome	0.2±0.4	0.2±0.4	0.2±0.4	0.6±1.3	0 (Normal)	MASLD
Co-treatment	0.3±0.0	0.2±0.4	0.0±0.0	0.2±0.4	0 (Normal)	MASLD
p-value	0.213	0.133	0.110	0.133	0.124	

p-values were calculated using the Kruskal-Wallis test.

inconsistent. The use of UC-MSCs secretome in this study is grounded in its cell-free nature, offering a safer and more practical approach while retaining the stem cell regenerative potential. Importantly, cell-free therapy overcomes major limitations of viable cell transplantation, such as maintaining cell viability and functionality within a hostile microenvironment as in diabetic MASLD.(33) Importantly, several *in vivo* studies have reported the therapeutic potential of UC-MSCs secretome in T2DM. Previous studies have shown that UC-MSCs secretome enhances pancreatic β-cell number and insulin secretory capacity (18), while intravenous administration of UC-MSCs exosomes combined with insulin increases the expression of membrane translocation of glucose transporter 4 (GLUT4) in muscle and promotes hepatic glycogen storage. (34) However in this finding, treatment with insulin, UC-MSCs secretome, or their combination did not significantly improve FBG levels. Although the combination therapy showed a trend toward greater FBG reduction, that is 16.4% lower FBG level than the untreated diabetic group at the final observation (276.0±99.8 *vs.* 330.2±44.0 mg/dL), this difference did not reach statistical significance.

Intriguingly, insulin monotherapy failed to significantly reduce FBG levels across the study period. An *in vivo* study showed that intermittent insulin injections resulted only transient and negligible glucose-lowering effects, even when long-acting insulin was used; effective glycemic control was achieved only through continuous insulin infusion.(35) Therefore, the limited therapeutic response observed in the Insulin group may partly reflect suboptimal insulin exposure, rather than an inherent lack of insulin efficacy. Future studies should employ clinically relevant insulin regimens to better represent standard therapeutic practice, such as more frequent administration or long-acting formulations.

Hence, we suggest that secretome may not act as a primary glucose-lowering agent in diabetic MASLD settings; instead, enhances metabolic responsiveness when paired with insulin, at least through the restoration of hepatic structure and function. This interpretation is supported by the observed reduction in hepatomegaly and Ishak fibrosis score as compared to the untreated diabetic group, indicating improved hepatic histopathological outcomes. Importantly, the combined treatment did not cause hypoglycemia or other adverse events, supporting the safety profile of secretome as an adjunctive therapy.

Similar with our finding, administration of embryonic stem cells secretome failed to result significant reductions in blood glucose levels. Instead, the secretome therapy reduced hepatic lipid accumulation and fibrosis.(36) Another study demonstrated that treatment of Wharton's jelly MSCs secretome could potentially ameliorate MASH by promoting anti-inflammatory IL-10 expression, lowering Caspase-3 and -9 levels to suppress apoptosis, and alleviating oxidative stress.(37)

Recognizing that combined insulin and secretome therapy mitigated hepatic structure and fibrosis in diabetic rats, the study further elucidated whether these effects were concomitant with modulation of hepatic oxidative stress. In diabetes, excessive reactive oxygen species (ROS) production and impaired antioxidant defense are central drivers of liver injury, as the liver serves as the main organ to detoxify oxidative stress. Systemic IR causes lipid accumulation in the liver, which results in persistent oxidative stress and triggers the secretion of inflammatory cytokines, such as IL-6, resulting in a low-constitutive inflammation state that exacerbates hepatic dysfunction. (7,11,38) In line with this, diabetic MASLD rats exhibited elevated hepatic IL-6 protein levels and reduced hepatic SOD activity compared to the Normal group, although the reduction in SOD activity was not statistically significant. Treatment with insulin, secretome, or their combination led to an increasing trend of hepatic SOD activity and reduced IL-6 protein levels. However, these changes did not reach statistical significance, suggesting that the observed histopathological improvements may not have been primarily mediated by direct alterations of SOD activity or pro-inflammatory IL-6 levels. Instead, although not directly investigated in the present study, MSCs secretome has been shown to exert anti-inflammatory effects by stimulating the production of hepatic anti-inflammatory cytokines (*e.g.*, IL-4, IL-5, and IL-10), rather than solely inhibiting pro-inflammatory mediators.(39)

Notably, previous characterization confirmed that the currently used UC-MSCs secretome contains abundant growth factors, particularly hepatocyte growth factor (HGF).(20) In another investigation, HGF has been reported to exert hepatoprotective and antifibrotic effects, as well as to improve hepatic lipid metabolism. Specifically, HGF has been shown to enhance the expression of LDL receptors, apolipoprotein B, and microsomal TG transfer protein, which are key components involved in the assembly and secretion of very low-density lipoprotein (VLDL), thereby facilitating triglycerides export from hepatocytes. (40) These reported mechanisms may partially explain the favorable lipid profile observed following UC-MSCs secretome administration, that is lower total cholesterol and higher HDL levels.

Nevertheless, further investigations are warranted to elucidate the precise mechanisms underlying the therapeutic effects of UC-MSCs secretome as an adjuvant to insulin therapy. The current study has a relatively small sample size and short treatment duration, which may have limited the statistical power to detect differences in several biochemical and histological outcomes, contributing to the relatively large variability observed in some parameters. In addition, only a single treatment dose and administration regimen were evaluated; therefore, dose-response relationships and the optimal therapeutic protocol remain to be determined. Direct assessment of IR, such as HOMA-IR, molecular characterization and comprehensive profiling of bioactive components in UC-MSCs secretome was also not performed in the current study. Future studies incorporating larger sample sizes, comprehensive mechanistic analyses, dose optimization, and longer follow-up periods are warranted to further validate these findings.

Conclusion

Combination of insulin and UC-MSCs secretome therapy in diabetic MASLD rat model was able to show a trend of FBG reduction, lipid profile improvements, AST and ALT reductions, increased SOD activity, and IL-6 reduction compared with the untreated diabetic MASLD rat model. The combination therapy was also significantly reduce hepatomegaly and improve hepatic fibrosis. Though several biochemical parameters did not show statistical significance, these findings suggest that UC-MSCs secretome might serve as a potential adjunct to insulin therapy.

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Authors Contribution

SG, SH, and HUB were involved in the conceptualization of the study. SH prepared the methodology. SH, OM, and JL performed the data curation. SG, OM, and JL did the investigation and validation. OM performed formal analysis and prepared the visualization. SG, SH, and HUB was involved in the funding acquisition and supervision. SG and OM prepared the original draft of the manuscript. All authors took parts in giving critical revision of the manuscript.

Ethical Statement

The Institutional Animal Care and Use Committee of Tarumanagara University approved the experimental procedures used in this study (approval no. 019.KEPH/UPPM/FK/VI/2024). All animal housing and experiments were conducted in strict accordance with the institutional Guidelines for Care and Use of Laboratory Animals at Tarumanagara University.

Conflict of Interest

The authors declare no conflict of interest.

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