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Regenerative therapeutic effects of conditioned medium from human umbilical cord-derived mesenchymal stem cells as an adjuvant to insulin therapy in a rat model of type 2 diabetes

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Abstract. Standard therapies for type 2 diabetes (T2DM) that utilize anti-diabetic drugs and insulin are well established; nevertheless, the incidence of complications and mortality associated with the disease remain relatively high. Conditioned medium (CM) from human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) has demonstrated regenerative effects, although these have yet to be properly investigated for the purposes of managing T2DM. Therefore, the present study aimed to evaluate the therapeutic potential of CM from hUC-MSCs in various animal models of T2DM. T2DM was established in rats using a high-fat diet and streptozotocin, and the rats were subsequently injected with insulin, CM or insulin + CM two times per week (subcutaneously for insulin; intraperitoneally for CM) over a 4-week period. Blood glucose levels were measured prior to each treatment up until 2 weeks after the final injection. At the end of the experiment, glucose tolerance, insulin sensitivity and the levels of glycated hemoglobin (HbA1c), serum insulin and insulin-like growth factor-1 (IGF-1) were measured. Histological analyses of pancreatic tissues were also performed, and the findings were compared among the groups. The CM-treated group was found to exhibit significantly improved levels of blood glucose, glucose tolerance and insulin sensitivity, whereas administering a combination of CM and insulin therapy to the rats led to even more robust effects in terms of blood glucose level management and reducing the level of HbA1c compared with the other treatment groups. CM treatment also led to an improvement in the IGF-1 serum level, although this was found to be reduced in the other diabetic groups. Furthermore, both the numbers of β-cells and their capability to secrete insulin were enhanced in the CM-treated group. Taken together, these findings underscore the regenerative effect of CM from hUC-MSCs in terms of T2DM therapy.

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Introduction

Diabetes mellitus (DM) is one of the global burden diseases and a major cause of mortality, with a reduced life expectancy for affected individuals (1). In 2021, there were approximately 537 million individuals worldwide affected by diabetes, according to the International Diabetes Federation; without proper medical implementation and effective prevention methods, however, these numbers are predicted to increase to as high as 783 million individuals by 2045 (1,2). In Indonesia, ~19.5 million adults were diagnosed with diabetes in 2021, of which 6.7 million succumbed due to the complications of this disease (2). Moreover, high management costs lead to the further worsening of the socio-economic conditions affecting this disease. Diabetes itself is a chronic, non-communicable disease that is characterized by hyperglycemia and impaired glucose tolerance. Specifically, type 2 diabetes (T2DM) is characterized by insulin resistance and impaired β-cell function, and this is mainly associated with β-cell death (3). However, recent studies have shown that this dysfunctionality occurs due to a more complex network of interactions among the environment and several molecular pathways in cell biology (4-6). Generally, T2DM develops as a consequence of unhealthy lifestyles, including poor diet and a lack of physical activity. This is also associated with an increase in the levels of markers of chronic systemic inflammation (low grade), including interleukin 6 and tumor necrosis factor- α (TNF-α), and this phenomenon induces metabolic inflammation (4,7-9).

Although therapies for T2DM comprising anti-diabetic medication and insulin therapy are well established, both the number of complications and the mortality rate remain relatively high. Hence, alternative management therapies for T2DM are urgently required. Over the past few years, regenerative medicine has yielded promising results, and the use of mesenchymal stem cells (MSCs) has garnered attention as a potential therapeutic approach. Human umbilical cord-derived MSCs (hUC-MSCs) have been demonstrated to have the ability to differentiate into various cell types, to modulate immune responses, and to secrete bioactive molecules that exert a role in tissue repair and regeneration (10,11). These secreted factors are found in the medium in which cells are cultured under certain conditions. Therefore, this medium is termed conditioned medium (CM) (12,13). CM provides an attractive alternative strategy for the management of T2DM due to its regenerative capability via its paracrine activities. Robust studies have previously explored the potential of CM for the treatment of type 1 diabetes (14); however, only a few of these studies have investigated the CM from hUC-MSCs as an alternative treatment for T2DM (15-17). Therefore, the present study aimed to evaluate the therapeutic potential of CM from hUC-MSCs as an alternative treatment for hyperglycemia management in T2DM-induced rats, and the findings demonstrated herein will highlight the regenerative effects of CM from hUC-MSCs in T2DM therapy.

Materials and methods

Ethical approval. All animal procedures performed in the present study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine, Tarumanagara University, Jakarta, Indonesia, with the ethical approval no. 019.KEPH/UPPM/FK/VI/2024. The duration of the animal experiments was 4 months, upon animal arrival until the study endpoint.

CM production. The CM used in the present study was derived from hUC-MSCs and produced in Tarumanagara Human Cell Technology Laboratory, Jakarta, according to a previously published protocol (18). In brief, MSCs were isolated from fresh umbilical cords obtained from caesarian delivery with parental consent (ethical approval no. PPZ20192062, obtained from the Universitas Tarumanagara Human Research Ethics Committee, Tarumanagara University). MSCs were cultured until they had reached 80% confluency at passages 5-6, and subsequently the medium was replaced with serum-free media [Gibco® Minimum Essential Medium Alpha (MEMα); Thermo Fisher Scientific, Inc.] and cultured under hypoxic conditions (in the presence of 5% O_2) for 3 days. Finally, the cultured medium was collected and filtered as a CM.

Animal experiments. Male Sprague-Dawley rats, aged 8-10 weeks (n=33), were obtained from the Indonesian Food and Drug Authority, Jakarta, Indonesia. The number of rats was accounted for using resource equation ANOVA (19,20), also taking into consideration the 3Rs principle (Replacement, Refinement and Reduction). The rats were group-housed and acclimatized to the laboratory conditions (12-h light:dark cycle, with access to water and feed provided *ad libitum*) for 1 week, and fed a standard feed (PT Surya Sains Indonesia) during this period, until their body weight (BW) had reached 180-200 g. The animals were maintained in a controlled environment and monitored daily. At the start of the experiment, individual housing was implemented to enable the accurate measurement of individual feed intake. Structural environmental enrichment was provided throughout the study period and animal behavior was monitored daily. Humane endpoints were employed to minimize animal suffering throughout the study. These included severe pain or distress indicated by the inability to eat or drink for >24 h, body weight loss >20% within 1 week or 10% within 24 h, persistent hunched posture for >8 h, or any other clinical signs indicating a poor prognosis, as determined by the attending veterinarian.

Development of the animal model of T2DM. The T2DM model was rendered in 28 rats, constructed as previously described (21,22). Essentially, T2DM induction was performed by feeding the rats a high-fat diet (HFD) comprising 33% fat, 37% carbohydrate and 13% protein content feed (PT Surya Sains Indonesia) combined with injections of streptozotocin (STZ) (MilliporeSigma). Rats receiving HFD feed were adapted for 3 days (details provided below) before being entirely administered HFD feed. During the adaptation process, feeds were administered in decreasing percentage ratios between standard feed and HFD for each consecutive day, namely 75:25, 50:50 and 25:75% (Fig. 1). Subsequently, the group fed the HFD was administered 100% HFD feed for 4 weeks. Both the condition of the animals and the feed intake were monitored on a daily basis. Fasting blood glucose (FBG) levels were examined in all animals using a glucometer (Accu-chek®; Roche Diagnostics), with a minimum of 6 h fasting prior to every blood glucose check. At the end of 4 weeks of providing the rats a HFD, the FBG level was examined as the baseline prior to a low dosage injection of STZ (30 mg/kg BW via the intraperitoneal route). The dosage of STZ administered was based on previous studies, although with modifications (21,22). STZ was freshly prepared by dissolving the compound in 0.09 M sodium citrate buffer (MilliporeSigma) under sterile and dark conditions. The FBG levels of the T2DM-modeled rats were monitored on day 3 and 9 post-STZ administration, in order to confirm the establishment of T2DM. Rats were confirmed to be diabetic if the FBG level was >200 mg/dl; re-injection of STZ (30 mg/kg BW, via the intraperitoneal route) was performed if the FBG level was <200 mg/dl. The level of FBG was subsequently monitored again on days 3 and 9 post re-injection. Rats with FBG levels >200 mg/dl were then included in the present study, whereas those with a FBG level that persisted <200 mg/dl were earmarked to be excluded from the study. Of the 28 rats induced with STZ, 8 were found dead 3 days after the first STZ injection possibly due to an acute STZ toxicity (23,24), leaving 20 rats eligible for treatment. During the study, appropriate medical interventions were applied when necessary, including insulin administration for FBG levels >400 mg/dl, subcutaneous fluid (Ringer's lactate solution, B. Braun) infusion for signs of dehydration, and paracetamol for indications of pain. Following this time point, rats were continuously fed a HFD throughout the remainder of the study period (Fig. 2).

Intervention of CM from hUC-MSCs, insulin and serum-free media. Rats were randomly divided into five experimental groups (n=5 rats per group), and the investigators were

Figure 1. Standard feed and HFD feed. 1, 100% standard feed; 2, 75% standard feed:25% HFD; 3, 50% standard feed:50% HFD; 4, 25% standard feed:75% HFD; 5, 100% HFD. HFD, high-fat diet.

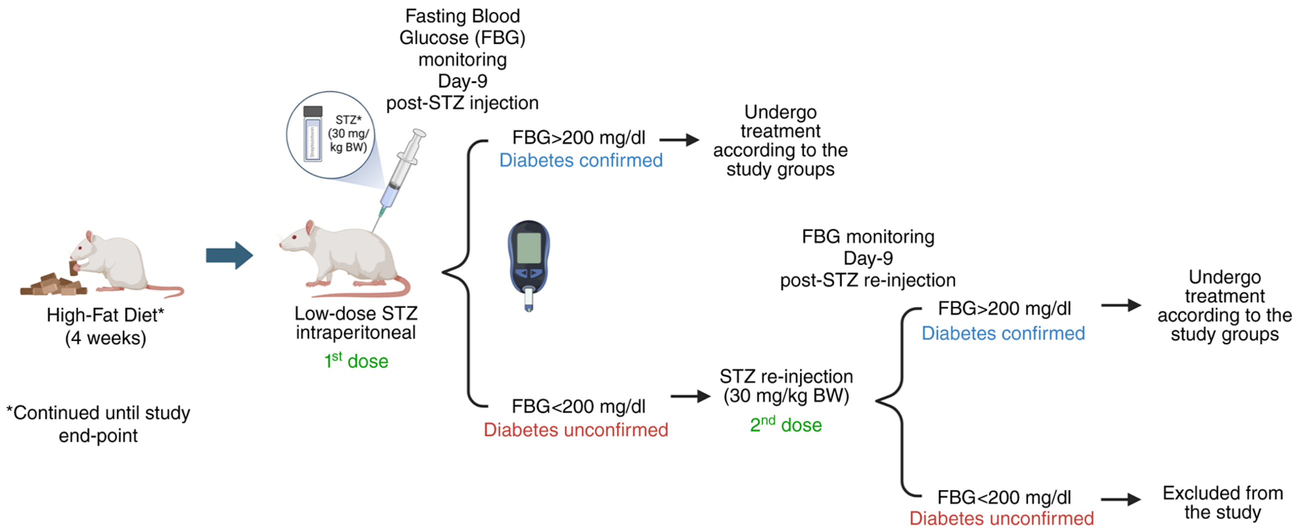

Figure 2. Schematic diagram of type 2 diabetes animal model development. STZ, streptozotocin; FBG, fasting blood glucose; BW, body weight.

blinded to the group allocation. The groups were designated as follows: i) The normal group, wherein the rats were fed without any diabetic interventions; ii) the control DM group, wherein diabetic rats were treated with serum-free medium (MEMα), administered 0.5 ml intraperitoneally; iii) the DM + insulin group, wherein diabetic rats were treated with insulin (NovoRapid®; Novo Nordisk), administered 1 unit subcutaneously; iv) the DM + CM group, wherein diabetic rats were treated with CM, administered 0.5 ml intraperitoneally (25,26); and v) the DM + insulin + CM group, wherein the diabetic rats were injected with insulin (1 unit subcutaneously) and CM (0.5 ml intraperitoneally). The treatments were started immediately following T2DM confirmation, and the rats received the treatments two times per week for 4 weeks (Fig. 3). The FBG level was examined prior to each treatment, and subsequently monitored for up to 2 weeks after the final treatment.

Performances of the oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (IPITT). At 2 weeks following the final treatment, an OGTT and IPITT were performed. For the OGTT procedure, the rats were fasted for at least 4 h, and the observed FBG level was regarded as the baseline. Subsequently, glucose solution (2 g/kg BW) was administered via oral gavage (17). Blood glucose levels were examined at 30, 60, 90 and 120 min post-glucose administration. IPITT was performed similarly to the OGTT procedure, with the exception that insulin (1 unit) was administered intraperitoneally instead of glucose solution. Blood glucose levels were then examined at 30, 60, 90 and 120 min post-insulin administration to observe the corresponding insulin sensitivity of the rats. To more effectively assess the overall glucose intolerance and insulin sensitivity, the area under the curve (AUC) (mg/dl·min) was calculated using the trapezoidal rule (27).

Euthanasia and blood analyses. On the following day, the rats were euthanized via an exsanguination process by cardiac puncture under deep anesthesia. Anesthesia was induced by intraperitoneal injection of 10% ketamine (80 mg/kg BW) and 2% xylazine (10 mg/kg BW) prior to blood collection (8-10 ml). Death was confirmed by the attending veterinarian or veterinary paramedic through noting the absence of a heartbeat or respiration. Whole blood was used to measure the glycated hemoglobin (HbA1c) level, and the serum was subjected to enzyme-linked immunosorbent assay (ELISA) to measure the levels of insulin (Elabscience® cat no. #E-EL-R3034; Elabscience Bionovation, Inc.) and insulin-like growth factor-1 (IGF-1) (Invitrogen® cat no. #ERIGF1; Thermo Fisher Scientific, Inc.). Homeostasis model assessment for insulin

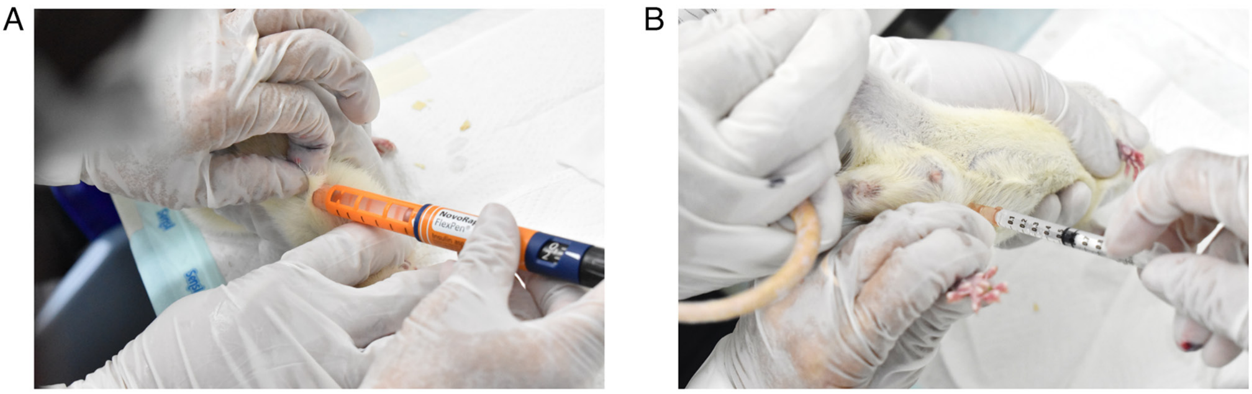

Figure 3. Administration of treatments according to the study groups. (A) Subcutaneous administration of insulin and (B) intraperitoneal injection of serum-free media or conditioned medium.

resistance (HOMA-IR) was also calculated using the FBG level (mg/dl) x the fasting insulin serum level (mU/l)/405 (22,28).

Hepatic inflammation analyses. At the endpoint of the experiment, liver tissue was obtained, weighed within an accuracy of ±0.1 g, and immediately stored in a deep freezer (-80˚C) prior to protein isolation. Protein isolation was performed according to the manufacturer's instructions. Cold radioimmunoprecipitation assay (RIPA) lysis buffer (Elabscience® cat no. #E-BC-R327; Elabscience Bionovation, Inc.) containing 1% protease inhibitor cocktail (cat no. #HY-K0010; MedChemExpress) was used to isolate the whole liver protein. The total protein concentration was subsequently measured using a Bradford kit assay (Elabscience® cat no. #E-BC-K168-M; Elabscience Bionovation, Inc.). Standardized protein samples were then subjected to ELISA to determine the levels of the pro-inflammatory marker, TNF-α (cat. no. #DY510-05; R&D Systems, Inc.).

Histopathological analyses. Pancreatic tissue was collected at the endpoint of the experiment, and fixed in 4% paraformaldehyde solution (4˚C, 72 h). The fixed pancreas tissues were stained with hematoxylin for 10 min at room temperature (ScyTek Laboratories Inc.) and eosin for 1 min at room temperature (Thermo Fisher Scientific, Inc.) (H&E) to observe the morphology of islets of Langerhans. Apart from H&E staining, the fixed pancreatic tissue was also stained with Gomori Aldehyde Fuchsin stains for 1 h at room temperature (Fuchsin: Gurr Certistain, BDH Laboratory; paraldehyde: MilliporeSigma) to identify the numbers of β-cells. The tissue was also subjected to immunohistochemical staining to measure the expression of insulin in a process that involved the use of specific antibodies to detect the presence of insulin in the tissue. This procedure was performed at the Pathology Laboratory, Primate Research Center, Bogor Agricultural University, Bogor, Indonesia.

Statistical analysis. All results are expressed as the mean ± SD. The normality of the data was assessed using the Shapiro-Wilk test. For multiple comparisons, one-way ANOVA or repeated measures ANOVA was used, and followed by Tukey's post-hoc test for normally distributed data. Otherwise, Kruskal-Wallis with pairwise comparisons followed by Dunn's post-hoc test was used. For comparison within groups, the paired Student's t-test was used for normally distributed data, otherwise the Wilcoxon signed rank test was used. $P<0.05$ was considered to indicate a statistically significant difference, whereas $P<0.01$ was considered to indicate a highly statistically significant difference.

Results

Successful induction of diabetes. Diabetes was successfully induced in the rats that were assigned to the diabetic group, with an 85% success rate obtained after the first dose of STZ, followed by the remaining 15% of the rats achieving the successful induction of T2DM after the second dose. Therefore, no animals were excluded in the present study. During the study period, BW, food and calorific intake were monitored daily. Although all diabetic groups exhibited a slight increase in BW between the post-STZ (namely, prior to the administration of the specific treatments) and post-treatment periods, these differences were found not to be statistically significant. Similarly, no significant changes were observed in either food or calorific intake comparing between the post-STZ and post-treatment periods, although an increase in calorie intake was observed in the DM + insulin and DM + CM experimental groups (Table I).

CM from hUC-MSCs reduces blood glucose levels in rats with T2DM. To determine whether the CM derived from hUC-MSCs could exert any therapeutic effects in the management of hyperglycemia in rats with T2DM, CM from hUC-MSCs was administered intraperitoneally according to the design of the various experimental groups. Repeated blood glucose monitoring revealed that CM-treated groups (namely, the DM + CM and DM + insulin + CM groups) exhibited lower blood glucose levels compared with the other groups (namely, the control DM and DM + insulin groups) (Fig. 4A). This decrease was found to be significant during the injection period. However, at 1 and 2 weeks following the final injection, no significant differences were observed among the groups. Rats in the DM + insulin + CM group exhibited the lowest blood glucose levels compared with those in the other groups, suggesting the positive effect of CM on blood glucose regulation in T2DM. In accordance with this finding, the DM + insulin + CM group also displayed

Table I. Body weight, food and calorie intake of the rats (n=5/group).

Groups	Body weight (g)			Food intake (g)		Calorie intake (kcal)		
	Post-STZ[a]	Post-treatment[a]	P-value[b]	Post-STZ	Post-treatment	Post-STZ	Post-treatment	P-value[c]
Normal	271.40±7.44	314.40±9.18	0.042[d]	20.00±0.00	19.40±1.34	60.59±0.00	58.77±4.06	0.317
Control DM	252.00±20.38	264.20±31.34	0.678	13.60±2.30	13.80±2.02	67.13±11.36	68.12±9.96	0.686
DM + insulin	263.80±33.98	279.40±39.22	0.532	11.00±3.94	13.70±0.57	54.30±19.43	67.63±2.81	0.216
DM + CM	243.80±19.60	245.20±19.03	0.957	10.60±3.05	12.70±2.36	52.32±15.05	62.69±11.66	0.225
DM + insulin + CM	259.60±20.91	275.60±15.93	0.329	12.80±3.70	12.80±1.48	63.18±18.27	63.18±7.32	0.892

Data are expressed as the mean ± SD. [a]Post-STZ, before treatment was administered; post-treatment, at 1 week after the final treatment. [b]P-value for body weight; analysed using a paired Student's t-test. [c]P-value for food intake and calorie intake; analysed using the Wilcoxon signed rank test. [d]P<0.05 compared within the group. STZ, streptozotocin; DM, diabetes mellitus; CM, conditioned medium.

the lowest HbA1c levels (6.5±1.6%) compared with the control group (7.6±0.6%) (P=0.358) (Fig. 4B). This highlights the additive effect of combining CM and insulin therapy in terms of reducing HbA1c levels in T2DM. On the other hand, fasting insulin serum levels did not exhibit any significant differences across all the treatment groups, and these were also similar to the normal group (Fig. 4C). The HOMA-IR score, which estimates insulin resistance, was shown to be significantly higher in all diabetic groups compared with the normal group (Fig. 4D). Finally, although not statistically significant, the DM + insulin + CM group achieved the lowest insulin resistance index (4.17±1.28) among the diabetic groups, suggesting a potential improvement of insulin sensitivity through the use of combined therapy.

CM from hUC-MSCs promotes glucose tolerance and insulin sensitivity in rats with T2DM. The glucose tolerance level and insulin sensitivity were subsequently assessed using an OGTT and IPITT, respectively. Blood glucose levels were measured at 0, 30, 60, 90 and 120 min following glucose administration (OGTT) or insulin injection (IPITT). In the OGTT, blood glucose levels in all diabetic groups were found to increase sharply during the first 60 min post-glucose administration. Furthermore, glucose levels in the normal group were relatively stable throughout the time point (Fig. 5A). Further analysis, performed by measuring the AUC, which provides an indication of glucose intolerance, revealed significantly higher AUC values (P<0.001) in all the diabetic groups compared with the normal group, confirming the existence of glucose intolerance under diabetic conditions (Fig. 5B). Among all the diabetic groups, the DM + insulin + CM group exhibited an improved glucose tolerance, although this improvement was found not to be statistically significant.

As regards the IPITT, blood glucose levels exhibited a decrease in all groups up to 90 min following insulin administration. By the 120 min time point, all groups exhibited a slight increase in blood glucose levels, with the exception of the control diabetic group, which maintained the same level as at 90 min (Fig. 5C). Although not statistically significant, the decreases in the level of blood glucose in the rats with T2DM treated with exogenous insulin alone (namely, the DM + insulin group) were less pronounced compared with those of the other groups (Fig. 5C). Accordingly, the AUC analysis demonstrated that all diabetic groups had significantly higher AUC values compared with the normal group, with the DM + insulin group displaying the largest AUC value, indicating its poor insulin sensitivity compared with the other diabetic groups (Fig. 5D). Although no significant differences were identified among the diabetic groups, both of the CM-treated groups (namely, the DM + CM and the DM + insulin + CM groups) demonstrated an improved insulin sensitivity compared with the DM + insulin group.

CM from hUC-MSCs exhibits an elevated level of IGF-1 secretion in rats with T2DM. IGF-1 is a key cytokine that regulates glucose uptake and insulin sensitivity. It is primarily produced by the liver, and released into the bloodstream. In T2DM, however, circulating IGF-1 levels are altered due to insulin resistance, which has been shown to impair the signaling pathways involved in IGF-1 production (29). In

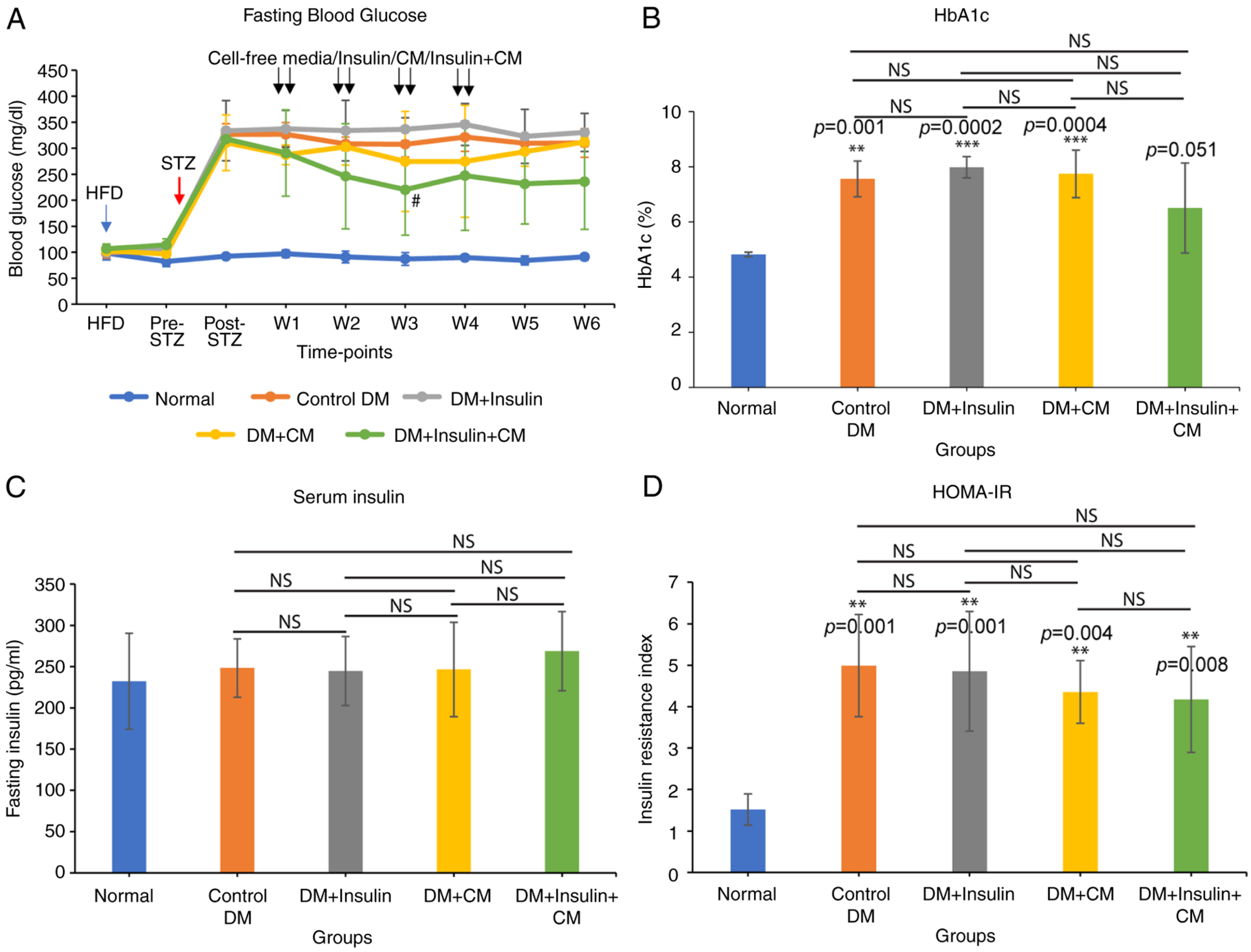

Figure 4. CM positively regulated blood glucose levels in rats with diabetes. Comparison of (A) fasting blood glucose levels, (B) HbA1c, (C) fasting serum insulin level, and (D) insulin resistance index between groups. HFD indicates the starting point of HFD feed; Pre-STZ represents 4 weeks post-HFD and before the STZ injection; Post-STZ represents diabetes confirmation; W1-W4 represents weeks 1-4 of treatment; W5-W6 represents weeks 5-6 (2 weeks after the final treatment). Data are expressed as the mean ± SD (n=5/group). $^{\#}$P<0.05, significant difference compared to the DM + insulin group, **P<0.01 and ***P<0.001, significant difference compared to the normal group (P-values are indicated above the bars); NS, not significant. Statistical analysis was determined using (A) repeated measures ANOVA and (B-D) one-way ANOVA. DM, diabetes mellitus; CM, conditioned medium; HFD, high-fat diet; HbA1c, glycated hemoglobin.

the present study, the analysis of serum IGF-1 levels revealed that the DM + CM group had the highest level of IGF-1 compared with the other diabetic groups, with a value of 1,085±343 pg/ml, almost comparable with that of the normal group (1,308±610 pg/ml) (Fig. 6A). By contrast, the groups treated with exogenous insulin exhibited no improvement in serum IGF-1 levels, where these remained the lowest.

CM from hUC-MSCs reverses hepatic inflammation in rats with T2DM. Improvements in blood markers would be representative of the therapeutic effects of insulin + CM therapy in a T2DM setting. As the primary site for gluconeogenesis, glyconeogenesis and glycogen storage, the liver has a central part in maintaining glucose homeostasis (30). Hence, from a mechanistic perspective, the present study examined whether CM from hUC-MSCs could protect the liver against inflammatory damage in rats with T2DM. In diabetic rats, the hepatic TNF-α level was found to be higher compared with that in normal rats (78.08±36.43 pg/ml in the DM group compared with 33.99±7.88 pg/ml in the normal group; P=0.029). Although the administration of insulin or CM alone was shown to reduce hepatic TNF-α levels (67.38±30.22 pg/ml in the DM + insulin group, and 62.69±17.85 pg/ml in the DM + CM group), administering the combination therapy of insulin + CM resulted in a further decrease in the TNF-α concentration to 39.36±10.07 pg/ml (Fig. 6B), which approached the level observed in the normal group. These results demonstrated the positive effect of CM in attenuating hepatic inflammation.

CM from hUC-MSCs results in increased numbers of pancreatic β-cells, with an improved ability to produce insulin. To assess the effects of CM from hUC-MSCs on pancreatic function under T2DM conditions, a microscopic evaluation was performed via the methods of H&E, Gomori and insulin staining to examine, in turn, the morphology of islets of Langerhans, the numbers of β-cells and insulin production, respectively. H&E staining of pancreatic tissue sections revealed a reduction in both the size and numbers of the islet of Langerhans in all diabetic rats compared with the normal group (Fig. 7). Infiltration by a small number of lymphocytes

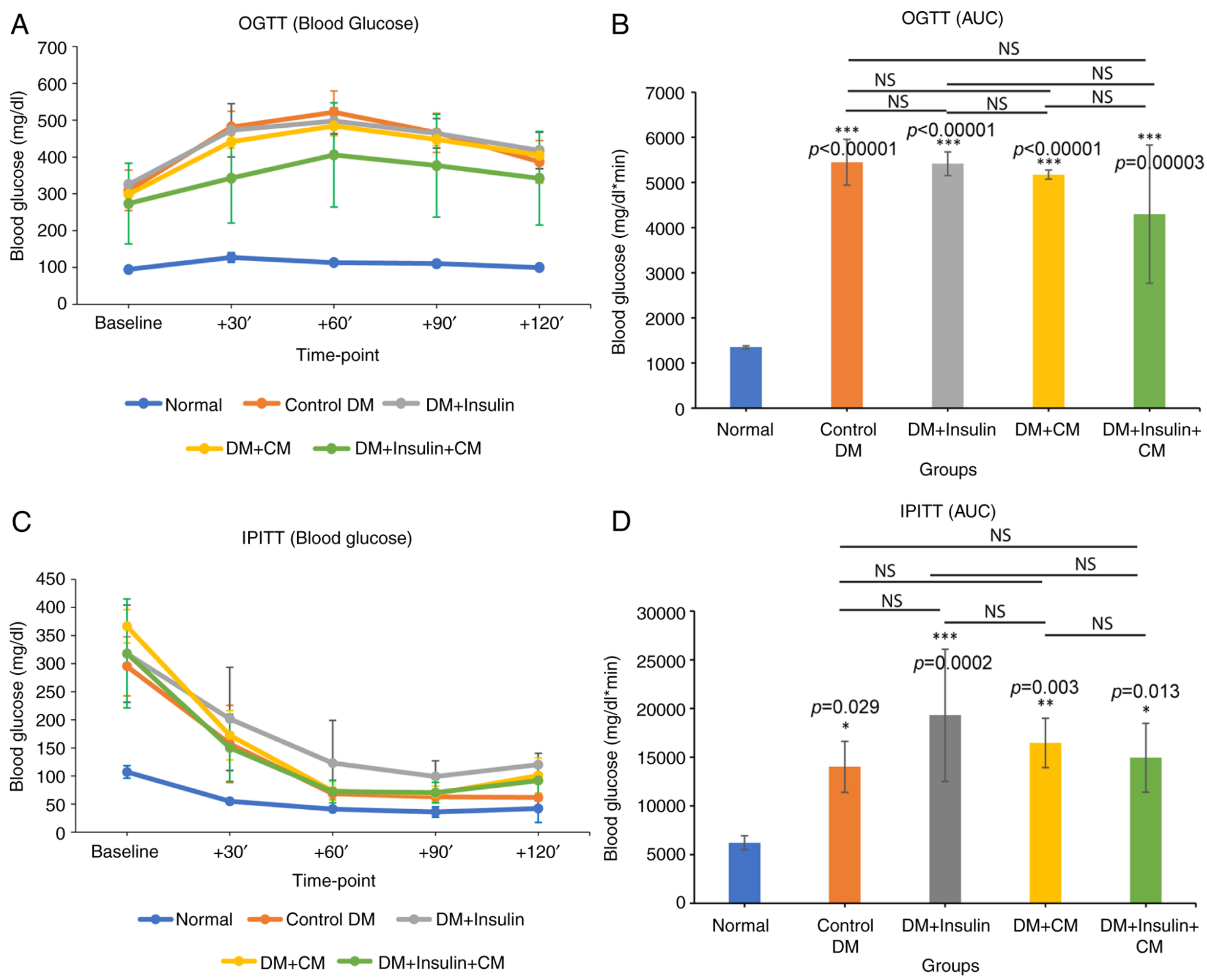

Figure 5. The glucose tolerance test and insulin sensitivity test. (A) OGTT of each group was determined at 0, 30, 60, 90 and 120 min post-glucose administration. (B) Area under the curve of OGTT. (C) IPITT of each group was determined at 0, 30, 60, 90 and 120 min post-insulin administration. (D) Area under the curve of IPITT. Data are expressed as the mean ± SD (n=5/group). *P<0.05, **P<0.01 and ***P<0.001, significant difference compared to the normal group (P-values are indicated above the bars); NS, not significant. Statistical analysis was determined using (A and C) repeated measures ANOVA and (B and D) one-way ANOVA. OGTT, oral glucose tolerance test; IPITT, intraperitoneal insulin tolerance test; DM, diabetes mellitus; CM, conditioned medium.

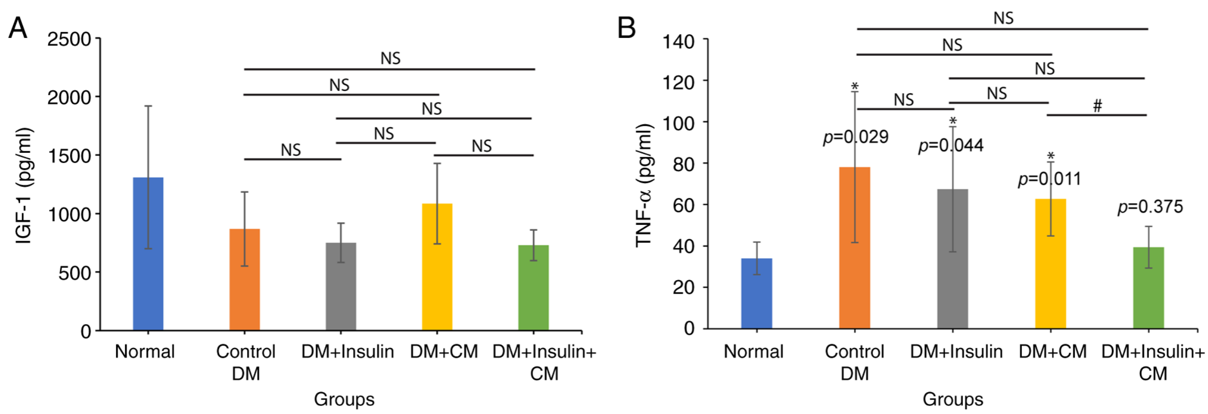

Figure 6. Measurement of serum IGF-1 and hepatic TNF-α protein levels. (A) Serum IGF-1 and (B) hepatic TNF-α protein levels were measured at the study endpoint using ELISA. Data are expressed as the mean ± SD (n=5/group). *P<0.05, compared to the normal group (P-values are indicated above the bars); #P<0.05, compared between indicated groups; NS, not significant. Statistical analysis was performed using one-way ANOVA. IGF-1, insulin growth factor-1; TNF-α, tumor necrosis factor-α; DM, diabetes mellitus; CM, conditioned medium.

was observed in the DM + insulin group, but not in the other experimental groups. The mean number of β-cells, as determined using Gomori staining, was found to be significantly higher in the DM + insulin + CM group compared with the control DM group (Table II). Similarly, the mean number of insulin-positive cells was also significantly higher in both

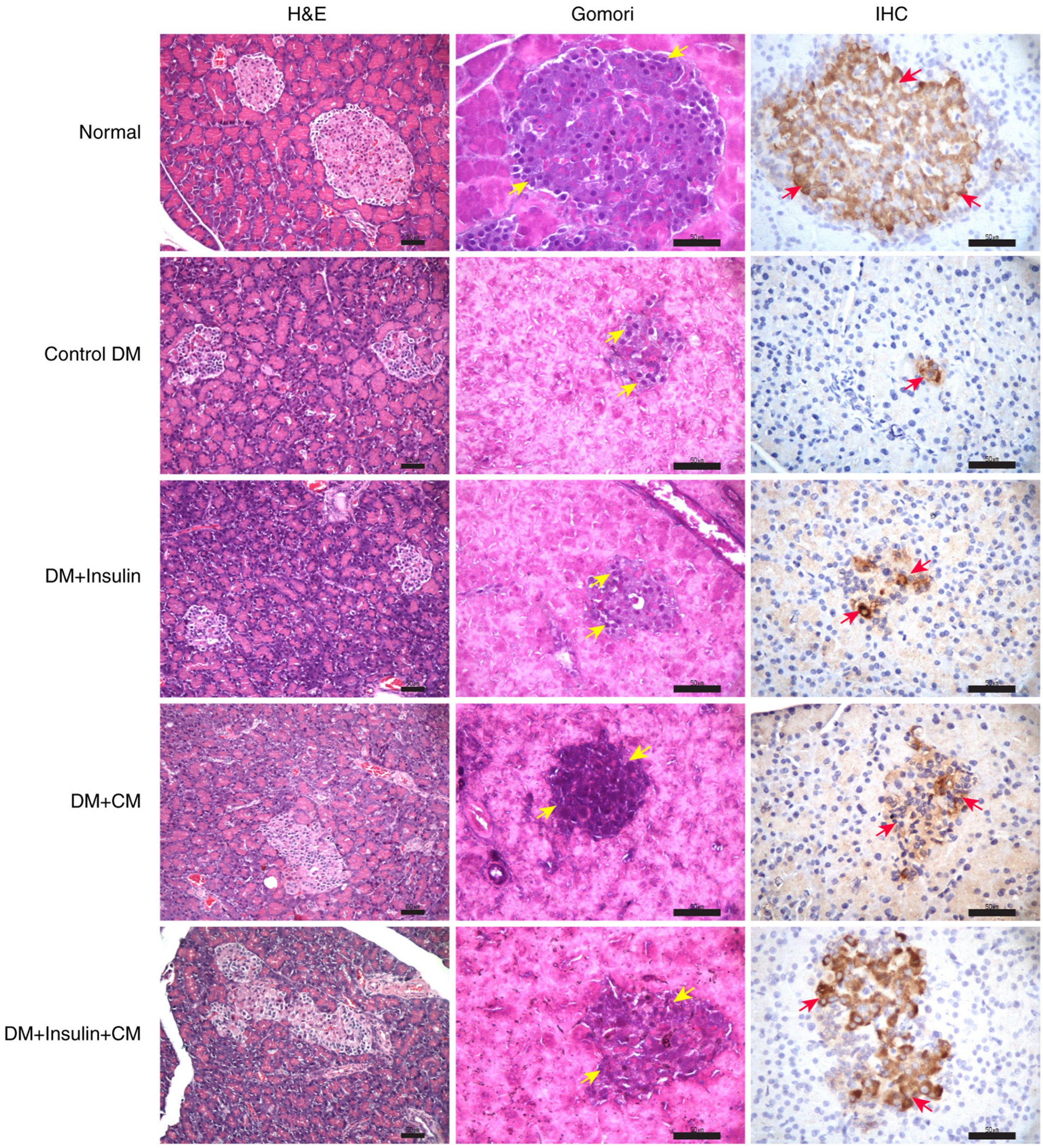

Figure 7. Histopathological analysis of islets of Langerhans on pancreatic tissue section stained with H&E and Gomori staining, and IHC for insulin. β-cells are represented as a dark blue color (yellow arrows) and insulin-positive cells are represented as brown colorization (red arrows). Magnification: x200 for H&E staining, and x400 for Gomori staining and IHC. Scale bars, 50 μm. H&E, hematoxylin and eosin. IHC, immunohistochemistry; DM, diabetes mellitus; CM, conditioned medium.

the CM-treated groups compared with the control DM and DM+ insulin groups (Table II). Taken together, these findings suggested that the intraperitoneal administration of insulin + CM led to beneficial effects in terms of enhancing pancreatic β-cell viability and function.

Discussion

Poor diet and a sedentary lifestyle are major contributors to the development of T2DM. As the underlying cause, insulin resistance over time results in the exhaustion of pancreatic β-cells, eventually leading to inadequate insulin production and elevated blood glucose levels (26-28). This condition is also associated with impaired glucose tolerance and low insulin sensitivity (34). At the same time, persistent hyperglycemia may lead to other complications, as for example, cardiovascular disease, nephropathy and neuropathy (3,35). The current standard therapy relies upon the use of exogenous insulin to achieve glycemic control; however, insulin resistance is associated with multiple organ dysfunctions, and merely lowering the level of blood glucose, as a therapeutic strategy, is insufficient to address the metabolic and inflammatory dysregulations that are observed in T2DM. Moreover, hyperinsulinemia induced by exogenous insulin can also exacerbate oxidative

Table II. Average number of β-cells per field of view and insulin-positive cells per field of view on the pancreatic tissue (n=5/group).

Groups	Gomori staining: No. of β-cells per field of view	Gomori staining: P-value	Immunohistochemistry: No. of insulin-positive cells per field of view	Immunohistochemistry: P-value
Normal	57±27		131±36	
Control DM	10±2		13±4	
DM + insulin	13±8		15±4	
DM + CM	20±11		27±10	0.009[b], 0.047[c]
DM + insulin + CM	27±15	0.037[a]	33±7	0.009[b], 0.009[d]

Data are expressed as the mean ± SD. Statistical analysis was determined using one-way ANOVA (Gomori staining) and the Kruskal-Wallis test (immunohistochemistry). [a]Significant at P<0.05 vs. control DM group, [b]significant at P<0.01 vs. control DM group; [c]significant at P<0.05 vs. DM+insulin group; [d]significant at P<0.01 vs. DM + insulin group. DM, diabetes mellitus; CM, conditioned medium.

stress and alter lipid metabolism, further worsening insulin resistance. As a result, insulin therapy fails to reduce the levels of inflammatory markers, with the consequence that neither cardiovascular burden nor mortality outcomes are effectively lowered in T2DM (36).

Stem cell therapy has emerged as a promising alternative for the treatment of various degenerative diseases through a more comprehensive approach. However, several challenges with stem cell therapy still remain, including immunogenicity risks, invasive procedures and low cell survival post-transplantation (37,38). To overcome these challenges, CM has garnered attention as a cell-free product with comparable regenerative properties. CM derived from MSCs is known to contain various bioactive molecules, including growth factors, extracellular vesicles (EVs), cytokines and exosomes (12,39). Recently, several studies have highlighted the regenerative capabilities of EVs or exosomes alone in a variety of disease models, including osteoarthritis, cardiovascular disease, neurodegenerative diseases and liver fibrosis, among others (35-41). Additionally, MSC-derived exosomes have been explored as therapeutic agents for the treatment of diabetes-related complications. The study by Li *et al* (47) demonstrated that hUC-MSC-derived exosomes shuffle the miRNA miR-17-3p, which targets the STAT1 signaling pathway, thereby alleviating inflammatory reactions and oxidative injury in diabetic retinopathy mice. Similarly, Yang *et al* (48) reported that exosomes incorporated into pluronic F-127 hydrogel could enhance diabetic wound healing through promoting wound closure, stimulating tissue regeneration, and upregulating the expression of vascular endothelial growth factor (VEGF) and transforming growth factor β-1. Despite its potential, however, the effects of CM have not yet been extensively investigated, particularly as regards the treatment of T2DM.

The present preliminary study explored the potential of whole CM as an adjuvant therapy to exogenous insulin for the management of hyperglycemia in rats with T2DM. In the present study, it was found that the intraperitoneal administration of insulin + CM alleviated the pancreatic dysfunction that arises under T2DM conditions, particularly by enhancing the proliferation of β-cells and their insulin-producing capability. These findings are consistent with those of previous studies, which demonstrated the positive effects of MSCs and their CM in terms of promoting β-cell proliferation and function (49,50). MSCs have the ability to regenerate pancreatic islet β-cells, to protect them from apoptosis, and even to improve insulin resistance in peripheral tissues, a process that is mainly driven by the activities of their paracrine factors (51,52). Moreover, insulin + CM therapy has previously been shown to exert protective effects against hepatic inflammation, as revealed by lower levels of the pro-inflammatory protein, TNF-α. In the context of T2DM, elevated hepatic TNF-α levels disrupt insulin receptor signaling, leading to impaired glucose regulation and enhanced insulin resistance (53,54).

The improvements in pancreatic function, and the reduction in hepatic inflammation, that resulted from insulin + CM treatment were consistent with an improved control of the blood glucose level in this experimental group (namely, the insulin + CM group) compared with the other diabetic groups. This was in spite of the fact that, during the 2 weeks following the final intervention, blood glucose levels in the CM-treated groups were found to fluctuate, suggesting a need for prolonged or continuous therapy. Furthermore, the HbA1c level was comparatively lower in the DM+ insulin + CM group; in fact, it exhibited the lowest insulin resistance index among all treatment groups. CM treatment, however, was found not to affect the fasting insulin serum levels. Taken together, these findings align with those of previous studies, where CM was demonstrated to reduce both the blood glucose and HbA1c levels in patients with type 1 diabetes (55), whereas it did not affect the fasting insulin serum concentration (17).

Herein, CM was also found to improve glucose tolerance and insulin sensitivity, as demonstrated by performing OGTT and IPITT, respectively. Typically, blood glucose levels peaked at 30 min following glucose administration, before gradually returning to the baseline level, as observed in the normal group. However, in a previously published study (56), in cases of impaired glucose tolerance (namely, the control DM group), blood glucose levels remained elevated for up to 60 min. The intervention of insulin + CM resulted in the lowest blood glucose level, thereby reflecting improved glucose tolerance compared with the other diabetic groups. All these observations demonstrated that combination therapy with CM and insulin may improve the glucose tolerance of patients with T2DM (27,57). Insulin sensitivity, another key issue in T2DM, was also shown to be elevated in the CM-treated groups. In line with these findings, Sun *et al* (17) demonstrated that exosomes from hUCM-MSCs, but not exogenous insulin, led to an increase in insulin sensitivity by activating the insulin-signaling pathway and enhancing glycogen synthesis.

As a key factor in determining insulin resistance, IGF-1 regulates insulin sensitivity and glucose uptake in peripheral tissues via pathways similar to the insulin-signaling pathway (53-55). Reductions in the IGF-1 level were found to lead to a deterioration in insulin resistance, especially in cases of T2DM (60). IGF-1 is mainly produced by the liver; hence, in conjunction with the suppression of hepatic inflammation, the results of the present study demonstrated that CM enhanced IGF-1 secretion. By contrast, treatment with exogenous insulin did not lead to any increases in IGF-1 secretion, and this finding corroborates those of previous studies, which identified that subcutaneous insulin treatment over an extended period may even reduce IGF-1 levels (61,62).

Notably, in the present study, rats treated with insulin alone had the poorest insulin sensitivity, accompanied by persistently elevated blood glucose levels throughout the study compared with other treatment groups. These findings were similar to those of a previous study, which reported that insulin-treated diabetic rats displayed glucose tolerance and insulin-sensitivity profiles that were comparable with those of the control DM group (17). This result may be attributed to the use of short-acting insulin, which was administered at a frequency equivalent to that of the CM regimen. The selection of insulin type and dosage were selected based on preliminary findings and previous studies (63,64), in which insulin administration exceeding 1 unit was shown to often lead to severe hypoglycemia and subsequent mortality. Notably, only the co-administration of insulin with CM led to disease alleviation, partly through repair of the functions of the pancreas and liver.

Previously, the authors have demonstrated that the CM from hUC-MSCs contains several growth factors, including VEGF, basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) (18). Although the specific mechanism through which hUC-MSC-derived paracrine activity alleviates the T2DM condition has not been clearly elucidated, these growth factors may play a major role in alleviating disease progression. Proangiogenic factors, such as VEGF, bFGF and HGF, are known to promote the proliferation of pancreatic β-cells, stimulating nutrient and blood flow delivery into these cells (60-62). Other *in vitro* studies (14,52) have demonstrated that CM from hUC-MSCs enhances glucose uptake through upregulating the expression of membranous glucose transporter 4 (GLUT4) and activating the insulin signaling pathway, which eventually leads to an improvement in insulin resistance. Similarly, Sun *et al* (17) found that exosomes from hUC-MSCs were responsible for an increase in the expression and the membrane translocation of GLUT4 in muscle, thereby enhancing glycogen storage in the liver to maintain glucose homeostasis. Moreover, it can reverse peripheral insulin resistance and relieve β-cell destruction. The administration of hUC-MSC exosomes has been shown to lower blood glucose levels and to enhance glucose uptake in skeletal muscle and liver cells (17,68,69). In the presence of insulin, these exosomes stimulate the expression of key insulin signaling proteins, including protein kinase B/AKT and insulin receptor substrate 1, which exert crucial roles in glucose transport and metabolism. Consequently, this process attenuates the development of insulin resistance (47,66-68). hUC-MSC-derived exosomes have also been shown to restore islet architecture and to inhibit the STZ-induced apoptosis of β-cells (68,69).

However, there were several limitations associated with the present study. Although the CM from hUC-MSCs demonstrated potential in alleviating T2DM, some of the results lacked statistical significance, probably due to the relatively small sample size of the study. Additionally, variability in CM composition between production batches may have represented a potential confounding factor. Factors such as the umbilical cord source and manufacturing process have previously been shown to markedly influence the quantity and types of factors secreted into the medium (73). Therefore, future studies with larger sample sizes are required to investigate the appropriate dosing and mechanisms of action. Furthermore, it is essential to analyze the components of CM that may contribute to immunogenic responses in order to assess its potential immunogenicity. In addition, standardizing the production process is also critical to ensure the efficacy and reliability of CM from hUC-MSCs for T2DM clinical applications. The half-lives of secreted factors in the CM are crucial for determining both the effective duration of CM use and the optimal dosing frequency *in vivo* (14). Taken together, however, these results have shown that CM from hUC-MSCs, when administered as an adjuvant therapy to insulin, may positively influence T2DM by lowering blood glucose and HbA1c levels, as well as improving insulin sensitivity and the secretion of IGF-1.

In conclusion, finding suitable therapies against T2DM is crucial, as T2DM is a debilitating disease, and effective blood glucose management is essential. CM derived from hUC-MSCs has the potential to reduce the levels of both blood glucose and HbA1c, particularly if administered as an adjuvant therapy to insulin. Additionally, CM may also improve glucose tolerance and insulin sensitivity, particularly when combined with insulin. It also has the potential to enhance IGF-1 secretion, which may partly decrease insulin resistance. Finally, CM may also increase both the numbers of pancreatic β-cells and their functionality, which deteriorate under diabetic conditions. The present study has provided a knowledge base for future research in terms of the application of CM as an adjuvant therapeutic approach in the management of T2DM.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

All authors (SH, JL, OM, LT, HUB and SG) were involved in the conception and design of the study. JL and OM were responsible for data collection and administration. SH, JL and OM were responsible for data calculation and analysis. SH and JL were responsible for the writing of the manuscript (the original draft preparation). SH, LT, HUB and SG were responsible for data interpretation. OM, LT, HUB and SG were responsible for the writing of the manuscript (reviewing and editing). and SH and HUB supervised the project. SH, SG confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal study protocol was approved by the Institutional Animal Care and Use Committee of Faculty of Medicine, Tarumanagara University, Jakarta, Indonesia, (grant no. 019.KEPH/UPPM/FK/VI/2024; date of approval: 11 June 2024). MSCs were isolated from fresh umbilical cords obtained from caesarian delivery with parental consent (ethical approval no. PPZ20192062, obtained from the Universitas Tarumanagara Human Research Ethics Committee, Tarumanagara University).

Patient consent for publication

Not applicable.

Competing interests

HUB is the Director of Baermed, Center of Abdominal Surgery, Zürich, Switzerland. The other authors declare that they have no competing interests.

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