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Human umbilical cord mesenchymal stem cell derived exosomes (HucMSC-ex) down regulate blood glucose of type 2 diabetes mellitus through reverse insulin resistance in insulin target tissue and relieve β cell destruction.

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Yaoxiang Sun†,‡, Hui Shi†,‡, Siqi Yin†,‡, Cheng Ji†, Xu Zhang†, Bin Zhang†, Peipei Wu†,

Yinghong Shi†, Fei Mao†, Yongmin Yan†, Wenrong Xu‡, Hui Qian*†

†. Jiangsu Key Laboratory of Medical Science and Laboratory Medicine, Institute of Stem Cell, School of Medicine, Jiangsu University, Zhenjiang, Jiangsu, 212013, China

‡. Department of Clinical Laboratory, The Affiliated Yixing Hospital of Jiangsu University, Yixing, Jiangsu, 214200, China.

ABSTRACT: Exosomes are nano-sized extracellular vesicles (EVs) that show great promise in tissue regeneration and injury repair as mesenchymal stem cell (MSC). MSC has been showed to alleviate diabetes mellitus (DM) in both animal models and clinical trials. In this study, we aimed to investigate whether exosomes from human umbilical cord MSC (hucMSC-ex) have therapeutic effect on type 2 DM (T2DM). We established a rat model of T2DM using high-fat diet (HFD) and streptozotocin (STZ). We found that the intravenous injection of hucMSC-ex reduced blood glucose level as main paracrine approach of MSC. HucMSC-ex partially reversed insulin resistance in T2DM indirectly to accelerate glucose metabolism. HucMSC-ex restored the phosphorylation (tyrosine site) of insulin receptor substrate1 (IRS-1) and protein kinase B in T2DM, promoted expression and membrane translocation of glucose transporter 4 (GLUT4) in muscle and increased storage of glycogen in liver to maintain glucose homeostasis. HucMSC-ex inhibited STZ induced β-cell apoptosis to restore the insulin secreting function of T2DM. Taken together, exosomes from hucMSC can alleviate
T2DM through reversing peripheral insulin resistance and relieving β-cell destruction which provide an alternative approach for T2DM treatment.

**KEYWORDS:** exosomes; mesenchymal stem cell; type 2 diabetes mellitus; insulin sensitivity; glucose metabolism.

Diabetes mellitus is a metabolic disease that affects an estimated of 500 million people worldwide.\(^1\) Type 2 diabetes mellitus (T2DM) accounts for 95% of diabetes cases. Peripheral insulin resistance, pancreatic β-cell mass loss and β-cell dysfunction are primary causes of T2DM, which results in glucose out of control and degenerative complications.\(^1,2\) Pump or daily insulin injection and chemical drugs such as sulfonylurea, metformin, and thiazolidinedione are the principal treatments for T2DM at present.\(^1\) However, these treatments can only temporarily control blood glucose level and have side effect like ametropia, subcutaneous nodule, diarrhea and obesity. In addition, exogenous insulin administration may result in the loss of insulin production and secretion in β-cells, making it hard to ameliorate peripheral insulin resistance and relieve the symptoms of diabetic complications.\(^1,3,4\)

The transplantation of tissues and stem cells has been used to improve diabetes care.\(^5,6\) However, the insufficient donation of tissues and low survival rate of stem cells *in vivo* make these approaches unsatisfactory.\(^7,8\) Mesenchymal stem cell (MSC) derived from different tissues such as bone marrow, umbilical cord, adipose have been currently under investigation for their potential in tissue regeneration.\(^9-12\) The intravenous infusion of bone marrow derived MSC can reverse hyperglycemia in high-fat diet (HFD) and streptozotocin (STZ) induced T2DM rat models through improving insulin secretion, activating insulin signaling pathway.
and increasing the expression and membrane transposition of glucose transporters (GLUT).\textsuperscript{13,14} The transfusion of adipose derived MSC can significantly lower blood glucose level \textit{via} promoting hepatic glycogen synthesis and inhibiting hepatic glucose production in T2DM rats.\textsuperscript{15} Moreover, T2DM patients transplanted with human umbilical cord MSC (hucMSC) have shown relatively stable blood glucose levels, some patients become insulin free at 25 to 43 months after treatment and the others only require low dose of insulin.\textsuperscript{16} Although there is evidence that MSC could be induced into islet endocrine lineages \textit{in vitro},\textsuperscript{17-20} there is also report showing that MSC cannot differentiate into β-cells \textit{in vivo},\textsuperscript{13,21} indicating that MSC may exert their activities in T2DM majorly through paracrine actions.

MSC derived factors have pleotropic roles in T2DM therapy. The culture medium from BM-MSC can reduce blood glucose level in T2DM animal models.\textsuperscript{14} The conditioned medium (CM) from adipose-derived MSC can reverse insulin resistance \textit{in vitro} through up-regulating GLUT4 level and reducing interleukin 6 (IL-6) and plasminogen activator Inhibitor-1(PAL-1) levels.\textsuperscript{22} Exosomes are extracellular nanoparticles secreted by cells and contained bioactive molecules including proteins, lipids and nucleic acids.\textsuperscript{10,23} Exosomes involved in glucose metabolism in diabetes is attracting attentions. Thomou \textit{et al.} have found that adipose-derived exosomes could transfer miR-99b to hepatic cells to regulate the expression of fibroblast growth factor 21 (FGF-21) and participate in glucose metabolism.\textsuperscript{24} Garcia \textit{et al.} have found that cardiomyocytes derived exosomes can directly transfer GLUT protein and glycolytic enzymes to endothelial cells to modulate glucose transport.\textsuperscript{25} These studies suggest that
exosomes carrying active contents may be the key approach of MSC that mediates their therapeutic effects in diabetes. As we all know, T2DM is a systematic disease, therapeutic effect of conventional oral medicines are always harmed because of the low water solubility or high clearance after “biotransformation”, as one kind of nanomedicine, the efficiency of exosomes should be considered about the physical barriers either. Before being internalized by target tissues or cells, exosomes need to escape the clearance of liver or kidney, to avoid the absorption of serum protein, to escape surveillance of immune system, to interact with vascular endothelial walls and extracellular matrix. After exosomes overcoming these barriers and getting to target tissue, the effect of exosomes internalized by target cells need to escape the degradation of endosome/lysosome which also depended on the size and structure of exosomes. The special characteristics of structure (“cup” or “dish” in transmission electron microscopy), size (30-150nm) and density (1.13-1.19g/ml) of exosomes make themselves easily to be absorbed, to cross blood brain barrier or to be internalized by target cells in caveolae route which is a much better way for exosomes to escape the degradation of endosome/lysosome, lipid bilayer membrane can protect carried components from degradation of physical environment and provides long-term releasing effect. These information remind that exosomes have more advantages than other nanoparticals and may be the potential “smart” nanomedicine for diabetes treatment. We have previously reported that hucMSC derived exosomes (hucMSC-ex) could repair liver fibrosis, acute renal injury, and cutaneous wound healing. We identified the contents of hucMSC-ex by LC/MS-MS.
and found that glucose metabolism associated proteins were enriched in hucMSC-ex.\textsuperscript{10,32} Therefore, we hypothesized that hucMSC-ex might also alleviate hyperglycemia in T2DM as observed in hucMSC.

In the current study, we exploit the feasibility and efficacy of using hucMSC-ex to alleviate T2DM rat model induced by HFD and streptozotocin.\textsuperscript{33} Results show that intravenous infusion of hucMSC-ex can decrease blood glucose level in T2DM rats by enhancing periphery organs insulin sensitivity and relieving islets destruction. HucMSC-ex can restore glucose homeostasis of T2DM by promoting the expression and membrane translocation of GLUT4 in muscle and glycogen storage in liver depending on insulin. HucMSC relieved insulin secreting dysfunction in T2DM by inhibiting STZ induced β-cell apoptosis. HucMSC-ex shows a desirable alleviating ability in T2DM rat models.

RESULTS AND DISCUSSION

Identification of hucMSC-ex and fat-fed/STZ induced T2DM rat model. HucMSC-ex and human lung fibroblast 1 derived exosomes (HFL1-ex) were purified from cell culture supernatant as previously described \textsuperscript{30-32} and characterized by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and Western blot. The results showed that hucMSC-ex displayed cup-like morphology (Fig S1a) with a mode diameter around 120 nm (Fig S1b) and expressed exosomal markers CD9 and CD81 (Fig S1c), HFL1-ex as control and had the similar features. We have previously analyzed the protein contents of hucMSC-ex by using LC/MS-MS and have identified glucose transporters (GLUT), pyruvate
kinase (PK), and lactic dehydrogenase (LDH) in hucMSC-ex.\textsuperscript{32} We confirmed the enrichment of GLUT4 in hucMSC-ex but not in HFL1-ex (Fig S1c).

To test the therapeutic effects of hucMSC-ex on T2DM, we established a rat model by using high fat diet (HFD) combined with streptozotocin (STZ).\textsuperscript{14,33} T2DM rats showed blood glucose levels higher than 20mmol/L but the normal group was lower than 10mmol/L no matter in fasted or re-fed (Fig S2a). Reduced insulin sensitivity, decreased islet size, and impaired serum insulin levels were presented by the results of oral glucose tolerance tests (OGTTs) (Fig S2b), intra-peritoneal insulin tolerant tests (IPITTs) (Fig S2c), pancreas histology (Fig S2d), and serum insulin tests (Fig S2e). In addition, the rats also appeared typical symptoms of T2DM, including polyphagia, polydipsia and polyuria. The data testified the successfully establishment of T2DM animal model.

**HucMSC-ex ameliorated hyperglycemia in T2DM rats.** To investigate whether hucMSC-ex has a therapeutic effect on T2DM as hucMSC, we infused hucMSC-ex (10mg/kg body weight (bw) was the maximum tolerated dose, Fig S3a), hucMSC, PBS into T2DM rats via tail vein, normal rats infused with PBS as control and detected the blood glucose level of different groups every 3 days in 1 month (Detail procedure were in supplementary table 1). The results of continuous blood glucose tests displayed that hucMSC-ex considerably ameliorated hyperglycemia in T2DM rats as hucMSC (Fig 1a). After the last infusion of hucMSC-ex, blood glucose levels of hucMSC-ex treated T2DM rats kept on decreasing and
maintained at 15.49±2.80mmol/L, which did not show significant differences compared with hucMSC group (16.73±3.35 mmol/L). On the contrary, the blood glucose level of control T2DM rats increased to 32.13±2.60mmol/L (Fig 1a).

Cell therapy has been suggested as a new strategy for tissue injury repair. The therapeutic effects of MSC on diabetes have been verified in animal models and clinical patients. However, MSC administration may have several disadvantages such as tumorigenic potential, thrombosis, and fever. The low survival time of MSC in vivo may also limit their application. Although gene modifications may improve the therapeutic efficiency of MSC, it may add more risk to the clinical application. In addition, it is still controversial whether hucMSC can differentiate into functional cells such as pancreatic β-cell in the pancreas. Thus it is needed to investigate an alternative approach of MSC for the therapy of T2DM. Paracrine actions are considered as the predominant mechanism for the roles of MSC in tissue repair. Exosomes are described as active nano-component that mediates the paracrine actions of their producing cells and we have already detected hucMSC-ex can down-regulate blood glucose of T2DM rats. To confirm that hucMSC-ex are the main paracrine approach of hucMSC to regulate hyperglycemia, we added concentrated whole hucMSC-CM (10mg/kg bw) and hucMSC-ex free CM (10mg/kg bw) to administrate T2DM rats and detected blood glucose level in the same way as hucMSC-ex. According to the results, hucMSC-CM also could down-regulate blood glucose of T2DM rats and maintain to 18.26±4.95 mmol/L. On the contrary, hucMSC-ex free CM could not decrease blood glucose...
of T2DM rats obviously as hucMSC-CM and the blood glucose level increased to 29.76±5.30 mmol/L. Besides, we pretreated hucMSC with noncompetitive N-SMase inhibitor GW4869 (5µM) which can inhibit exosomes secretion. We detected the protein concentrations of exosomes derived from GW4869 pretreated hucMSC by BCA analysis kit and the CD63 level by Western blot, GW4869 could temporary inhibit exosomes secretion in hucMSC for about 7 days in vitro (Fig S4a, b). We infused hucMSC-GW4869 into T2DM rats just like hucMSC. The results of continuous blood glucose tests revealed that hucMSC-GW4869 could not decrease blood glucose of T2DM rat as fast as hucMSC (Fig S4C) which provided more evidence for hucMSC-ex could be the substitution of hucMSC in T2DM treatment.

We administrated T2DM rats with HFL1-ex as control to investigate whether hucMSC-ex had the special effect in hyperglycemia intervention. The continued blood glucose level detection showed that HFL1-ex could stabilize the blood glucose level to 27.41±5.00mmol/L but not increasing unlimited as T2DM control group even though there had no significant differences between two groups (Fig 1b). We also treated normal rats with hucMSC-ex, no differences with normal control were detected (Fig 1b).

In this study, we demonstrated that hucMSC-ex could decrease blood glucose level in HFD/STZ induced T2DM rats as hucMSC and hucMSC-CM. On the contrary, exosomes-depleted culture medium from hucMSC, hucMSC with impaired exosome-producing function and HFL1-ex had minimal effect in hyperglycemia intervention, suggested that hucMSC-ex had special effects in hyperglycemia amelioration as a main component of hucMSC paracrine.
**HucMSC-ex enhanced glucose uptake in myotube and hepatocyte in vitro.** Skeletal muscle and liver are essential tissues and organs for glucose metabolism. To verify the target tissue or cells of hucMSC-ex in reversing hyperglycemia of T2DM rats, we used rat L6 skeletal muscle cell (L6-myoblasts) differentiated skeletal muscle cell (L6 cell) (Fig S5a) and human L02 cell (L02 cell) as cell models to evaluate the effect of hucMSC-ex on glucose uptake. After treated with hucMSC-ex for 24h, the uptake of fluorescent glucose analog 2-NBDG by rat L6 cell and L02 cell were detected. The results showed that hucMSC-ex could promote the uptake of 2-NBDG into L6 cell in a concentration dependent manner and 400µg/ml may be the optimal concentration (Fig 2a and 2b). We also treated L6 cell with HFL1-ex (400µg/ml) and hucMSC-ex free CM (400µg/ml) and results revealed that hucMSC-ex was more efficient than HFL1-ex and hucMSC-ex free CM in stimulating 2-NBDG uptake. HFL1-ex could promote glucose uptake slightly while hucMSC-ex free CM had no effect on 2-NBDG uptake (Fig 2c). We detected 2-NBDG uptake in L6 cell treated with insulin (100nM) and hucMSC-ex. HucMSC-ex combined with insulin led to more uptake of 2-NBDG by L6 cell than insulin or hucMSC-ex alone (Fig 2d). HucMSC-ex also increased the uptake of 2-NBDG in L02 cell (Fig 2e). These findings indicated that hucMSC-ex could promote glucose uptake in skeletal muscle cell and liver cell either alone or together with insulin.

**HucMSC-ex increased insulin sensitivity in T2DM rats and insulin resistant cell model.** Peripheral insulin resistance is responsible for the pathogenesis of T2DM. We wanted to know whether hucMSC-ex affected insulin sensitivity of T2DM rats to regulate
blood glucose. We compared the blood glucose levels of T2DM rats treated with PBS, hucMSC-ex, insulin and hucMSC-ex combing with insulin. The blood glucose level was lower in hucMSC-ex combing with insulin group than hucMSC-ex or insulin groups, indicating that hucMSC-ex could increase insulin sensitivity in T2DM rats (Fig 3a). We then detected the OGTTs and IPITTs levels of rats at 2 weeks after the last time of hucMSC-ex injection. The results of OGTTs presented that glucose metabolism was significantly ameliorated by hucMSC-ex administration (Fig 3b). The results of IPITTs also showed that there was a significant improvement in insulin sensitivity after treated with hucMSC-ex in T2DM rats (Fig 3c). The homeostasis model assessment of insulin resistance (HOMA-IR) index in hucMSC-ex treated group was much lower than that in control group and exogenous insulin could not influence the individual insulin sensitivity (Fig 3d).

To determine whether hucMSC-ex could improve insulin sensitivity \textit{in vitro}, we established palmitic acid (PA) induced insulin resistant cell models.\textsuperscript{46} The fluorescent intensity of 2-NBDG was weaker in PA-induced insulin resistant L02 cell than that in insulin sensitive cell. Insulin could promote the uptake of 2-NBDG in insulin sensitive but not insulin resistant L02 cell (Fig S5b). The induced expression of tyrosine phosphorylation of insulin receptor substrate1 (p( tyr)-IRS-1) by insulin was inhibited in the resistant L02 cell (Fig S5c). We then detected the effect of hucMSC-ex on the uptake of 2-NBDG in PA-induced insulin resistant L02 cell in the presence or absence of insulin by using imaging flow cytometry. PA impaired glucose up-took of L02 cell can be partially reversed by hucMSC-ex and this effect was more
remarkable combined with insulin (Fig 4a). Both of the mean fluorescence intensity of 2-NBDG and the percentage of cell that up-took 2-NBDG in insulin resistant cells were increased by hucMSC-ex (Fig 4b, c). Furthermore, images of L02 cell pretreated with PA had lower fluorescence intensity than non-PA group and could not be reversed by insulin alone but could be reversed by insulin combined with hucMSC-ex (Fig 4d). Additionally, HucMSC-ex also promoted glycogen synthesis in PA-induced insulin resistant L02 cell, either alone or in combination with insulin (Fig S6a). These results indicated that hucMSC-ex could increase insulin sensitivity both in T2DM rats and insulin resistant cell model.

**HucMSC-ex activated insulin signaling to promote insulin sensitivity.** IRS-1 tyrosine phosphorylation and insulin signaling pathway activation are critically involved in glucose transport and glucose metabolism in liver and skeletal muscle. The impaired tyrosine phosphorylation of IRS-1 and the inactivation of protein kinase B (AKT) are hallmarks of insulin resistance in T2DM.\textsuperscript{13,47,48} To elucidate whether hucMSC-ex has the ability to active insulin signaling directly, we detected the expression of p(tyr)-IRS-1 and p-AKT in the liver and muscle of rats starved overnight in four groups (Normal+PBS, Normal+hucMSC-ex, T2DM+PBS, T2DM+hucMSC-ex). The expression of p(tyr)-IRS-1 had no differences between normal groups or T2DM groups both in liver or muscle, the expression of p-AKT were much lower in T2DM groups compared with normal groups (Fig 5a,b,c,d). HucMSC-ex could not increase the expression of p(tyr)-IRS-1 and p-AKT directly without insulin. We then detected p(tyr)-IRS-1 and p-AKT in insulin (2IU/kg bw) pretreated groups, the expression of p(tyr)-
IRS-1 and p-AKT were dramatically increased in normal groups and no differences were detected between PBS or hucMSC-ex treatment (Fig 5a, b, c, d). Insulin could increase the expression of p(tyr)-IRS-1 and p-AKT in T2DM group treated with hucMSC-ex but not in PBS control (Fig 5a, b, c, d). Additionally, insulin and AMP activated protein kinase (AMPK) signaling pathway\textsuperscript{43,49} are cross-linked and we’d like to verify if AMPK signaling pathway were also activated. According to the results, the activation of AMPK only could be detected in normal groups and insulin was required, hucMSC-ex could not influence the expression of p-AMPK no matter in normal groups or T2DM groups (Fig 5a, b, c, d). \textit{In vitro}, hucMSC-ex also could increase the activation of p(tyr)-IRS-1 and p-AKT of PA-induced insulin resistant L02 cell with insulin treatment (Fig S6b).

In general, chronic inflammation in tissue is the main inducement of insulin resistance which lead to inflammatory cells secrete pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α) or interleukin 6 (IL-6) to inhibit insulin signaling pathway activation,\textsuperscript{50,51} so we collected the serum of animal models in different groups and detected the level of TNF-α and IL-6, serum level of TNF-α in hucMSC-ex treated T2DM rats was much lower than PBS control (Fig S7a), but no significant difference in serum level of IL6 was detected between two groups (Fig S7b). These data indicated that hucMSC-ex might inhibit secretion of pro-inflammatory cytokine like TNF-α to reverse insulin resistance in T2DM and increase the activation of insulin/AKT signaling pathway but not insulin/AMPK signaling pathway indirectly.
HucMSC-ex promoted glucose up-take of skeletal muscle and glycogen storage of liver.

Skeletal muscle and liver play essential roles in maintaining blood glucose balance through the regulation of glucose up-take, storage, production and consumption. STZ and HFD may dramatically decrease glucose uptake through inhibiting the expression and membrane translocation of GLUT4 which is the main glucose transporter in muscle\textsuperscript{13,52} and influence glycogen synthesis in liver.\textsuperscript{15} To investigate the effects of hucMSC-ex in glucose uptake, we detected the membrane translocation of GLUT4 by immune-fluorescence in muscle of T2DM rats treated with or without insulin before executed in different groups. HucMSC-ex treated T2DM rats showed more GLUT4 translocation on membrane of muscle than PBS control (Fig 6a), and insulin was required. We also detected expression of GLUT4 related protein in membrane of muscle by Western blot after insulin stimulating. HucMSC-ex treated T2DM groups had more GLUT4 on membrane than PBS control (Fig 6b). The expression of total GLUT4 in T2DM groups were decreased, hucMSC-ex could up-regulate it both in protein and gene level no matter with or without insulin pre-treated in normal group or T2DM group (Fig 6a, c, d, e). However, hucMSC-ex couldn’t significantly increase membrane translocation of GLUT4 in normal group (Fig 6a, b) which mean hucMSC-ex might influence the expression of GLUT4 but the membrane translocation of GLUT4 still depending on insulin signaling pathway. Besides, in insulin treated T2DM groups, hucMSC-ex up-regulated the phosphorylation of TBC1D4 (AS160) which is an important protein in the regulation of
GLUT4 translocation and can be activated by insulin/AKT signaling pathway \(^{53}\) (Fig 6c, d). These results indicated that hucMSC-ex promoted muscle uptake glucose depending on reversing insulin resistance and increasing GLUT4 expression of T2DM rats.

Glucose also can be up-took by liver but in insulin in-dependent way and relies on glucose-sensitive transporter GLUT2 \(^{54}\) which can be increased by STZ. We detected the expression of hepatic GLUT2 with immune-histochemical staining and Western blot. Normal groups and T2DM rats treated with hucMSC-ex had lower translocation of GLUT2 in membrane (Fig S8) which indicated that the blood glucose level in hucMSC-ex treated group was lower than that in T2DM control.

To verify the effect of hucMSC-ex in regulating glucose metabolism of T2DM rats, we determined the level of glycogen deposits in liver and muscle sections by using periodic acid Schiff staining (PAS). HucMSC-ex treated T2DM group showed more glycogen accumulation in liver (Fig 6f). The expression of glycogen synthesis related protein p-GSK3β and glycogen synthase in liver were detected by Western blot, hucMSC-ex treated T2DM rats had higher expression of p-GSK3β and glycogen synthase (Fig 6g, h) than those in control group. HucMSC-ex could not change the glycogen synthesis in normal group (Fig 6f, g, h). \textit{In vitro}, the higher levels of p-GSK3β, glycogen synthase (Fig S6b) were also verified in insulin resistant cell model L02 either alone or in combination with insulin. However no differences of glycogen level between normal groups and T2DM groups or PBS control and hucMSC-ex treated groups were observed in muscle (Fig 6f). We next assessed whether hucMSC-ex
treatment influenced the expression of enzymes that were involved in glucose metabolism such as citrate synthase (CS), isocitrate dehydrogenase (ICDH), glucokinase (GCK), pyruvate kinase (PK). In liver, PK hardly could be detected, the levels of GCK and CS in T2DM groups were lower than normal groups and we had no evidence to prove hucMSC-ex can influence their expression (Fig 6i). The expression of PK was greatly decreased in the skeletal muscle of T2DM rats while was up regulated by hucMSC-ex treatment which mean hucMSC-ex might intervene the glycolysis in muscle of T2DM rats, minimal GCK could be detected in muscle and the expression of CS and ICDH had no significant difference between normal groups and T2DM groups with or without hucMSC-ex treated (Fig 6j). These results indicated that hucMSC-ex activated the insulin signaling to improve glycogen synthesis in the T2DM rats, restore glucose homeostasis in liver but promote glucose uptake and glycolysis in skeletal muscle.

**HucMSC-ex promoted insulin secretion and islet regeneration by inhibiting STZ induced cell apoptosis.** In T2DM, the release of insulin by pancreatic β-cells is impaired.\(^1\)\(^,\)\(^4\) HFD/STZ induced T2DM rats have destructed pancreatic islet, which leads to an impaired insulin production. In our study, we indicated that hucMSC-ex reversed impaired insulin secreting function of re-fed T2DM rats by serum insulin level detection and insulin releasing tests (IRTs) (Fig 7a,b). The results of H-E staining and IHC staining for pancreatic tissue sections revealed that the count and area of islet in hucMSC-ex treated T2DM group were higher than that in T2DM control (Fig 7c-e). HucMSC-ex could not influence the insulin
secretion, islet structure or numbers in normal groups (Fig 7a-e). We also detected the islet
structure of low dose STZ injection induced S-D rats pancreatic damage with early (7days) or
later (14days) hucMSC-ex (10mg/kg bw, signal dose) infusion, there was no obvious
difference in area between early and later hucMSC-ex infusion, but we observed that structure
of islet with early hucMSC-ex infusion was more uniform than the later one, and later
hucMSC-ex infusion or without hucMSC-ex treatment had more vacuolar degeneration (Fig
S9a). These results indicated that hucMSC-ex could relieve STZ-induced pancreatic damage
in T2DM rats by protecting against pancreatic islet destruction in the early phase. To figure
out the approach that hucMSC-ex restoring the islets, we executed the T2DM rats 1weeks
after accepted single dose hucMSC-ex treatment. We detected the proliferation-related protein
proliferating cell nuclear antigen (PCNA) and apoptosis related indicator Caspase3 in pancreas
serial sections. There was no difference of PCNA between normal group and T2DM groups,
but Caspase3 was up-regulated in T2DM rats and hucMSC-ex could decrease it (Fig 7f). We
also detected the protein of pancreas from 3 groups (Normal+PBS, T2DM+PBS, T2DM+hucMSC-ex) with Western blot, T2DM groups had much higher expression of
Caspase3 than other groups, and hucMSC-ex treated T2DM group had no difference with
normal group (Fig 7g, h). The level of PCNA in three groups had no significant differences
(Fig 7g, h). These data verified that hucMSC-ex promoted insulin secretion and islet
regeneration by inhibiting STZ induced cell apoptosis.
Exosomes have been proved can be an emerging source of biomaterials in impaired tissue repair.\textsuperscript{29-32} Exosomes from cardiomyocytes and adipose tissues have been reported to regulate glucose metabolism.\textsuperscript{24, 25} Neutral ceramidase-enriched exosomes could not only inhibit PA-induced cell apoptosis but also rescue PA-induced insulin resistance.\textsuperscript{46,55} MiR-containing exosomes secreted by adipose tissue macrophage (ATM) in obese mice can cause glucose intolerance and insulin resistance in lean mice. On the contrary, ATM-ex obtained from lean mice improved glucose tolerance and insulin sensitivity when administered to obese recipients.\textsuperscript{51} The therapeutic effects of exosomes on the complications of diabetes mellitus had also been reported. For example, exosomes from non-diabetic plasma could repair ischemic heart disease in T2DM through activating cardioprotective signaling.\textsuperscript{56} Exosomes secreted from human urinary stem cells could ameliorate diabetic kidney disease by reducing podocyte apoptosis and enhancing glomerular endothelial cell proliferation.\textsuperscript{57} Exosomes derived from MSC transported miR-133a to promote axonal remodeling and neuronal outgrowth in diabetes-induced central nervous system damage.\textsuperscript{58} In addition, exosomes derived from MSC could deliver Fas shRNA and miR-375 antagonist to modulate immune response and improve islet transplantation.\textsuperscript{59} These studies suggest that exosomes derived from healthy tissues or cells can be used as a potential tool for therapeutic intervention.

In our study, hucMSC-ex decreased blood glucose level in HFD/STZ induced T2DM rats just like hucMSC or hucMSC-CM, exosomes-depleted culture medium from hucMSC and exosome-producing impaired hucMSC had no effects. These results indicated that exosomes
were one of key factors for the therapeutic effects of hucMSC on hyperglycemia. HucMSC-ex could improve periphery insulin sensitivity, increase the activation of IRS-1 (tyrosine site), AKT and downstream signaling pathway in T2DM indirectly. This progress was possibly caused by hucMSC-ex decreased the serum pro-inflammatory cytokine TNF-α which plays an important role in the development and progression of individual insulin resistance. In muscle, hucMSC-ex promoted the expression of GLUT4 both in physiological status and T2DM but only increased the membrane translocation of GLUT4 in T2DM groups. Additionally, hucMSC-ex treatment protected the STZ induced destruction of islets in T2DM rats by inhibiting β-cell apoptosis. These findings indicated that hucMSC-ex might regulate glucose metabolism of T2DM not only relying on single approach or single component but depending on multiple-components. We had analyzed the components by LC/MS-MS and found that hucMSC-ex contained thousands proteins and most of them were different with proteins from HFL1-ex. These proteins take part in lots of signaling pathways especially the metabolism related insulin pathway, pyruvate metabolism, pentose phosphate pathways. Our previous work also found that hucMSC-ex could deliver glutathione peroxidase1 to improve hepatic oxidant injury recovery and Wnt4 to mediate β-catenin signaling pathway in cutaneous wound healing repairmen, which reminded us hucMSC-ex carried proteins might also be the efficient component in T2DM intervention. Exosomes’ characteristics insure that they can be internalized efficiently as we analyzed based on previously work of Sun and Yameen, in our research, we discovered the bio-distribution of hucMSC-ex in T2DM rats and normal rats
24h after infusion, most of the hucMSC-ex spread to liver, lung, pancreas, spleen and kidney (Fig S10a). HucMSC can be easily derived from umbilical cord and the donation of umbilical cord is enough \(^{60}\) which can be a stable source of exosomes, hucMSC-ex can be purified through 30% sucrose/D\(_2\)O cushions density gradient centrifugation which guarantee the quantity and purity,\(^{10,61}\) these features provided the basis for the application of hucMSC-ex in T2DM and indicated that hucMSCs-ex could be a potential nanomedicine for type 2 diabetes mellitus intervention.

Given the safety evaluation of hucMSC-ex, relevant researches had been carried out. We treated healthy rabbits, guinea pigs and rats with hucMSC-ex,\(^{62}\) no evidence proved that hucMSC-ex could lead to tumorigenesis, there was no obvious hemolysis, systemic anaphylaxis, liver and renal toxicity in different kind of animals either.\(^{62}\) We also detected the body weight, liver and renal function of rats in hucMSC-ex or PBS treated normal or T2DM rats 2 months after last infusion of hucMSC-ex to figure out whether hucMSC-ex change the function of healthy tissues in T2DM or normal rats. HucMSC-ex infusion did not influence the weight, liver or renal function of normal rats compared with PBS control (Fig S11). In T2DM rats, the body weight of hucMSC-ex treated T2DM rats was heavier than control group which reminded that hucMSC-ex treatment could relieve the rapid emaciation of T2DM rats (Fig S11a). No significant differences of alanine aminotransferase (Fig S11b), alanine aminotransferase /aminotransferase (ALT/AST) (Fig S11c), blood urea nitrogen (BUN) (Fig S11d), creatinine (CREA) (Fig S11e) were found between hucMSC-ex treated T2DM groups
and normal groups either. These data indicated that hucMSC-ex hardly damaged healthy
tissues which provided partial evidences for the safety of future clinic application. For clinic
transformation of hucMSC-ex, other elements are necessary such as develop more
standardized methods for purifying therapeutic level of exosomes to stabilize curative effects,
using hucMSC-ex in combination with proper anti-diabetic drugs or modify hucMSC-ex to
further improve its efficacy. All of these further researches can be carried out based on our
study.

CONCLUSION

In conclusion, we reported for the first time that hucMSC-ex could effectively alleviate
hyperglycemia in HFD/STZ-induced T2DM rats by promoting insulin sensitivity, increasing
glucose uptake and metabolism in peripheral tissues, and protecting pancreatic islet from
damaging by inhibiting STZ induced β-cell apoptosis. Our study provided a basis for the
application of hucMSCs-ex as another approach of the therapy for type 2 diabetes mellitus.

METHODS

All experimental protocols were approved by the Medical Ethics Committee of Jiangsu
University (2012258).

Cell culture. HucMSC was isolated as previously described.60 The cells in passage 3-4
were used for experiments. For exosomes secretion inhibition, hucMSC was pre-treated with
the noncompetitive N-SMase inhibitor GW4869 (Santa Cruz Biotechnology, 5µM) for 24 h.
Human lung fibroblast1 (HFL1), rat skeletal muscle cell (L6-myoblast) and human L02 cell
(L02 cell) were purchased from the Cell Bank of Chinese Academy of Sciences and maintained in a high-glucose Dulbecco’s modified Eagle medium (DMEM) or RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, USA) at 37°C with 5% CO2. L6-myoblast were plated in 6-well plates and induced to differentiate into myotube (L6 cell) as previously described. L02 cell was incubated with palmitic acid (Sigma-Aldrich, 0.25mM) for 24 h to establish insulin resistant cell model.

**Exosomes purification and characterization.** HucMSC-ex and HFL1-ex were purified from cell culture supernatant as previously described. The exosomes-depleted supernatant (hucMSC-ex free CM) was passed through a 0.22µm filter and stored at -70 °C for future use. The protein contents of the isolated exosomes and hucMSC-ex free CM were determined by using a BCA protein assay kit (CWBIO). The final concentration of hucMSC-ex for *in vitro* use was 400µg/ml and 10mg/kg for *in vivo* studies. Exosomal markers CD9 and CD81 were determined by using Western blot. The morphology and size distribution of exosomes were identified by using transmission electron microscopy (TEM, FEI Tecnai 12, Philips, Netherlands) and nanosight tracking analysis (NanoSight, Amesbury, UK).

**Type 2 diabetes mellitus animal model.** Eight weeks male Sprague-Dawley (S-D) rats (200-250g) were purchased from the Animal Centre of Chinese Academy of Sciences (Shanghai, China). The rats were housed with a 12:12h of light/dark cycle at an ambient temperature of 22-25 °C for 5 days. The rats were fed with 45% high fat diet (HFD) for 5 weeks. After being fasted for 12h with free access to water, HFD fed rats were injected with
STZ (35mg/kg in 0.1 M citrate buffered saline, pH=4.5) via tail vein to induce T2DM. STZ-treated rats were kept on feeding with high-fat diet for another week and then subjected to 12h of fasting before blood glucose test. The rats showed fasting glucose levels of more than 16.7mmol/L were considered to be T2DM rats. For islet impair study, the rats were injected with STZ (35mg/kg in 0.1M citrate buffered saline, pH=4.5) via tail vein 7 days before hucMSC-ex treatment, for demonstrated the effect of hucMSC-ex on STZ induced β-cell impairment. Normal rats were fed with normal diets.

**HucMSC and hucMSC-ex administration.** T2DM rats were divided into 9 groups (n=6/group): PBS, hucMSC-ex, hucMSC, hucMSC-GW4869, hucMSC-ex free CM, hucMSC-CM, HFL1-ex, insulin, hucMSC-ex combined with insulin. HucMSC (3×10^6) pre-treated with or without GW4869 were suspended in 0.2ml PBS and injected into T2DM rats via tail vein at 7 days, 13 days and 19 days after STZ injection. HucMSC-ex, HFL1-ex, hucMSC-CM, hucMSC-ex free CM in 0.2ml PBS (10mg/kg bw), were injected into rats via tail vein every 3 days for 5 cycles. The rats in insulin group were given 2IU/kg/d of insulin. The rats in hucMSC-ex combined with insulin were not only administrated with hucMSC-ex (10mg/kg bw) every 3 days but also given 2IU/kg/d of insulin. Normal groups were divided into 2 groups, one group infused with 0.2ml PBS and the other one infused with same concentration of proteins as that of hucMSC-ex (see supplementary table 1 for more detailed experiment procedure). For islet impair study, the eight weeks male S-D rats were injected with STZ (35mg/kg), then treated with hucMSC-ex (10mg/kg bw) in 0.2ml PBS via tail vein at early-
phase (7 days after STZ injection) and late-phase (14 days after STZ injection) of experiment, and executed 28 days after STZ injection.

**Location of hucMSC-ex in vivo and in vitro.** *In vivo,* hucMSC-ex (10mg/kg bw in 200µL) was stained with fluorochrome DiR (10µM, Themo Fisher, USA) and infused into T2DM rats or normal rats, 24h later we take the lung, liver, kidney, spleen of the rats and the location of hucMSC-ex *in vivo* was detected *via* IVIS® Lumina LT Series III (PerkinElmer, USA) (wavelength=720nm).

**Serological analysis.** One week after STZ injection, fasted and re-fed blood glucose level, oral glucose tolerance tests (OGTTs), intra-peritoneal insulin tolerant tests (IPITTs), insulin release tests (IRTs) were performed to confirm the establishment of T2DM rat model. Two weeks after the last infusion of hucMSC-ex, OGTTs, serum insulin level, IPITTs, IRTs, serum IL-6 level, serum TNFα level were performed to determine the effect of infused hucMSC-ex. The rats were starved for 3h before the measurement of blood glucose levels. Tail capillary blood glucose levels were monitored with a gluco-meter ACCU-CHEKA performa (Roche Diagnostics GmbH, Mannheim, Germany). Serum insulin level was measured by using enzyme-linked immune-sorbent assay (ELISA) (Millipore, Billerica, MA, USA). serum IL-6 and TNFα level was measured by using enzyme-linked immune-sorbent assay (ELISA) (Excel, China) according to the manufacturer’s protocols. For OGTTs, the rats were fasted overnight and intra-gastric administrated of glucose (2g/kg bw) and the blood glucose levels were detected at 0, 30, 60, 90, 120min after administration. IPITTs was done by injecting
glucose (2g/kg bw) into rats intraperitoneally followed by administration of insulin (2IU/kg
bw) immediately and the blood glucose levels were detected at 0, 30, 60, 90, 120 min after
administration. For IRTs, the rats were intra-gastric administrated of glucose (2g/kg bw) and
the serum insulin levels were detected at 0, 30, 60, 90, 120, 180 min after administration. 2
months after the last infusion of hucMSC-ex, the rats were starved for 12 h and serum AST,
ALT, ALP, BUN, CREA were detected.

**Histological analysis.** The liver, muscle and pancreas tissues were fixed in 4% para-
formaldehyde, gradually dehydrated, embedded in paraffin, cut into 4µm sections, and
subjected for hematoxylin/eosin staining. Periodic acid Schiff stain was performed according
to the manufacturer’s protocols (Jkchem, China) on liver and muscle sections. Immunohistochemical staining was performed according to the manufacturer’s protocols
(Boster, China). Liver sections were incubated with rabbit anti-rat GLUT2 (1:100, Proteintech,
USA), Pancreas sections were incubated with rabbit anti-rat PCNA antibody (1:1000, CST,
USA), rabbit anti-rat caspase3 antibody (1:100, bioworld, USA) at 4 °C overnight. For
immunohistofluorescence, muscle sections were incubated with rabbit anti-rat GLUT4
antibody (1:100, SAB, USA), pancreas were incubated with rabbit anti-rat insulin antibody
(1:100, Bioworld, USA) at 4 °C overnight, respectively. The sections were then washed and
incubated with Alexa Fluor 555 conjugated donkey anti-rabbit IgG, or FITC conjugated goat
anti-Rabbit IgG (Invitrogen) for 1 h. Subsequently, sections were stained with Hoechst 33342
at 0.5µg/mL before observed under microscope.
**Glucose uptake assay.** Glucose uptake in L6 cell and L02 cell was analyzed by 2-NBDG method (Life Technologies, USA). Briefly, the differentiated L6 cell and L02 cell seeded in 6 well plates were starved for 12h and treated with different doses of hucMSC-ex (0, 50, 100, 200, 400µg/ml) in serum-free medium for 24h. The medium was discarded and the cells were washed with PBS for three times. Using insulin (100nM) or not to verify the effect of hucMSC-ex in regulating insulin sensitivity (incubated for 30min). Then, 2-NBDG (100µM) was added into the cells. After one hour, the cells were collected and subjected to flow cytometric analysis (BD FACS Calibur) and microscopic observation (Nikon, Tokyo, Japan). HLF1-ex and hucMSC-ex free CM were used to demonstrate the specific effect of hucMSC-ex on 2-NBDG uptake.

**Western blot.** Total protein was extracted from tissues and cells by using RIPA lysis buffer. For insulin signaling pathway analysis, animals were starved overnight and treated with insulin (2IU/kg bw) or PBS (control). Cell membrane protein was prepared from skeletal muscle and liver by using Proteo-Prep Membrane Extraction Kit (Beyotime Biotechnology, China). The protein concentration was determined by using a BCA protein assay kit. Equal amounts of protein were separated on 12% SDS-PAGE gel and then transferred onto polyvinylidene fluoride (PVDF) membranes. After blockade with 5% skim milk for 1 h, the membranes were incubated with primary and the HRP-conjugated secondary antibodies and detected by using ECL detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). The primary antibodies were as follows: CD9 (1:500; Bioworld, USA), CD81 (1:500, Abcam,
UK), GLUT4 (1:400, SAB, USA), GLUT2 (1:400, Proteintech, USA), AKT (1:500, CST, USA), Phospho-Akt (1:500, CST, USA), IRS-1(1:200, SAB, USA), IRS-1 (Phospho-tyr896) (1:500, SAB, USA), GSK-3β (1:500, SAB, USA), GSK3β (phospho-Ser9) (1:500, SAB, USA), AMPK (1:500; Bioworld, USA), p-AMPK (1:500, Bioworld, USA), Glycogen synthase2 (1:500, Bioworld, USA), PCNA (1:5000, CST, USA), caspas3 (1:400, bioworld, USA), p-TBC1D4 (1:400, bioworld, USA), GAPDH (1:2000, CWBIO, China), β-actin (1:2000, CWBIO, China). The secondary antibodies were HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies (1:2000, CWBIO, China).

QRT-PCR. Total RNA was extracted from the skeletal muscle, liver, L6 cell and L02 cell. The expression levels of target genes were determined by using reverse and real-time quantitative PCR. β-actin was used as the internal control. The primers were provided by Invitrogen (Shanghai, China) and their sequences were shown in Table 2.

Statistical analysis. All the data are shown as mean ± standard deviation (SD). The statistically significant differences between groups were assessed by two-way ANOVA with Bonferroni Comparisons, Repeated Measures ANOVA or unpaired t-test using Prism software (GraphPad, San Diego, USA). P<0.05 was considered significant.
FIGURES:

Figure 1

Figure 1. HucMSC-ex down-regulated blood glucose level in T2DM rats as the main paracrine approach of hucMSC. (a) HucMSC-ex, hucMSC-CM, hucMSC-ex free CM (protein concentration: 10mg/kg bw), HucMSC(3×10^6 cells/dose) in 0.2ml PBS and 0.2ml PBS were infused into 5 groups of T2DM rats respectively by tail intravenous and the blood glucose levels were determined after 3h starved, normal rats treated with PBS as control (each group compared with T2DM+PBS). (b) HucMSC-ex, HFL1-ex (protein concentration: 10mg/kg bw) in 0.2ml PBS and 0.2ml PBS were infused into 3 groups of T2DM rats by tail intravenous and the blood glucose levels were determined, normal rats treated with hucMSC-ex (protein concentration: 10mg/kg bw) in 0.2ml or 0.2ml PBS as control (all groups compared with each other). (Values of a-b are means ± SE; n=6 rats per group; ns>0.05, ** p < 0.01, ***p < 0.001 determined by Repeated Measures ANOVA.)
Figure 2. HucMSC-ex enhanced glucose uptake in muscle cell (L6 cell) and hepatic cell (L02 cell). Cell models pre-treated with hucMSC-ex, HFL1-ex or hucMSC-ex free CM for 24h and stimulated with insulin or not for 30min before incubated with 2-NBDG, DMSO as control: (a) Concentration-dependent effect of hucMSC-ex on 2-NBDG uptake in L6 cell was determined with fluorescence microscopy (Scale bar=400µm). (b) Effect of hucMSC-ex on 2-NBDG uptake in L6 cell was confirmed by flow cytometry (FCM) (wave length=488nm). (c) Effect of hucMSC-ex, HFL1-ex, hucMSC-ex free CM (protein concentration: 400µg/ml) on 2-NBDG uptake in L6 cell was detected by FCM (wave length=488nm). (d) Effect of insulin (100nM), hucMSC-ex, hucMSC-ex+insulin on 2-NBDG in L6 cell uptake was analyzed by FCM (wave length=488nm). (e) Effect of insulin, hucMSC-ex, hucMSC-ex+insulin on 2-NBDG in L02 cell were observed with Fluorescence microscopy (Scale bar=200 µm). (Values of b-d are means ± SE; n=3 per group; ** p < 0.01, ***p < 0.001 determined by One-way ANOVA.)
Figure 3. HucMSC-ex ameliorated the insulin resistance in T2DM rats. T2DM rats were randomly divided into 4 groups and respectively accepted: PBS (0.2ml), hucMSC-ex (10mg/kg bw), insulin (2IU/kg bw), hucMSC-ex/insulin intervention, normal group treated with PBS as control: (a) Blood glucose levels of each group were determined every 3 days (each group compared with T2DM+hucMSC-ex+insulin). (b) Individual glucose tolerance was assessed by oral glucose tolerant tests (OGTTs), fasted rats were administrated 2g glucose /kg body weight by intra-gastric and blood glucose levels were determined in 0, 30, 60, 90, 120min (each group compared with T2DM+hucMSC-ex+insulin). (c) Individual insulin tolerance was evaluated by intra-peritoneal insulin tolerance tests (IPITTs), by injecting 2g glucose /kg body weight (bw) immediately followed by insulin administration at a dose of 2IU/kg bw, blood glucose level were detected in 0, 30, 60, 90, 120min and compared with 0 in (each group compared with T2DM+hucMSC-ex+insulin). (d) IR index of each group, HOMA-IR index=(FBG [in mmol/L] × FINS [in units/L])/22.5. Blood glucose level of each group was detected after 3 hours fasted. (Values of a-d are means ± SE; n=6 rats per group; * p < 0.05, ** p < 0.01, ***p< 0.001 determined by (a), (b), (c): Repeated Measures ANOVA, (e): One-way ANOVA.)
Figure 4. HucMSC-ex increased insulin sensitivity in palmitic acid (PA) induced insulin resistant cell model. L02 cell was divided into 7 groups and respectively accepted: PA(-), PA(-)+insulin (100nM), PA (0.25mM), PA+hucMSC-ex (400µg/ml), PA+insulin, PA+insulin+hucMSC-ex pretreated. Cells were treated with PA (PBS as control) for 24h and washed for 3 times by PBS and serum starved for 12h. HucMSC-ex (PBS as control) was added and incubated for 24h and culture medium were removed. After washed by PBS for 3 times, insulin (PBS as control) were added 30min before incubated with 2-NBDG (1h), cells treated with DMSO as blank control. (a) Effect of L02 cell uptake 2-NBDG in different groups was detected by flow sight, Scatter diagram show the fluorescence intensity distribution of cell (X-axis:2-NBDG Height; Y-axis: SSC Height; wave length=488nm). (b) The rate of cell uptake 2-NBDG in different groups of L02 cell. (c) Mean fluorescence intensity of cell uptake 2-NBDG in different groups of L02 cell. (d) Images of L02 cell uptake 2-NBDG were taken by imaging streaming (wave length=488nm, Scale bar=20µm). (Values of b, c are means ± SE n =3 per group; * p < 0.05, ** p< 0.01 determined by One-way ANOVA.)
Figure 5. HucMSC-ex activated the insulin signaling pathway in liver and muscle of T2DM rats. Protein of liver and muscle from normal rats (treated with PBS or hucMSC-ex), T2DM rats (treated with PBS or hucMSC-ex) was extracted. Rats in each group accepted 2IU/kg bw insulin or PBS (as control) injection before executed. (a) Expression of p-(tyr)-IRS-1, p-AKT, p-AMPK in liver were detected by Western blot. (b) Expression of p-(tyr)-IRS-1, p-AKT, p-AMPK in muscle were detected by Western blot. (c) Quantification of p-(tyr)-IRS-1, p-AKT, p-AMPK in (a). (d) Quantification of p-(tyr)-IRS-1, p-AKT, p-AMPK in (b). (Values of c, d are means ± SE, n =3 per group; * p < 0.05, ** p < 0.01, *** p < 0.001 determined by One-way ANOVA.)
Figure 6-2

(f) PAS staining of liver and muscle tissues from different groups.

(g) Western blot analysis of GYS-2, p-GSK3β, GSK-3β, and β-actin expression in liver tissues from different groups.

(h) Quantitative analysis of GYS-2 and p-GSK3β protein levels in liver tissues from different groups.

(i) mRNA expression levels of GCCK, CS, and ICDH in liver tissues from different groups.

(j) mRNA expression levels of PK, CS, and ICDH in muscle tissues from different groups.
Figure 6. HucMSC-ex promoted glucose uptake and glycolysis in muscle and glycogen storage in liver in T2DM rats. (a): Muscle histology from four groups (normal group treated with PBS or hucMSC-ex, T2DM groups treated with PBS or hucMSC-ex) pretreated with PBS or insulin (2IU/kg bw) were studied in immune-fluorescence to detected the membrane translocation of GLUT4 (red), representative images of muscle sections were observed under Fluorescence microscopy (Scale bar=200µm). (b) Expression of GLUT4 relative membrane protein of muscle from rats in four groups (normal group treated with PBS or hucMSC-ex, T2DM groups treated with PBS or hucMSC-ex) pretreated with insulin were detected by Western blot. (c) Expression of p-TBC1D4, GLUT4 of muscle from rats in four groups pretreated with insulin was detected by Western blot. (d) Quantification of p-TBC1D4, GLUT4 in (c). (e) Expression of GLUT4 mRNA in muscle was analyzed by quantitative RT-PCR. (f) Liver and muscle histology was studied in serial 4 µm, representative images of periodic acid Schiff staining (PAS) of liver and muscle sections were observed under light microscopy (Scale bar = 200µm). (g) Expression of p-GSK3β and glycogen synthase2 (GYS-2) in liver was detected by Western blot. (h) Quantification of p-GSK3β, GYS-2 in (g). (i) Expression of glucokinase (GCK), citrate synthase (CS), isocitrate dehydrogenase (ICDH) mRNA in liver was analyzed by quantitative RT-PCR. (j) Expression of pyruvate kinase (PK), CS, ICDH mRNA in muscle was analyzed by quantitative RT-PCR (Values of b,c are means ± SE n = 6 rats per group; *p< 0.05, **p< 0.01, ***p< 0.001 determined by One-way ANOVA)
Figure 7-1

(a) Serum insulin levels in fasting and refeeding conditions.

(b) Serum insulin levels over time.

(c) Histological images showing HE staining.

(d) Bar graphs showing numbers of fat cells.

(e) Bar graphs showing area of fat (100μm²).
Figure 7-2

Figure 7. HucMSC-ex relieved the destructed islets in T2DM rats by inhibit STZ induced apoptosis. (a) Individual insulin levels of fasting and re-fed rats in four groups (normal group

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treated with PBS or hucMSC-ex, T2DM groups treated with PBS or hucMSC-ex) were evaluated by ELISA. (b) Individual insulin level in four groups was assessed by insulin release test (IRT), involving administration of 2g glucose/kg body weight and determination of serum insulin levels in 0, 30, 60, 120,180min. (c) Morphology of pancreatic islets in four groups stained with hematoxylin/eosin(H-E) and β-cells were characterized by immune-histochemistry(IHC) staining according to the presence and distribution of insulin (yellow) (Scale bar=200 µm). (d) Amounts of pancreatic islets in four groups observed in H-E stained sections were quantified. (e) Area of pancreatic islets in four groups observed in IHC stained sections were quantified. (f) Serial sections of pancreas from different group (T2DM groups treated with PBS or hucMSC-ex, normal group treated with PBS as control) were prepared and detected insulin to location β-cells in immune-fluorescence (green), Caspase3 and PCNA were detected in immune-histochemical staining (brown, red arrow point) (Scale bar = 200 µm). (g) Protein of pancreas from animal models (T2DM groups treated with PBS or hucMSC-ex, normal group with PBS as control) was extracted and the expression of Caspase3, PCNA were detected with Western blot. (h) Quantification of Caspase3, PCNA in (g). (Values of a-e are means ± SE n=6 rats per group; * p < 0.05, ** p < 0.01, *** p<0.001 determined by One-way ANOVA)

ASSOCIATED CONTENT

Supporting Information:

Figure S1. Characterization of exosomes from in vitro cell culture.

Figure S2. Identification of fat-fed/STZ-induced T2DM rat model.

Figure S3. Maximum tolerated dose of hucMSC-ex in T2DM intervention.

Figure S4. Validity period of GW4869 in hucMSC-ex secretion inhibition and blood glucose regulation of hucMSC-GW4869 in T2DM rats.

Figure S5. Identification of cell models.

Figure S6. HucMSC-ex contributed to hepatic glycogen synthesis and activated the insulin signaling pathway in vitro.
Figure S7. HucMSC-ex decreased the level of TNFα but not IL6 in serum of T2DM rats.

Figure S8. HucMSC-ex treated T2DM rats have lower hepatic GLUT2 translocation in membrane.

Figure S9. HucMSC-ex inhibited the STZ induced apoptosis of β cells in pancreas.

Figure S10. Bio-distribution of hucMSC-ex in T2DM rats.

Figure S11. Physiology index of rats in different groups 2 months after last infusion of hucMSC-ex.

Supplementary Table 1: Time point of different treat to animal models.

Supplementary Table 2: Sequences of real-time PCR primers

AUTHOR INFORMATION

Corresponding Author:

Hui, Qian, Ph.D., Professor, School of Medicine, Jiangsu University, 301 Xuefu Road, Zhenjiang, Jiangsu 212013, China

Tel: +86 511 85038334

Fax: +8651185038483

E-mail: lstmmmlst@163.com

Wenrong, Xu, Ph.D., Professor, School of Medicine, Jiangsu University, 301 Xuefu Road, Zhenjiang, Jiangsu 212013, China

Tel: +86 511 85038215

Fax: +86 511 85038483
E-mail: icls@ujs.edu.cn

Author Contributions

# Yaoxiang, Sun, Hui, Shi, Siqi, Yin have contributed equally to this work.

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REFERENCES


(61) Van, D. J.; Mestdagh, P.; Sormunen, R.; Cocquyt, V.; Vermaelen, K.; Vandesompele, J.; Bracke, M.; De, W. O.; Hendrix, A. The Impact of Disparate Isolation Methods for

