Reports of Biochemistry & Molecular Biology
Vol.12, No.1, Apr 2023

Original article

www.RBMB.net

Bone Marrow-Derived Mesenchymal Stem Cells and Pioglitazone or Exendin-4 Synergistically Improve Insulin Resistance via Multiple Modulatory Mechanisms in High-Fat Diet/Streptozotocin-Induced Diabetes in Rats

Mohamed Mesbah Mohamed[1], Laila Ahmed Rashed[2], Noha Ahmed El-Boghdady[3], Mahmoud Mohamed Said*[1]

Abstract

Background: Diabetes mellitus (DM) is a metabolic disease, characterized by hyperglycemia resulting from defects in insulin secretion and/or insulin action. The current study was designed to assess the therapeutic potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) alone and in combination with pioglitazone (Pz) or exendin-4 (Ex) in high-fat diet/streptozotocin (HFD/STZ)-induced diabetes in rats.
Methods: The rats were subjected to the HFD for three weeks before being injected with a single low dosage of STZ (35 mg/kg bw). The animals were assigned to different treatment groups after type II diabetes mellitus (T2DM) induction was confirmed.
Results: Severe insulin resistance was verified in untreated HFD/STZ T2DM rats, along with the exaggeration of oxidative stress, inflammation, apoptosis, and autophagy suppression in the adipose tissues. Monotherapy of HFD/T2DM rats with BM-MSCs and Pz or Ex alleviated diabetic complications by increasing insulin sensitivity, decreasing apoptosis and inflammation as evidenced by a decrease in serum tumor necrosis factor-alpha, caspase-3, and nuclear factor-kappa B (NF-κB) genes expression and Janus kinase (JNK) protein expression, and enhancing autophagy as revealed by upregulation in beclin and LC3, as well as peroxisome proliferator-activated receptor-γ coactivator-1 alpha (PGC-1α) genes expression in the adipose tissues. An augmented ameliorative efficacy was recorded in combined treatments. The biochemical and molecular results were confirmed by histological investigation of pancreatic tissues.
Conclusions: Combining Pz or Ex with BM-MSCs is a synergistic therapeutic option that reduces insulin resistance and subsequent complications in T2DM via multiple molecular mechanisms.

Keywords: Exendin-4, High-fat diet, Mesenchymal stem cells, Pioglitazone, Streptozotocin, Type II diabetes mellitus.

Introduction

Diabetes is a dangerous chronic disease that has a significant global impact on people's lives, families, and communities. It is one of the top ten causes of adult mortality. In 2019, 463 million people worldwide were anticipated to have diabetes, with that number expected to rise to 578 million by 2030 and 700 million by 2045. In a very short period of time, the prevalence of diabetes in Egypt has risen dramatically, from roughly 8.9 million

1: Biochemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt.
2: Department of Medical Biochemistry, Faculty of Medicine, Cairo University, Cairo, Egypt.
3: Department of Biochemistry, Faculty of Pharmacy, Cairo University, Cairo, Egypt.
***Corresponding author: Mahmoud Mohamed Said; Tel: +20 1148871573; E-mail: mahmoudmsaid@sci.asu.edu.eg.**
Received: 10 Nov, 2022; Accepted: 18 Jan, 2023

[Downloaded from rbmb.net on 2024-10-12]
[DOI: 10.52547/rbmb.12.1.42]

in 2019 to 11.9 million in 2030. By 2045, it is anticipated that this number will have risen to 16.9 million (1).

Diabetes is characterized by the body's failure to produce insulin hormone or its resistance to its activity, resulting in a reduction in glucose metabolism and an increase in glucose levels in the blood, a condition known as hyperglycemia. Chronic hyperglycemia can damage tiny blood vessels, resulting in failure in organs, including the kidneys, eyes, and nerves. An autoimmune reaction against pancreatic β-cells causes type I diabetes mellitus (T1DM). Insulin production is affected when pancreatic β-cells are destroyed by the immune system (2).

Type II diabetes mellitus (T2DM) is a metabolic condition marked by insulin resistance and hyperglycemia that progresses over time. In T2DM, inadequate insulin levels fail to satisfy the increased demand brought on by insulin resistance. Most T2DM medications now seek to either reduce insulin resistance or increase insulin levels by improving β-cell function. In view of the possibility of an exhausting impact on β-cells, more basic research is focusing on mechanisms for regenerating β-cell mass or preserving β-cell function. To achieve this, a detailed knowledge of the role and dynamics of β-cell mass and function in insulin resistance compensation and progression to T2DM is required (3).

Insulin metabolism and blood glucose homeostasis are controlled by three major insulin-targeting tissues: the liver, adipose tissue, and skeletal muscles. Macrophages and other innate immune cells produce proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) that reduce insulin sensitivity. β-Cell dysfunction is another prominent feature of T2DM, and it often develops in individuals within a few years of diagnosis (4).

Insulin sensitizers like sulfonylurea and meglitinide are used in T2DM patients to stimulate insulin production from pancreatic β-cells. By stimulating AMP-activated protein kinase, biguanide improves insulin sensitivity in the liver and peripheral tissues; α-glucosidase inhibitors delay glucose absorption; thiazolidinedione (TZD) improves insulin sensitivity in peripheral tissues and the liver by activating peroxisome proliferator-activated receptor-gamma receptors (5).

Mesenchymal stem cells (MSCs) have a number of properties that make them a promising cell-based therapy for diabetes, including the ability to differentiate into insulin-producing cells (IPCs), promote pancreatic islet β-cell regeneration, protect endogenous pancreatic islet β-cells, and reduce insulin resistance (6).

Autophagy governs pancreatic β-cell activity and insulin-target tissues (skeletal muscle, liver, and adipose tissue) and is related to poor pancreatic β-cell function and the development of insulin resistance. According to Yang et al. (7), increased autophagy appears to protect pancreatic cells from oxidative stress (OS). Constitutively activated autophagy is toxic to pancreatic β-cells, and prolonged autophagy stimulation leads to autophagic cell death (8).

The current study aims to assess the efficiency of mesenchymal stem cell therapy with and without pioglitazone or exendin-4 in alleviating pathogenic alterations in T2DM, as well as to investigate some of the underlying molecular mechanisms of action.

Materials and Methods

Animals

Throughout this investigation, adult male inbred Sprague Dawley rats were employed. The animals were housed in plastic cages and subjected to regular temperature, humidity, and 12-hour light/dark cycles. As an acclimatization period, they were given unlimited access to water and standard laboratory chow for one week prior to the commencement of the experiment.

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

Chemicals

Streptozotocin (STZ) and exendin-4 (Ex) were provided by Sigma-Aldrich (St. Louis, Mo, USA), pioglitazone (Pz, commercially known as Actos®) from The Arab Pharmaceutical Manufacturing Co. Ltd. (Jordan), Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% fetal bovine serum (FBS), trypsin, EDTA and 1% penicillin/streptomycin from GIBCO/BRL (USA), Ficoll-Paque from Pharmacia (USA) and culture flasks from Thermo Fisher Scientific (USA).

Drugs preparation

STZ was dissolved in 0.1 M citrate buffer (pH 4.5), and each rat received a single 35 mg/kg bw (body weight) ip injection (9). Pioglitazone was dissolved in water, and each rat was given an oral dosage of 10 mg/kg bw (body weight) every day for 30 days (10). Exendin-4 was dissolved in water, and each rat was given a daily ip injection of 1 nmol/kg body weight for 7 days (11).

Induction of high fat diet (HFD) type II diabetes mellitus in rats

As an acclimatization period, all rats were fed the standard basal diet for one week prior to the commencement of the experiment. The standard basal diet was made up of 69% carbohydrate, 19.6% protein, 4.6% fat, and 4.8% crude fiber, whereas the high-fat diet (HFD) was made up of 10.6% carbohydrate, 19.4% protein, 60% fat, and 4.7% crude fiber (12). In brief, rats were subjected to the HFD for three weeks, then fasted overnight before receiving a single ip injection of freshly prepared STZ (35 mg/kg bw). To reduce hypoglycemia caused by abrupt insulin surges from pancreatic β-cells during the first 24 hours following STZ injection, rats were given a 5% glucose solution. In rats, hyperglycemia was confirmed by measuring fasting blood glucose levels 72 hours after STZ injection (12,13).

Isolation and propagation of bone marrow-derived mesenchymal stem cells (BM-MSCs) from rats

The bone marrow (BM) of a 6-week-old male Sprague Dawley rat was extracted by flushing the tibiae and femurs with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. After nucleated cells were isolated using a density gradient Ficoll-Paque, they were resuspended in complete DMEM culture medium supplemented with 1% penicillin/streptomycin and incubated in a humidified atmosphere at 37 °C and 5% CO_2 for 12-14 days as a primary culture or upon formation of large colonies, with culturing media changed every 2-3 days. After forming large colonies (80-90% confluence), the cultures were washed twice with phosphate-buffered saline (PBS), and the cells were trypsinized for 5 minutes at 37 °C with 0.25% trypsin in 1 mM EDTA. Cell suspensions were collected in a Falcon tube, centrifuged at 400 ×g for 5 minutes, and cell pellets were resuspended in serum-supplemented DMEM medium before being cultured in 50 cm^2 culture flasks at 37 °C and 5% CO_2 in a humidified environment. The cultures that were obtained were referred to as first passage cultures (14). The adhering colonies of cells were trypsinized and counted on day 14 (Fig. 1A).

Identification of BM-MSCs

Flow cytometry (Beckman Coulter Inc., CA, USA) was used to identify cultured cells as mesenchymal stem cells (MSCs) based on their morphology, adherence, and the identification of CD29, CD34, and CD90 as surface markers of rat MSCs using monoclonal antibodies provided by Santa Cruz Biotechnology, Inc. (TX, USA) (Fig. 1B-D).

Labeling of BM-MSCs with PKH26

Isolated and propagated BM-MSCs were tagged before to injection into rats using the PKH26 Red Fluorescent Cell Linker Kit from Sigma-Aldrich (St Louis, MO, USA) and injected intraperitoneally. The pancreatic slices were inspected using a fluorescence microscope after one month to track BM-MSCs labelled with PKH26 (Fig. 1E).

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

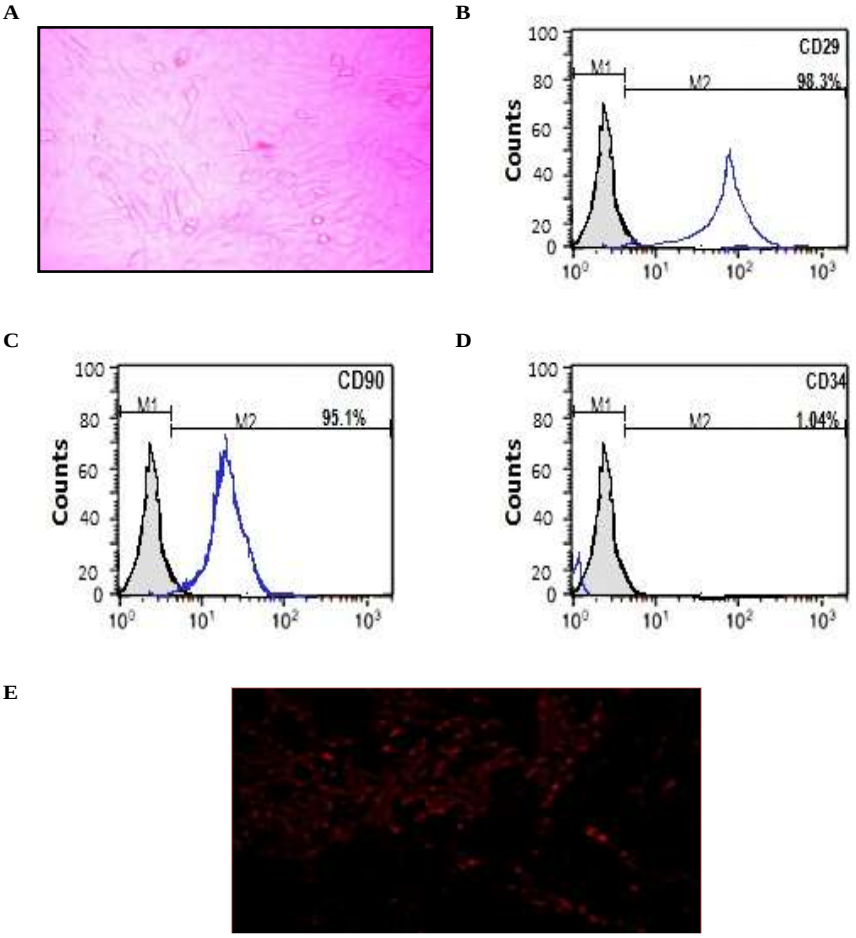

Fig. 1. Confluent mesenchymal stem cells (MSCs) are spindle-like or star-like (A). Flow cytometric characterization analysis of bone marrow derived MSCs uniformly positive for CD29 (B) and CD90 (C) but negative for CD34 (D). Bone marrow derived MSCs labeled with PKH26 fluorescent dye homing in the pancreatic tissue (E).

Experimental protocol

A total of 56 adult male Sprague Dawley rats were randomly allocated into seven equal-sized groups: Control (C), where rats received a daily ip injection of 1 ml/kg bw 0.1 M citrate buffer (pH 4.5) for 30 days; High-fat diet (HFD) type II diabetes mellitus (HFD-T2DM, D), where rats were assigned to the high-fat diet for 3 weeks, fasted overnight, and then injected with a single ip dose of freshly prepared STZ (35 mg/kg bw); HFD-T2DM with mesenchymal stem cells (D+MSC), in which diabetic rats received a single ip injection of BM-MSCs (1×10^6 cells/ml) beginning on the seventh day after STZ injection (15); HFD-T2DM with pioglitazone (D+Pz), in which diabetic rats were orally treated with 10 mg/kg bw pioglitazone (Pz) for 30 days beginning on the seventh day after STZ injection (10); HFD-T2DM with exendin-4 (D+Ex), in which diabetic rats were intraperitoneally injected with 1 nmol/kg bw exendin-4 daily for one week beginning on the seventh day after STZ injection (11); HFD-T2DM with mesenchymal stem cells and pioglitazone (D+MSC+Pz), in which diabetic rats were initially intraperitoneally injected with a single dose of BM-MSCs (1×10^6 cells/ml) beginning on the seventh day after STZ injection, followed by oral administration of Pz (10 mg/kg bw) 2 hours later for 30 days; HFD-T2DM with mesenchymal stem cells and exendin-4 (D+MSC+Ex), in which diabetic rats were intraperitoneally injected with a single dose of BM-MSCs (1×10^6 cells/ml) beginning on the seventh day following STZ injection, followed by an ip injection of exendin-4 (1 nmol/kg bw) 2 hours later for 7 days.

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

Blood collection and tissue sampling

The rats were anesthetized and subsequently exsanguinated from the abdominal aorta by the end of the experiment. The blood was allowed to coagulate before being centrifuged and the serum was separated and stored at -20 ºC until biochemical analysis. Sections of visceral adipose tissue were dissected during necropsy, washed in ice cold sterile isotonic saline, blotted dry using filter paper, and stored at -80 ºC for future examination. Furthermore, the pancreas was excised and stored in 10% phosphate-buffered formalin for histological examination.

Preparation of tissue homogenate

A 10 % whole tissue homogenate was prepared on ice by homogenizing a portion of the adipose tissue in 10 volumes of ice-cold isotonic saline in an electric homogenizer (Glas-col., Terre Haut, USA). The whole 10% homogenate was centrifuged at 12,000 ×g for 10 minutes at 4 ºC, and the supernatant was separated and kept at -20 ºC.

Biochemical analysis

Serum glucose level was assayed by a colorimetric method of Trinder (16) using a commercial assay kit (BioMed, Egypt). Serum insulin and tumor necrosis factor-alpha (TNF-α) levels were measured using rat-specific ELISA kits from My BioSource, Inc. (San Diego, USA) and Ray Biotech Inc. (Georgia, USA), respectively. Nitric oxide (NO), malondialdehyde (MDA), total antioxidant capacity (TAC) and total protein levels were colorimetrically assayed in adipose tissue homogenates by the methods of Miranda et al. (17), Yoshioka et al. (18), Koracevic et al. (19) and Bradford (20), respectively.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from frozen adipose tissue samples with the RNeasy® Mini Kit (Qiagen, Hilden, Germany), and first-strand complementary DNA (cDNA) was generated with a Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific Inc, UK). Real-time PCR reactions were carried out on an optical 96-well plate in an ABI PRISM 7500 Fast Sequence Detection System Thermal Cycler (Applied Biosystems, California, USA) using Power SYBR® Green PCR Master Mix (Applied Biosystems, USA). The amplification process included a 15-second denaturation step at 95 ºC, followed by 40 cycles (60 seconds at 60 °C and 60 seconds at 72 °C). Table 1 shows the primers used for caspase-3, beclin, light chain 3 (LC-3), peroxisome proliferator-activated receptor-coactivator-1 alpha (PGC-1α), nuclear factor-kappa B (NF-κB), and β-actin (endogenous reference gene). The comparative threshold cycle approach of Livak and Schmittgen (21) was used to compute the relative expression of the genes under consideration.

Table 1. Primer sequences for selected genes.

Primer	Sequence
LC3	Forward: 5′- CCTTGCCTCTAAGCCTTTGC -3′ Reverse: 5′- GCCCTCCAGAAGTGGTCATT -3
Beclin	Forward: 5'- AACCCCATGCTGTCCTTTCC -3′ Reverse: 5' CAACTGTGTGCCACAAGCATC 3′
NF-κB	Forward: 5'- GATGCTGGTGCTGAGTATGTCG -3' Reverse: 5’- GTGGTGCAGGATGCATTGCTGA -3
PGC-1α	Forward: 5′- AACCCCATGCTGTCCTTTCC -3′ Reverse: 5′- CAACTGTGTGCCACAAGCATC -3′
β-Actin	Forward 5′-CGCTCATTGCCGATAGTGAT-3′ Reverse 5′-TGTTTGAGACCTTCAACACC-3

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

Western Blot Analysis of adipose tissue Janus kinase (JNK)

On ice, adipose tissue samples were coarsely homogenized in 0.5 ml of ice-cold RIPA lysis buffer (BIO BASIC INC., Ontario, Canada) containing phenylmethylsulfonylfluoride (PMSF, a protease inhibitor, 2 mM). The lysate was centrifuged for 30 minutes at 4 °C at 16,000 ×g. The protein content in the cytosolic supernatant of whole cell lysate was determined using the Bradford Protein Assay kit (BIO BASIC INC., Ontario, Canada). Protein solutions were mixed with 2× Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), boiled at 95 °C for 5 minutes, and separated by SDS-PAGE using a Bio-Rad Minigel equipment (Bio-Rad Laboratories, Hercules, CA, USA), before being electrotransferred onto nitrocellulose membranes (22). The membranes were blocked for 1 hour with 3% bovine serum albumin (BSA), then washed twice with BSA/0.3% Tween-20 before being incubated overnight at 4 °C with the monoclonal mouse anti-rat JNK antibody (1:20). (Thermo Fisher Scientific, Rockford, IL, USA). Following incubation, the membranes were washed three times in TBS-T buffer before being incubated for 1 hour at 4 °C with the goat anti-mouse alkaline phosphatase-conjugated secondary antibody (1:2,000). (Thermo Fisher Scientific, Rockford, IL, USA). To visualize protein bands, a chemiluminescent kit (ClarityTM Western ECL Substrate, BIO-RAD, USA) was utilized, and the chemiluminescent signals were captured using a CCD camera-based imager. The Chemi Doc MP imager was used for densitometry analysis to normalize band intensities against β-actin.

Histological examination

Samples of fixed pancreas were processed and embedded in paraffin. Light microscopy was used to analyze serial 4-µm thick slices stained with hematoxylin and eosin.

Statistical analysis

The Shapiro-Wilk normality test (P> 0.05) revealed that all data were normally distributed. The statistical analysis of differences in mean values was performed using one-way analysis of variance (ANOVA), followed by the Bonferroni's test for multiple comparisons. For all analyses, SPSS statistical software 22.0 for Windows (SPSS, Inc., Chicago, IL, USA) was used.

Results

Effect of different treatments on serum glucose, insulin and TNF-α levels

The induction of diabetes mellitus in male rats produced a significant rise in serum glucose (201.87%), insulin (137.27%) and TNF-α (246.13%) levels, compared to control rats. Injection of diabetic rats with mesenchymal stem cells (MSCs) significantly reduced serum blood glucose (48.93%), insulin (42.93%) and TNF-α (49.29%) levels, compared to untreated diabetic rats. Treatment of diabetic rats with either pioglitazone or exendin-4, on the other hand, significantly decreased serum glucose (43.14 & 39.17%), insulin (38.74 & 35.86 %), and TNF-α (22.20 & 30.44%), respectively, compared to untreated diabetic rats. Co-administration of pioglitazone or exendin-4 to diabetic rats implanted with MSCs corrected blood glucose and insulin levels. When compared to untreated diabetic rats, serum TNF-α levels in pioglitazone-treated MSC-injected diabetic rats decreased significantly (52.55%), whereas serum TNF-α levels rebounded to normal in exendin-treated MSC-injected diabetic rats (Fig. 2).

Effect of different treatments on adipose tissues' oxidative stress

T2DM induction in male rats resulted in severe oxidative stress in the adipose tissue, as evidenced by the sharp significant rise in MDA (1515.84%) and NO (2644.20%) levels, accompanied by a considerable depletion in total antioxidant capacity (TAC) (79.76%),

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

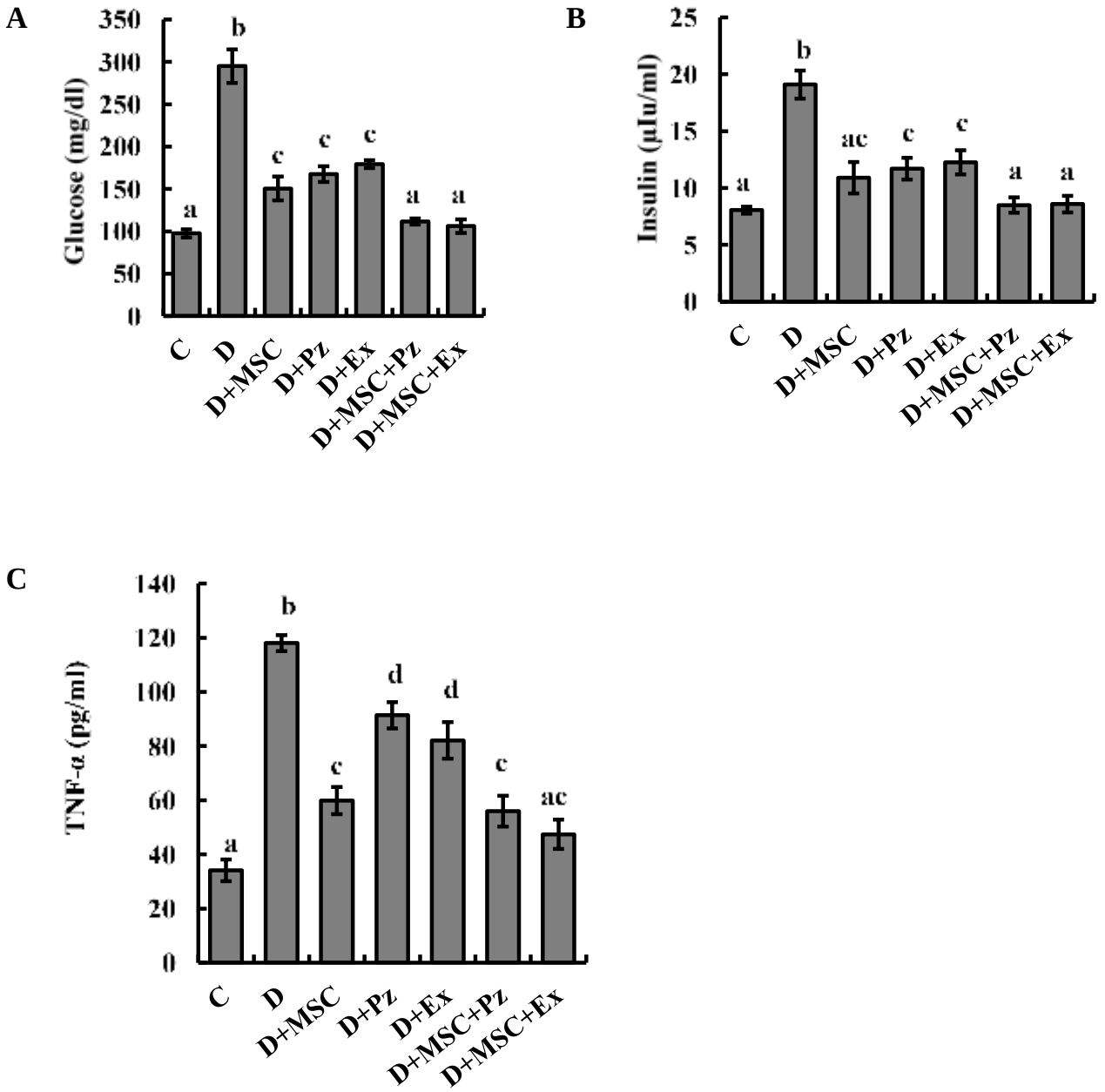

Fig. 2. Changes in serum glucose (A), insulin (B) and tumor necrosis factor-alpha (TNF-α) (C) levels in diabetic rats treated with mesenchymal stem cells (MSC), pioglitazone (Pz) and/or exendin-4 (Ex). Each column represents the mean ± standard error of 6 rats. Values with different letters are considered significantly different (P< 0.05). Control (C); Diabetic (D).

compared to control rats. In comparison to diabetic rats, injection of MSCs significantly reduced tissue MDA (68.94%) and NO (76.42%) levels, while dramatically increased TAC (132.98%). Treatment of diabetic rats with either pioglitazone or exendin-4, on the other hand, significantly decreased MDA (36.78 & 33.32%, respectively) and NO (59.39 & 49.30%, respectively) levels in the adipose tissue of both groups, while increased TAC (127.47%) in the adipose tissue of exendin-4-treated MSC-injected diabetic rats. All the aforementioned parameters were normalized by co-administration of pioglitazone or exendin to MSC-injected diabetic rats (Fig. 3).

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

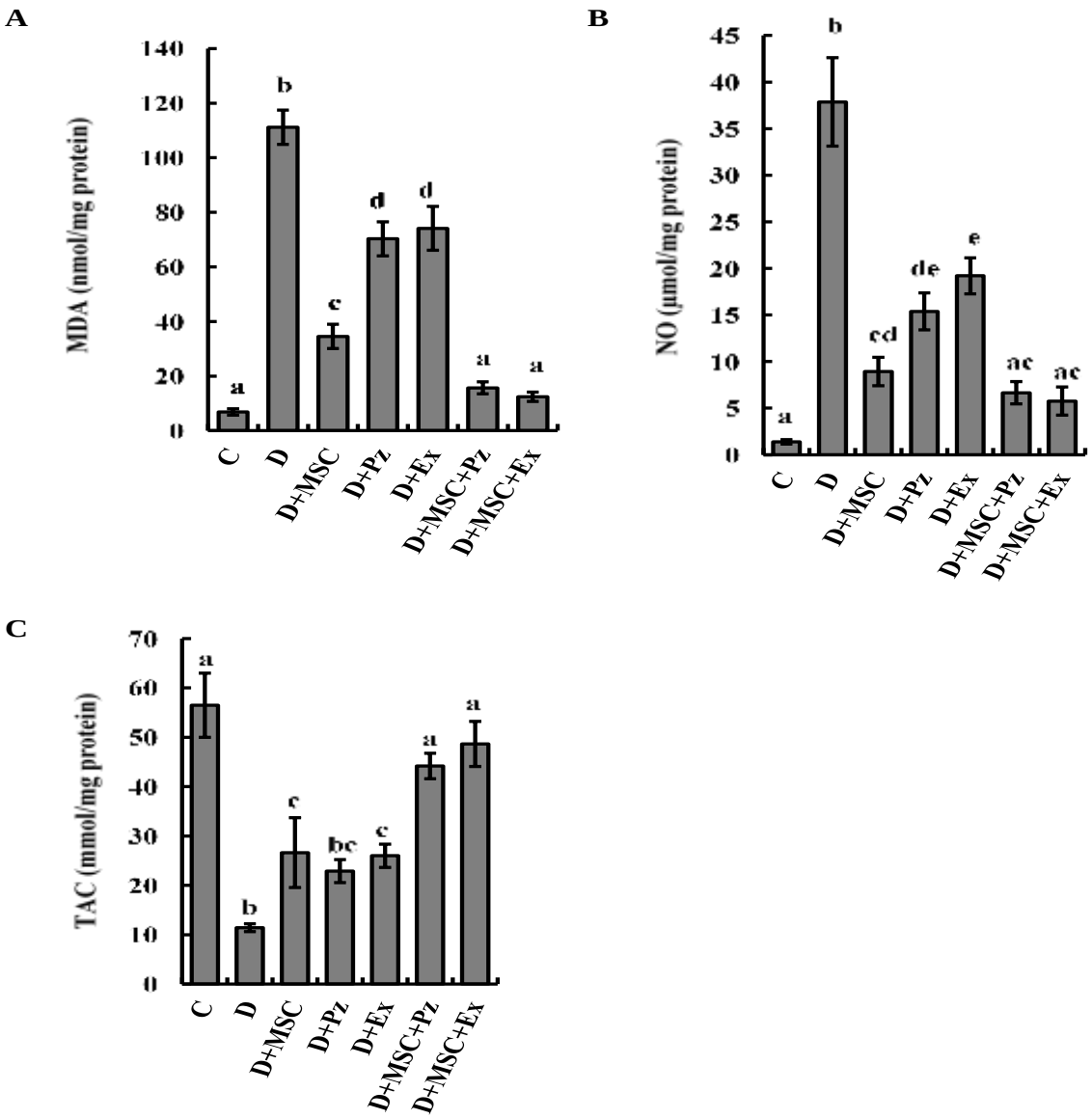

Fig. 3. Changes in malondialdehyde (MDA) (A), nitric oxide (NO) (B) and total antioxidant capacity (TAC) (C) levels in the adipose tissue of diabetic rats treated with mesenchymal stem cells (MSC), pioglitazone (Pz) and/or exendin-4 (Ex). Each column represents the mean ± standard error of 6 rats. Values with different letters are considered significantly different (P< 0.05).

Effect of different treatments on apoptotic, inflammatory and autophagy markers in adipose tissues

T2DM induction in rats resulted in severe apoptosis in adipose tissues, as evidenced by a substantial increase in caspase-3 gene expression compared to control rats. In diabetic rats, injections of MSCs, pioglitazone, or exendin-4, either alone or in combination, dramatically down-regulated caspase-3 gene expression in adipose tissues compared to diabetic rats (Fig. 4A). Similarly, when compared to control rats, the expression of the transcription factor NF-κB gene was significantly upregulated in the adipose tissues of T2DM-induced rats, whereas injection of diabetic rats with MSCs, pioglitazone, or exendin-4, either alone or in combination, significantly downregulated NF-κB gene expression in the adipose tissues (Fig. 4B). On the other hand, autophagy was suppressed in the adipose tissues of T2DM-induced rats, as revealed by a substantial decrease in beclin and LC3 genes expression as compared to control rats. By contrast, treatment of diabetic rats with MSCs, pioglitazone, or exendin-4, alone or in combination, resulted in a considerable upregulation in the expression of the

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

aforementioned autophagy markers as compared to diabetic rats (Fig. 4C&D). When compared to control rats, the expression of the PGC-1α gene was downregulated in the adipose tissues of T2DM-induced rats, whereas injection of diabetic rats with MSCs, pioglitazone, or exendin-4, either alone or in combination, produced a sharp significant upregulation in the expression of PGC-1α as compared to diabetic rats (Fig. 4E).

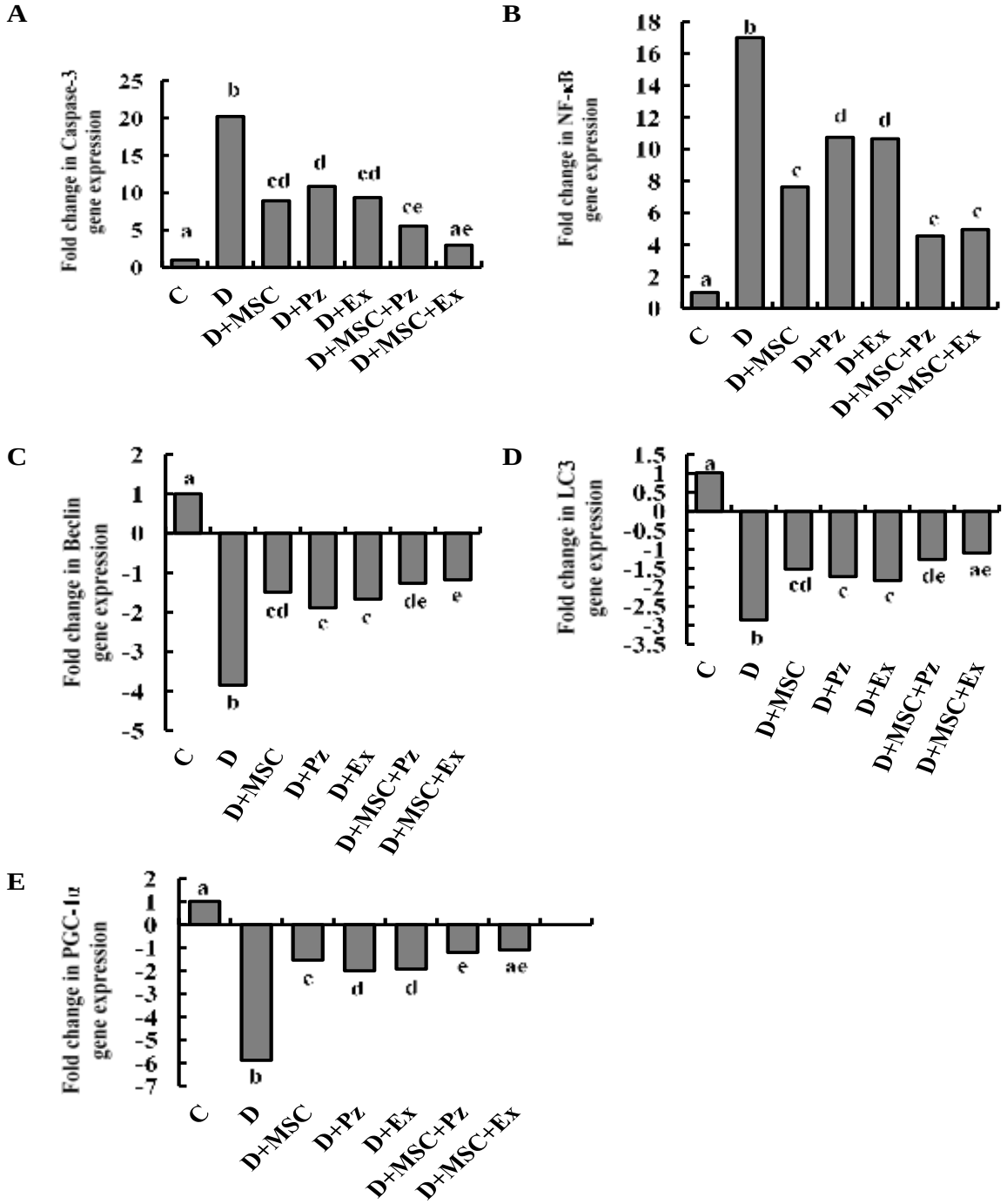

Fig. 4. The fold change in gene expression of caspase-3 (A), nuclear factor-kappa B (NF-κB) (B), beclin (C), light chain 3 (LC3) (D) and proliferator-activated receptor-coactivator-1 alpha (PGC-1α) (E) in the adipose tissues of diabetic rats treated with mesenchymal stem cells (MSC), pioglitazone (Pz) and/or exendin-4 (Ex). Each column represents the average of 6 rats. Values with different letters are considered significantly different ($P < 0.05$). The relative quantification (RQ) of mRNA expression of selected genes was compared to that of β-actin. In the case of gene downregulation, the fold change in the treatment group was calculated as follows: $\frac{-1}{RQ}$.

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

Effect of different treatments on JNK protein expression in adipose tissues

T2DM induction in male rats significantly increased JNK protein expression in adipose tissues, compared to control animals. By contrast, a significant reduction in JNK protein expression was observed in the adipose tissue of MSCs-inoculated, pioglitazone or exendin-4-treated diabetic rats, compared to untreated diabetic rats, whereas a rebound to normal levels was observed in JNK protein expression in the adipose tissue of MSC-injected-pioglitazone or exendin-4-treated diabetic rats (Fig. 5).

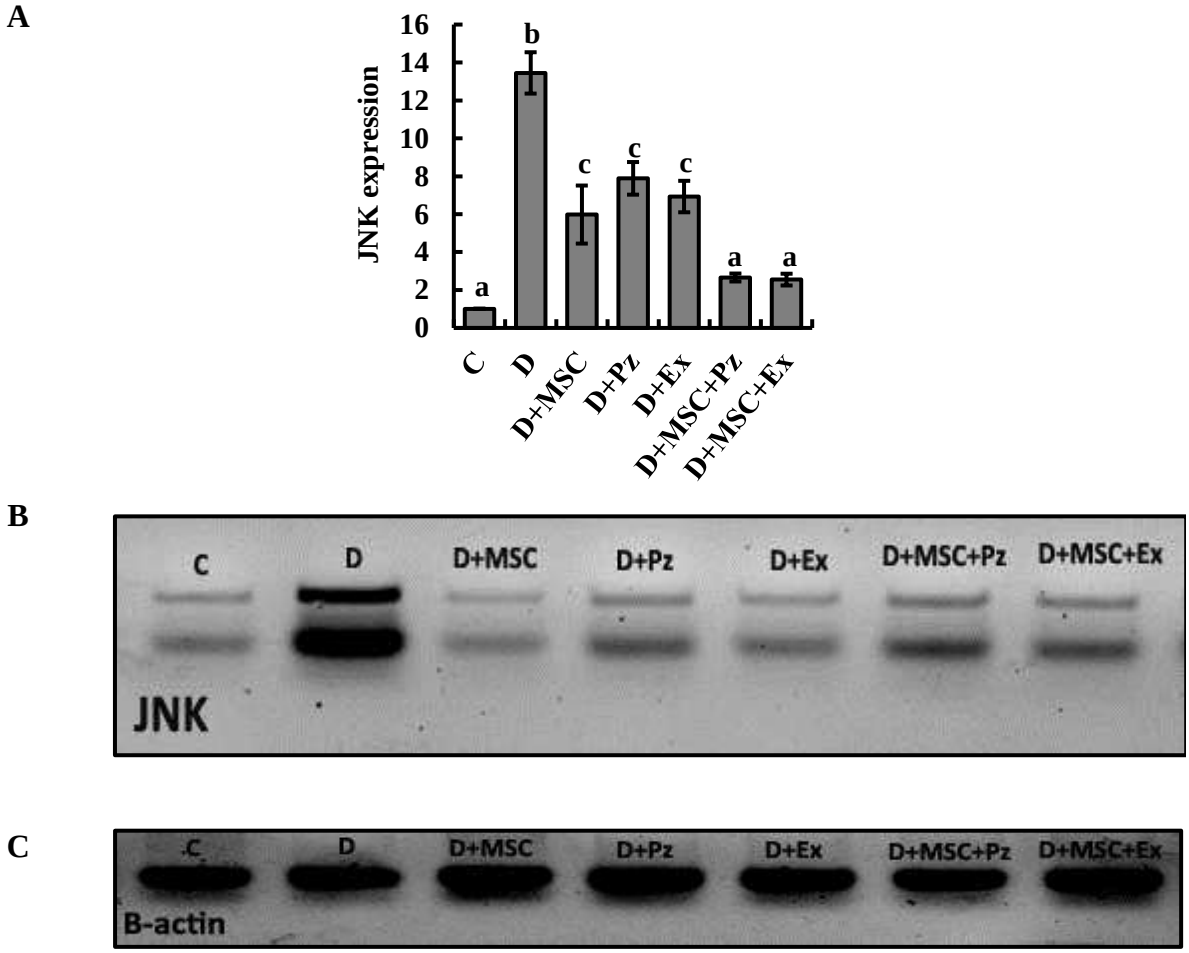

Fig. 5. Fold change in Janus kinase (JNK) protein expression in the adipose tissues of diabetic rats (D) treated with mesenchymal stem cells (MSC), pioglitazone (Pz) and/or exendin-4 (Ex). Each column represents the average of 6 rats. Values with different letters are significantly different ($P< 0.05$) (A). Western blots of JNK (B) and β-actin (C).

Effect of different treatments on pancreatic histology

In control rats, the histological structure of the islets of Langerhans cells as an endocrine component and the surrounding acini with the duct system as an exocrine portion was normal (Fig. 6A). T2DM induction with STZ in male rats resulted in islet of Langerhans atrophy (Fig. 6B), while infusion of BM-MSCs in diabetic rats retained the intact structure of the islets of Langerhans (Fig. 6C). Treatment with pioglitazone or exendin-4 alleviated STZ-induced toxicity in diabetic rats' pancreas (Fig. 6C&D), and a more apparent synergistic amelioration was seen after treatment of MSC-injected diabetic rats with pioglitazone or exendin-4 (Figs. 6 E&F).

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

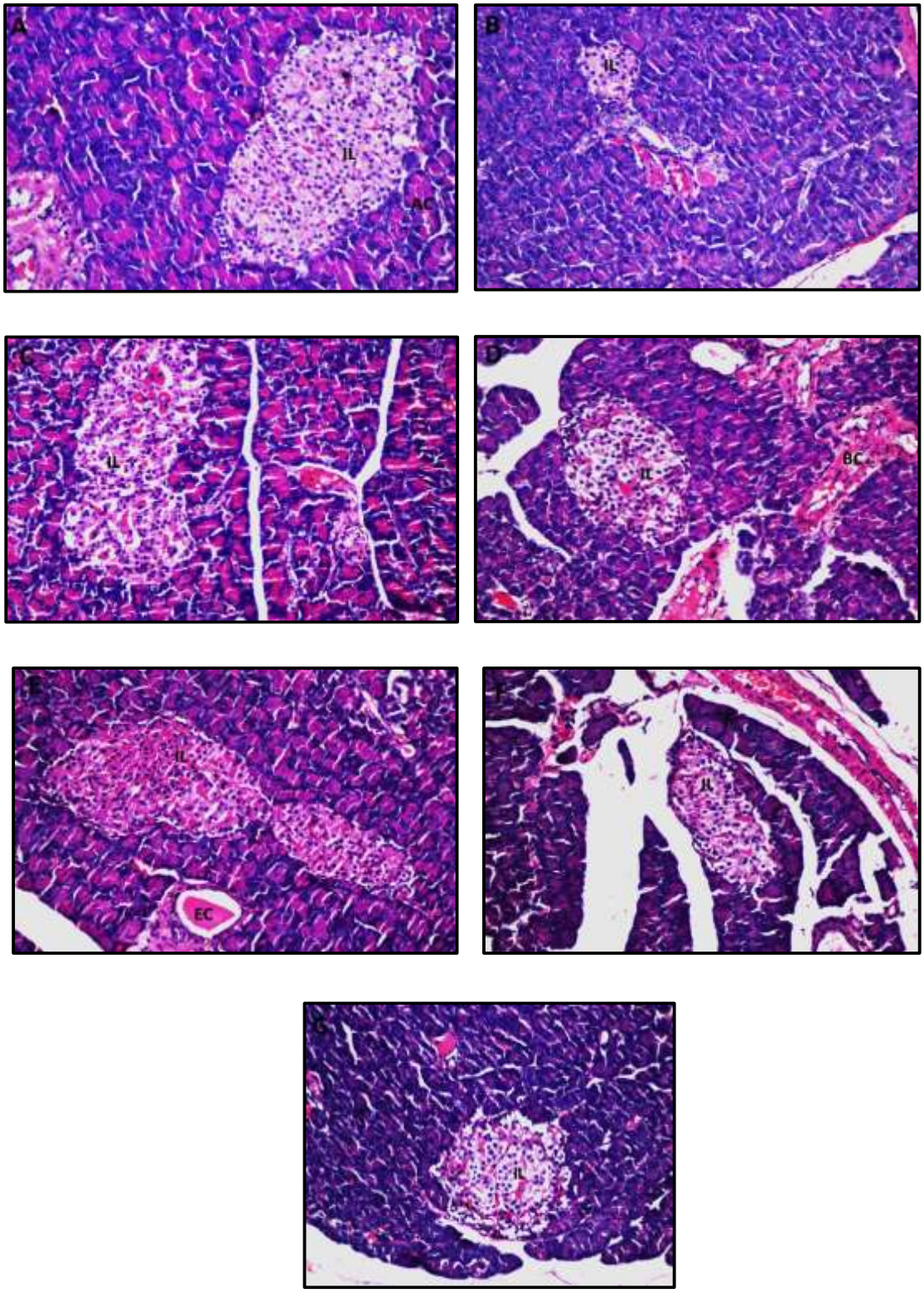

Fig. 6. Photomicrographs of pancreatic sections from a control rat (A) showing the normal histological structure and size of the islets of Langerhans (IL) formed of compact spherical masses of pale-staining cells surrounded by intact pancreatic acini (AC); a diabetic rat (B) with small size atrophied islets of Langerhans (IL); an MSC-injected diabetic rat (C) showing intact islets of Langerhans (IL) with normal size; pioglitazone-treated diabetic rats (D) with moderate size islets of Langerhans (IL) and congestion in the blood capillaries (BC); exendin-4-treated diabetic rats (E) showing normal histological structure of the islets of Langerhans (IL) associated with eosinophilic casts (EC) in the lumen of the ducts; MSC-injected pioglitazone-treated (F) or exendin-4-treated (G) diabetic rats with moderate sizes of the islets of Langerhans (IL) (hematoxylin and eosin stain, ×40).

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

Discussion

Insulin resistance and β-cell dysfunction are demonstrated in the current T2DM model using animals given a high-fat diet (HFD) and injected with streptozotocin (STZ). Obesity is related with decreased insulin sensitivity and an activated β-cell compensatory mechanism in the early stages of T2DM. This explains the elevated glucose and insulin levels in the current HFD/STZ-induced T2DM rat model. Furthermore, increasing glucose levels in T2DM enhance the creation of reactive oxygen species (ROS) and free radicals by glucose oxidation, non-enzymatic protein glycation, and increased lipid peroxidation, leading in a direct decrease in antioxidant enzymes activity. As a result, oxidative stress (OS) in tissues increases the disparity between ROS generation and the anti-oxidative protection system, resulting in a direct decrease in antioxidant enzymes activity. Consequently, hyperglycemia was related with increased ROS formation, lipid peroxidation, NO production, and apoptosis, as well as a decrease in total antioxidant capacity in diabetic adipose tissues. Furthermore, oxidative stress is linked to the etiology of T2DM and is a risk factor for insulin resistance due to disruption of insulin signals and adipocytokines modulation (23,24).

Obesity is associated with persistent, low-grade inflammation in adipose tissue, which aids in the progression of insulin resistance, a key cause of metabolic syndrome, including T2DM. Immune cells enter adipose tissue, promoting inflammatory responses and contributing to the pathogenesis of insulin resistance (25). Chronic release of proinflammatory mediators such as TNF-α and IL-6 from adipocytes and adipose tissue macrophages (ATM) induces insulin resistance by exerting a direct inhibitory effect on the insulin signaling pathway via down-regulating insulin receptor substrate (IRS-1) and AKT expressions, as well as inhibiting insulin-stimulated glucose uptake by adipose tissues through down-regulating glucose transporter type 4 (GLUT4) mRNA and protein expressions, and decreasing insulin-stimulated auto-phosphorylation of insulin receptors and phosphorylation of IRS-1. Furthermore, the activation of stress signaling pathways results in the production of a number of inflammatory mediators that are connected to insulin resistance via a variety of molecular processes (26,27).

It has been found that HFD intake in rats increases inflammation and activation of the transcription factor NF-κB, which is important in the TNF-α-induced upregulation of adipocytokines in adipocytes. Furthermore, ROS linked with hyperglycemia and advanced glycosylated end products exacerbated the signal transduction pathway for NF-κB activation, and enhanced expression of inducible nitric oxide synthase (iNOS) accelerated the generation of high levels of NO. In addition, activation of the NF-κB and JNK signaling pathways stimulates the production of inflammatory cytokine and chemokine genes, and both pathways are associated to insulin resistance in T2DM. Furthermore, hyperactivation of the NF-κB pathway has been reported in Zucker diabetic fatty rats' white adipose tissue. JNK1- and NF-κB-deficient animals are protected against HFD-induced insulin resistance because inflammatory cytokine production is inhibited. JNK promotes insulin resistance in obesity by inhibiting phosphorylation of insulin receptor substrates as well as stimulating metabolic inflammation. Exaggerated inflammation is established in the present HFD/STZ-induced T2DM rat model by a substantial increase in serum TNF-α, upregulation of NF-κB mRNA gene expression, and stimulation of JNK protein expression in adipose tissues (28-30).

Autophagy functions as a clearing mechanism, destroying large intracellular organelles or protein aggregates to modify cellular structure during differentiation or to provide critical nutrients in the event of an energy deficiency. When autophagy is initiated, a double-membraned vesicle called an autophagosome engulfs these components and combines with lysosomes to complete destruction. Multiple autophagy-related (ATG) proteins control the elongation and

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

shape of the autophagosome, including the mammalian homolog of yeast ATG8, LC3, which is commonly used as an autophagy marker because it is conjugated with the lipid phosphatidylethanolamine upon autophagy activation, resulting in the formation of microtubule associated protein light chain 3 (LC3-II). Beclin-1, the first discovered mammalian autophagy effector, is a mammalian homolog of yeast Atg6/Vps30. It is required for the formation of the beclin-1-interacting complex, which is required for the onset of autophagy. Many cellular functions, including inflammation, oxidative stress, and innate and acquired immunological response, have been demonstrated to be influenced by autophagy. Autophagy is known to regulate cytokines such as IL-1, IL-6, IL-18, and TNF-α (31,32).

Autophagy and oxidative stress play important roles in pancreatic β-cell dysfunction and the pathogenesis of T2DM. Defective β-cell function during lipid overload and T2DM is associated with lysosomal dysfunction and autophagy dysregulation. However, autophagy suppression is also known to induce inflammation in macrophages located in adipose tissue and has a significant impact on its inflammatory condition (31,33). We postulate that autophagy inhibition in the present HFD/STZ-induced T2DM rat model is a protective response against obesity-associated inflammation and insulin resistance in adipose tissues, as well as to reduce proinflammatory cytokines production.

Mesenchymal stem cells (MSCs) are multipotent adult stem cells capable of differentiating into any type of cell in damaged tissues, modulating their local environment, activating endogenous progenitor cells, and secreting a variety of substances. The molecular mechanism of action of bone marrow-derived MSCs (BM-MSCs) involves their ability to develop into insulin producing cells (IPCs), which is thought to be the major mechanism by which MSCs alleviate hyperglycemia in T2DM. MSCs migrate to endogenous damaged pancreatic β-cells and induce their regeneration by secreting various growth factors and cytokines with paracrine and autocrine activities, resulting in improved pancreatic islet function, increased blood insulin secretion, and hyperglycemia control. Furthermore, the immunosuppressive features of MSCs reduce the autoimmune mechanisms that trigger the destruction of pancreatic islet β-cells. Also, MSCs increase islet survival against oxidative stress and hypoxia conditions (34).

In the current investigation, we confirmed the homing of PKH26-labeled BM-MSCs in pancreatic tissues, where they participated in the healing process of injured pancreatic β-cells, resulting in increased viability and lower apoptosis. Furthermore, MSC treatment of HFD/STZ-induced T2DM rats reduced oxidative stress, apoptosis, and inflammation-mediated cellular injuries in the adipose tissues, as evidenced by reduced NO generation and lipid peroxidation, increased TAC, downregulation of caspase-3 and NF-κB gene expression, and JNK protein expression.

According to Si et al. (34), infusion of BM-MSCs in rats with HFD/STZ-induced T2DM alleviated hyperglycemia by activating the insulin receptor substrate (IRS)-1 signaling pathway, which resulted in increased translocation and expression of GLUT-4 and glucose uptake, resulting in BM-MSC-mediated improvement of insulin resistance in peripheral insulin target tissues. Hughey et al. (35) demonstrated that glucose uptake was increased in skeletal muscle and adipose tissues of MSC-treated mice with myocardial infarction, and that this increased glucose uptake was associated with improved insulin signaling as measured by Akt phosphorylation and GLUT-4 expression in these tissues. Furthermore, classically activated proinflammatory macrophages (M1) were reprogrammed into activated anti-inflammatory phenotype (M2) in the peripheral insulin target tissues of umbilical cord-derived MSCs-injected T2DM rats, along with the regulation of the expression of the NLRP3 inflammasome, which ultimately alleviated insulin resistance (6).

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

Several studies have found evidence that MSCs improve autophagy, making it an ideal strategy for T2DM treatment. Zhao et al. (36) found that the preventive effect of BM-MSCs on INS-1 cells was mediated by activation of autophagy via stimulation of autophagosome and autolysosome production. Following the infusion of BM-MSCs, autophagy was induced in the adipose tissue of HFD/STZ-induced T2DM rats in the current study, as evidenced by the elevation of the expression of certain autophagy markers, including beclin and LC3 genes.

The nuclear receptor peroxisome proliferator-activated receptor-gamma (PPARγ), an essential transcription factor in adipose tissue formation and function, participates in biological processes of adipocyte differentiation, insulin sensitivity, and inflammation. PPARγ is active during differentiation and stimulates the expression of downstream adipose-specific genes involved in adipose phenotype and glucose metabolism. PPARγ antagonists block the activation of NF-κB, a transcription factor that regulates the expression of several genes involved in immunological and inflammatory responses, lowering the production of pro-inflammatory cytokines and decreasing the inflamed condition of adipose tissue (37).

Thiazolidinediones (TZDs) have been used to treat hyperglycemia in T2DM patients since 1997. Pioglitazone (Pz), a thiazolidinedione drug and PPARγ agonist, reduces insulin resistance by binding to and activating PPARγ in adipose tissues, which promotes preadipocyte differentiation and increases the number of small adipocytes that are more sensitive to insulin, resulting in increased glucose uptake and improved insulin sensitivity in adipose tissues, as well as apoptosis of hypertrophic large fat cells. Also, pioglitazone reduces oxidative stress, inflammation, and apoptosis. Furthermore, TZDs stimulate PPARγ in macrophages, which is expected to lower macrophage populations in adipose tissue and suppress the production of the proinflammatory cytokines TNF-α and IL-6. IRS-2 expression, a protein that plays a facilitatory function in the insulin signaling pathway, is raised in adipose tissue cultured with PPARγ agonists, resulting in greater insulin sensitivity (38-40).

Glucagon-like peptide-1 (GLP-1), a gut incretin hormone secreted from L-cells of the small intestine in response to food intake, is known to have many glucose-lowering actions, including potentiating glucose-dependent insulin secretion, enhancing pancreatic β-cell proliferation, inhibiting pancreatic β-cell apoptosis, and decreasing glucagon production in response to food intake. Furthermore, GLP-1 enhances insulin sensitivity in T2DM patients and animal models (25,41). GLP-1 reduces macrophage infiltration and inflammatory cytokine production in ob/ob mouse adipose tissue. It inhibits inflammatory signaling pathways in adipocytes and ATM, leading in a reduction in inflammatory cytokine levels in adipose tissue (25). Exendin-4, a native GLP-1 receptor agonist with insulinotropic properties, has been shown to increase insulin-stimulated glucose uptake and thus insulin sensitivity and amplifies insulin signaling by inducing basal insulin receptor, IRS-1, and GLUT4 abundance in 3T3-L1 adipocytes. Exendin-4 has been shown to raise adiponectin levels and inhibit the generation of inflammatory adipokines in 3T3-L1 adipocytes (42). When mice are fed a high-fat diet, the expression of autophagy-related genes is decreased. Furthermore, mice fed a high-fat diet developed an autophagosome/lysosome fusion disorder (43). Similarly, autophagy markers, beclin and LC3, were decreased in rats in the present HFD/STZ-induced T2DM model. These markers, however, were significantly enhanced transcriptionally in diabetic rats given pioglitazone or exendin 4. Exendin-4 has been shown to enhance the rate of autophagosome and autophagolysosome production, as well as the autophagic flux, and to trigger critical autophagy protein makers at both the mRNA and protein levels (44).

PPAR gamma coactivator 1-alpha (PGC-1α) coactivates PPARα to control the expression of genes involved in the electron

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

transport chain, mitochondrial biogenesis, fatty acid oxidation, and glucose oxidative metabolism (45). In the current study, PGC-1α expression is downregulated in the adipose tissues of HFD/STZ-induced T2DM rats, which may be linked to decreased oxidative phosphorylation and mitochondrial dysfunction. By contrast, the different implicated treatment methods enhanced mitochondrial biogenesis, as evidenced by increased PGC-1α gene expression.

In conclusion, monotherapy treatment of HFD/STZ-induced T2DM rats with either BM-MSCs or anti-diabetic medications exhibited favorable anti-inflammatory, anti-oxidative, and anti-apoptotic potentials, as well as increased autophagy and suppressed insulin resistance. Furthermore, dual treatment with BM-MSCs and pioglitazone or exendin-4 was more effective than either treatment alone, indicating that the combined-therapy strategy had a synergistic effect in protecting against pancreatic damage and β-cell dysfunction, as well as alleviating insulin resistance and inflammation in adipose tissues.

Ethical Approval

The Local Ethical and Scientific Committee of Cairo University's Faculty of Pharmacy approved all experimental procedures (Registration Number 2485).

Acknowledgements

The authors would like to acknowledge the valuable histological comments provided by Dr Adel M. Bakeer, Professor of Pathology, Faculty of Veterinary Medicine, Cairo University, Egypt.

Funding

This research received no specific grant from any funding agency in the public, commercial, private, or not-for-profit sectors.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

References

1. Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, et al. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045. Diabetes Res Clin Pract. 2019;157:107843.
2. Joudaki R, Setorki M. The protective effect of Satureja bachtiarica hydroalcoholic extract on streptozotocin-induced diabetes through modulating glucose transporter 2 and 4 expression and inhibiting oxidative stress. Pharm Biol. 2019;57(1):318-327.
3. Chen C, Cohrs CM, Stertmann J, Bozsak R, Speier S. Human beta cell mass and function in diabetes: Recent advances in knowledge and technologies to understand disease pathogenesis. Mol Metab. 2017;6(9):943-957.
4. Ota T. Chemokine systems link obesity to insulin resistance. Diabetes Metab J. 2013;37(3):165-72.
5. Caballero AE. Long-term studies of treatments for type 2 diabetes. Postgrad Med. 2017;129(3):352-365.
6. Zang L, Hao H, Liu J, Li Y, Han W, Mu Y. Mesenchymal stem cell therapy in type 2 diabetes mellitus. Diabetol Metab Syndr. 2017;9:36.
7. Yang JS, Lu CC, Kuo SC, Hsu YM, Tsai SC, Chen SY, et al. Autophagy and its link to type II diabetes mellitus. Biomedicine (Taipei). 2017;7(2):8.
8. Demirtas L, Guclu A, Erdur FM, Akbas EM, Ozcicek A, Onk D, Turkmen K. Apoptosis, autophagy & endoplasmic reticulum stress in diabetes mellitus. Indian J Med Res. 2016;144(4):515-524.
9. Thulesen J, Orskov C, Holst JJ, Poulsen SS. Short-term insulin treatment prevents the diabetogenic action of streptozotocin in rats. Endocrinology. 1997;138(1):62-8.
10. Ahire YS, Ghaisas MM, Dandawate P. Beneficial effects of co-administration of PPAR-γ agonist with melatonin on cardiovascular complications associated with diabetes. Young Sci. 2013;4(1): 59-68.
11. Wang A, Li T, An P, Yan W, Zheng H, Wang

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

B, Mu Y. Exendin-4 Upregulates Adiponectin Level in Adipocytes via Sirt1/Foxo-1 Signaling Pathway. PLoS One. 2017;12(1):e0169469.
12. Ghelani H, Razmovski-Naumovski V, Nammi S. Chronic treatment of (R)-α-lipoic acid reduces blood glucose and lipid levels in high-fat diet and low-dose streptozotocin-induced metabolic syndrome and type 2 diabetes in Sprague-Dawley rats. Pharmacol Res Perspect. 2017;5(3):e00306.
13. Duan B, Zhao Z, Lin L, Jin J, Zhang L, et al. Antidiabetic effect of Tibetan medicine tang-kang-fu-san on high-fat diet and streptozotocin-induced type 2 diabetic rats. Evid Based Complement Alternat Med. 2017;2017:7302965.
14. Alhadlaq A, Mao JJ. Mesenchymal stem cells: isolation and therapeutics. Stem Cells Dev. 2004;13(4):436-48.
15. Abdel Aziz MT, Wassef MA, Ahmed HH, Rashed L, Mahfouz S, et al. The role of bone marrow derived-mesenchymal stem cells in attenuation of kidney function in rats with diabetic nephropathy. Diabetol Metab Syndr. 2014;6(1):34.
16. Trinder P. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J Clin Pathol. 1969;22(2):158-61.
17. Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide. 2001;5(1):62-71.
18. Yoshioka T, Kawada K, Shimada T, Mori M. Lipid peroxidation in maternal and cord blood and protective mechanism against activated-oxygen toxicity in the blood. Am J Obstet Gynecol. 1979;135(3):372-6.
19. Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V. Method for the measurement of antioxidant activity in human fluids. J Clin Pathol. 2001;54(5):356-61.
20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
22. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A. 1979;76(9):4350-4.
23. Mercuri F, Quagliaro L, Ceriello A. Oxidative stress evaluation in diabetes. Diabetes Technol Ther. 2000;2(4):589-600.
24. Tangvarasittichai S. Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. World J Diabetes. 2015;6(3):456-80.
25. Lee YS, Park MS, Choung JS, Kim SS, Oh HH, Choi CS, et al. Glucagon-like peptide-1 inhibits adipose tissue macrophage infiltration and inflammation in an obese mouse model of diabetes. Diabetologia. 2012;55(9):2456-68.
26. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am J Physiol Endocrinol Metab. 2001;280(5):E745-51.
27. Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. A central role for JNK in obesity and insulin resistance. Nature. 2002;420(6913):333-6.
28. Tuzcu M, Sahin N, Orhan C, Agca CA, Akdemir F, Tuzcu Z, et al. Impact of chromium histidinate on high fat diet induced obesity in rats. Nutr Metab (Lond). 2011;8:28.
29. Rodríguez-Calvo R, Serrano L, Coll T, Moullan N, Sánchez RM, Merlos M, et al. Activation of peroxisome proliferator-activated receptor beta/delta inhibits lipopolysaccharide-induced cytokine production in adipocytes by lowering nuclear factor-kappaB activity via extracellular signal-related kinase 1/2. Diabetes. 2008;57(8):2149-57.
30. Solinas G, Becattini B. JNK at the crossroad of obesity, insulin resistance, and cell stress response. Mol Metab. 2016;6(2):174-184.
31. Jansen HJ, van Essen P, Koenen T, Joosten LA, Netea MG, Tack CJ, Stienstra R. Autophagy activity is up-regulated in adipose tissue of obese individuals and modulates proinflammatory cytokine expression. Endocrinology. 2012;153(12):5866-74.
32. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature. 2011;469(7330):323-35.

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]

33. Wang Y, Li YB, Yin JJ, Wang Y, Zhu LB, Xie GY, Pan SH. Autophagy regulates inflammation following oxidative injury in diabetes. Autophagy. 2013;9(3):272-7.
34. Si Y, Zhao Y, Hao H, Liu J, Guo Y, Mu Y, et al. Infusion of mesenchymal stem cells ameliorates hyperglycemia in type 2 diabetic rats: identification of a novel role in improving insulin sensitivity. Diabetes. 2012;61(6):1616-25.
35. Hughey CC, Ma L, James FD, Bracy DP, Wang Z, Wasserman DH, et al. Mesenchymal stem cell transplantation for the infarcted heart: therapeutic potential for insulin resistance beyond the heart. Cardiovasc Diabetol. 2013;12:128.
36. Zhao K, Hao H, Liu J, Tong C, Cheng Y, Xie Z, et al. Bone marrow-derived mesenchymal stem cells ameliorate chronic high glucose-induced β-cell injury through modulation of autophagy. Cell Death Dis. 2015;6(9):e1885.
37. Tontonoz P, Spiegelman BM. Fat and beyond: the diverse biology of PPARgamma. Annu Rev Biochem. 2008;77:289-312.
38. Yki-Järvinen H. Thiazolidinediones. N Engl J Med. 2004;351(11):1106-18.
39. Mahmoudi A, Ghatreh Samani K, Amini SA, Heidarian E. Effects of Pioglitazone on the Lipid Profile, Serum Antioxidant Capacity, and *UCP1* Gene Expression in Mouse Brown Adipose Tissue. Rep Biochem Mol Biol. 2019;8(1):15-20.
40. Smith U, Gogg S, Johansson A, Olausson T, Rotter V, Svalstedt B. Thiazolidinediones (PPARgamma agonists) but not PPARalpha agonists increase IRS-2 gene expression in 3T3-L1 and human adipocytes. FASEB J. 2001;15(1):215-220.
41. Zander M, Madsbad S, Madsen JL, Holst JJ. Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. Lancet. 2002;359(9309):824-30.
42. Kim Chung le T, Hosaka T, Yoshida M, Harada N, Sakaue H, Sakai T, Nakaya Y. Exendin-4, a GLP-1 receptor agonist, directly induces adiponectin expression through protein kinase A pathway and prevents inflammatory adipokine expression. Biochem Biophys Res Commun. 2009;390(3):613-8.
43. Liu HY, Han J, Cao SY, Hong T, Zhuo D, Shi J, et al. Hepatic autophagy is suppressed in the presence of insulin resistance and hyperinsulinemia: inhibition of FoxO1-dependent expression of key autophagy genes by insulin. J Biol Chem. 2009;284(45):31484-92.
44. Sharma S, Mells JE, Fu PP, Saxena NK, Anania FA. GLP-1 analogs reduce hepatocyte steatosis and improve survival by enhancing the unfolded protein response and promoting macroautophagy. PLoS One. 2011;6(9):e25269.
45. Mostafa Tork O, Ahmed Rashed L, Bakr Sadek N, Abdel-Tawab MS. Targeting Altered Mitochondrial Biogenesis in the Brain of Diabetic Rats: Potential Effect of Pioglitazone and Exendin-4. Rep Biochem Mol Biol. 2019;8(3):287-300.

[Downloaded from rbmb.net on 2024-10-12]

[DOI: 10.52547/rbmb.12.1.42]