



Original Article

Mesenchymal stem cell conditioned medium ameliorates diabetic serum-induced insulin resistance in 3T3-L1 cells

Avinash Sanap^{a,d}, Ramesh Bhonde^b, Kalpana Joshi^{c,*}

^a Interdisciplinary School of Health Sciences, Savitribai Phule Pune University, Pune 411007, India

^b Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune 411018, India

^c Department of Biotechnology, Sinhgad College of Engineering affiliated to Savitribai Phule Pune University, Pune 411041, India

^d Regenerative Medicine Laboratory, Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune 411018, India

Received 3 April 2020

Available online 22 October 2020

Abstract

Background: Pharmacological factors used to induce insulin resistance (IR) in *in vitro* models may not mimic the full *in vivo* features of type 2 diabetes mellitus (T2DM). This study aimed to examine the ability of diabetic serum (DS) to induce IR and investigate whether adipose-derived mesenchymal stem cell conditioned medium (ADMSC-CM) reverses DS-induced IR.

Methods: DS was obtained from newly diagnosed T2DM patients. IR was induced in differentiated 3T3-L1 cells by employing dexamethasone, tumor necrosis factor alpha (TNF- α), palmitate and DS. Glucose uptake (2-[N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl] amino]-2-deoxyglucose(2-NBDG) uptake assay), intracellular levels of reactive oxygen species (ROS), and superoxide radicals (O²⁻) (fluorescence microscopy and fluorometry) were analyzed in control and experimental samples. mRNA expression of key genes involved in glucose transport and inflammation were analyzed by using reverse transcription polymerase chain reaction (RT-PCR). Pro-inflammatory cytokines and phospho-insulin receptor substrate (IRS) (Ser-307) protein expression were analyzed by fluorescence activated cell sorter analysis. Statistical significance was determined by using one-way ANOVA followed by Tukey's multiple comparison tests.

Results: ADMSC-CM significantly increased the DS-mediated decrease in 2-NBDG uptake (11.01 \pm 0.50 vs. 7.20 \pm 0.30, $P < 0.01$) and reduced DS-driven ROS (fluorescence count, 6.35 \pm 0.46 vs. 9.80 \pm 0.10, $P < 0.01$) and O²⁻ (fluorescence count, 3.00 \pm 0.10 vs. 4.60 \pm 0.09, $P < 0.01$) production. Further, the ADMSC-CM restored DS-induced down regulation GLUT4 (1.52-fold, $P < 0.05$) as well as the up-regulation of PPAR γ (0.35-fold, $P < 0.01$), and IKK β (0.37-fold, $P < 0.01$) mRNA, and phospho-IRS (Ser-307) protein expression compared to the baseline (median fluorescence intensity, 88,192 \pm 2720 vs. 65,450 \pm 3111, $P < 0.01$). DS induced IR, similar to the traditionally used pharmacological factors, namely dexamethasone, TNF- α , and palmitate, which can be attributed to the significantly higher pro-inflammatory cytokines levels (TNF- α (2.28 \pm 0.03 pg/mL vs. 2.38 \pm 0.03 pg/mL, $P < 0.01$), interleukin 6 (IL)-6 (1.94 \pm 0.02 pg/mL vs. 2.17 \pm 0.04 pg/mL, $P < 0.01$), IL-17 (2.16 \pm 0.02 pg/mL vs. 2.22 \pm 0.002 pg/mL, $P < 0.05$), and interferon gamma (IFN- γ) (2.07 \pm 0.02 pg/mL vs. 2.15 \pm 0.04 pg/mL, $P < 0.05$)) in DS.

* Corresponding author. Department of Biotechnology, Sinhgad College of Engineering, Vadgaon (Bk), Savitribai Phule Pune University, Pune 411041, India.

E-mail address: kalpanajoshi1788@gmail.com (K. Joshi).

Peer review under responsibility of Chinese Medical Association.



Production and Hosting by Elsevier on behalf of KeAi

Conclusions: DS can be explored as a novel inducer of IR in *in vitro* studies with further standardization, substituting the conventionally used pharmacological factors. Our findings also affirm the validity of ADMSC-CM as a prospective insulin sensitizer for T2DM therapy.

Copyright © 2020 Chinese Medical Association. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Keywords: Insulin resistance; Inflammation; Mesenchymal stem cells; Oxidative stress; Type 2 diabetes mellitus

Introduction

The incidence of type 2 diabetes mellitus (T2DM) is accelerating at an alarming rate and is expected to reach 640 million people worldwide in 2040, warranting new therapeutic approaches.^{1,2} Insulin resistance (IR) develops at an early stage of T2DM and is defined by loss of insulin sensitivity, resulting in deranged glucose uptake from blood.^{3,4} Among its multiple etiological factors, inflammation and oxidative stress are believed to be key events promoting IR. The expression of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6), and interferon gamma (IFN- γ), is increased in adipose tissue, which is associated with systemic inflammation and contributes to IR. I κ B kinase β (IKK- β) is a central coordinator of inflammatory responses through the activation of nuclear factor κ B (NF- κ B),⁵ whereas mitochondrial dysfunction arises as a result of abnormal generation of reactive oxygen species (ROS) and superoxide radicals (O²⁻).^{3,6,7} These factors activate serine kinases, which promote inhibitory phosphorylation of serine residues at insulin receptor substrate 1 (IRS-1) interfering with normal insulin signaling.⁸

Recent evidence in clinical trials suggests that management of IR through mesenchymal stem cell (MSC)-based therapies prevents progression of T2DM and associated secondary complications.⁹ *In vitro* studies on adipose-derived MSC conditioned medium (ADMSC-CM) have shown its potential to reverse TNF α -, IL-6- and palmitate-induced IR in 3T3-L1 cells.¹⁰ However, the partial understanding of the cellular and molecular targets of ADMSC-CM limits its full potential and development as a therapeutic agent for T2DM.¹¹

To explore new therapeutic targets *in vitro*, 3T3-L1 cells are believed to be a good *in vitro* model.¹² Several pharmacological agents and factors are known to induce IR in differentiated 3T3-L1 cells (TNF α ,¹³ IL-1,¹⁴ IL-6,¹⁵ free fatty acids,¹⁶ dexamethasone,¹⁷ high insulin,¹² glucosamine,¹⁸ growth hormone,¹⁹ and

hypoxia²⁰), however, the ability of these pharmacological agents or their combinations to activate the molecular pathways involved in the development of IR is limited. It is unclear whether each of these pharmacological inducers or their combination would be able to mimic the complete *in vivo* features of IR.²¹

Diabetic serum (DS) is composed of cellular metabolites, hormones, pro-inflammatory cytokines, fatty acids, steroids, and represents the diabetic environment. Since, DS mimics the *in vivo* diabetic environment; it could trigger most of the molecular pathways involved in the pathogenesis of IR. Also, the use of DS is economical as compared to the routinely used artificial pharmacological inducers, which are expensive and impose solubility issues.

In the present study, we examined the ability of DS to induce IR and the potential of ADMSC-CM to reverse DS-induced IR in 3T3-L1 cells.

Methods

Ethical approval

This study involving human blood serum and isolation of ADMSC was approved by the Institutional Ethics Committee of Sinhgad College of Engineering, Pune (India). Informed consent was obtained from the participants before blood sample collection.

ADMSC culture and characterization

ADMSCs were obtained from the School of Regenerative Medicine, Bengaluru (India). Cells were isolated from the subcutaneous depots of healthy volunteers undergoing cosmetic surgeries. Passage 4 cells were characterized for the expression of CD90, CD73, CD105 (1:1000 dilution, PE Tagged), CD45, CD34, and HLA-DR (1:1000 dilution, FITC Tagged) (BD Biosciences, CA, USA) as described in our previous study.¹⁰ Cells were further characterized for their chondrogenic (Stempro-A1007101, Gibco, MD, USA), adipogenic (Stempro-A1007001, Gibco, MD, USA),

and osteogenic (Stempro-A1007201, Gibco, MD, USA) potential as shown earlier.²²

The conditioned medium was prepared from ADMSCs as described earlier.¹⁰ ADMSCs were seeded in 35 mm cell culture plates at the density of 1×10^6 cells per well. Once cells were 80%–85% confluent, fresh α -MEM medium (32561037, Gibco, MD, USA) (without serum) was added and incubated for 48 h at 37 °C & 5% CO₂. After incubation, the culture medium was collected and filtered through 0.22 μ mol/L filters. ADMSC-CM was stored at –80 °C until further use. It was diluted to 50% concentration with α -MEM (without serum) and used for all the experiments.

Blood collection

Fasting blood samples were collected from newly diagnosed diabetic volunteers (HbA1c >9%) and healthy controls (non-diabetic, (HbA1c <5%)). To avoid the interference of diabetic medications with the *in vitro* assays, we collected samples from the study participants who had not received any diabetic medications. Collected blood samples were allowed to clot at 25–30 °C and centrifuged at 1800 \times g for 10 min. Serum was separated and filtered through 0.22 μ mol/L syringe filters and stored at –80 °C until further use.

3T3-L1 cell culture and differentiation

3T3-L1 cells were obtained from National Center for Cell Sciences, (Pune, India). Cells were cultured in Dulbecco's Modified Eagle Medium (11965092, Gibco, MD, USA) containing 10% fetal bovine serum (10500064, Gibco, MD, USA) and 1% antimycotic-antibiotic solution (15240062, Gibco, MD, USA). For differentiation, 90%–95% confluent cells were treated with differentiation medium [1 μ mol/L dexamethasone (CAS 50-02-2, Merck, NJ, USA), 500 μ mol/L IBMX (CAS 28822-58-4, Merck, NJ, USA), and 100 nmol/L insulin (41400045, Gibco, MD, USA)] for 48 h. Later, cells were treated with maintenance medium containing 100 nmol/L insulin from day two to seven as described previously.¹⁰

Pro-inflammatory cytokine analysis

Normal serum and DS were subjected to cytokine analysis by using the MACS Plex Cytokine 12 kit (Miltenyi Biotec, NJ, USA) on fluorescence activated cell sorter as per the manufacturer's instructions. Serum samples were mixed with MACS Plex Cytokine Capture

Beads and incubated for 2 h on a shaker in the dark. After incubation, the pellet of beads was obtained by centrifugation. The pellet was washed with 200 μ L MACS Plex buffer twice followed by incubation with MACS Plex Cytokine 12 detection reagent for 1 h on a shaker in the dark. Samples were centrifuged, and the pellet was suspended in 200 μ L MACS Plex buffer, which was acquired on Attune NxT acoustic focusing cytometer (Thermo Fisher). Results obtained from the cytometer were converted into pg/mL after correlating with standards. Cytokine levels were normalized to the mg of protein/well.

Glucose uptake assay

On day 7, 3T3-L1 cells were treated with (a) vehicle (Control), (b) non-diabetic serum (N-DS), (c) DTP [1 μ mol/L Dexamethasone, 1 ng/mL TNF-alpha (Sigma, NY, USA) and 500 μ mol/L Palmitate(Sigma, NY, USA)], (d) 1 μ mol/L DTP + 1.5 mmol/L Metformin(Sigma, NY, USA), (e) 5% non-diabetic serum (f) 5% DS, (g) 5% DS + 50% ADMSC-CM for 48 h. To estimate glucose uptake, cells were glucose starved for 2 h in krebs ringer phosphate buffer containing 0.5% bovine serum albumin (Gibco) at 37 °C. Cells were washed with phosphate-buffered saline (PBS) and incubated with 100 nmol/L insulin for 15 min. Then, cells were washed with PBS and 2-[N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl] amino]-2-deoxyglucose (2-NBDG) dye(Molecular Probes, Life Technologies, CA, USA) was added at a concentration of 50 mmol/L for 20 min. After the PBS wash, fluorescence was measured at a wavelength of 460/540 nm. 2-NBDG uptake was normalized to the mg of protein present per well to reduce the variation caused by cell seeding density and cell death during the assay.

Estimation of intracellular ROS and O²⁻ radicals

3T3-L1 cells were treated with (a) vehicle (Control), (b) DTP, (c) 5% DS, and (d) 5% DS + 50% ADMSC-CM for 48 h as described above. Further, the levels of ROS and O²⁻ were estimated by using Enzolive ROS-ID® ROS/RNS Detection Kit (Enzo Life Sciences, NY, USA) as per the manufacturer's instructions. The images were captured under a fluorescence microscope (Nikon Eclipse TE 200-S, Chiyoda-Ku, Japan). Fluorometric measurements of ROS (Ex/Em: 490/525 nm) and O²⁻ (Ex/Em: 550/620 nm) radicals were also performed. Fluorescence intensity was normalized to the mg of protein present per well.

Gene expression studies (Reverse transcription polymerase chain reaction)

As shown in “*Glucose uptake assay*”, 3T3-L1 cells were treated with (a) vehicle (Control), (b) DTP, (c) 1 $\mu\text{mol/L}$ DTP + 1.5 mmol/L Metformin, (d) 5% DS & (e) 5% DS + 50% ADMSC-CM for 48 h. RNA was isolated using the Trizol (Gibco, MD, USA) method as described previously.²² Using High-capacity cDNA reverse transcription (Thermo Fisher Scientific, CA, USA), total RNA was reverse transcribed into complementary DNA (cDNA). RT-PCR reaction conditions and primer sequences for β -Actin (internal control), GLUT4, PPAR γ , and IKK β are shown in Table 1. RT-PCR was performed on the ABI Step One Plus system using SYBR Green chemistry PCR (Applied Biosystems). All experiments were performed in triplicates. Relative expression was calculated using the $\Delta\Delta\text{Ct}$ method.

Flow cytometric analysis of IRS1-307 expression

To assess the expression of IRS1-307, differentiated 3T3-L1 cells were treated with (a) vehicle (Control), (b) 5% DS, (c) 5% DS + 50% ADMSC-CM for 48 h. Cells were fixed, permeabilized in FIX/PERM buffer (Bio legend, CA, USA), and stained with IRS-307 antibody (Invitrogen- PA1-1054, MD, USA) followed by FITC conjugated secondary antibody. Flow cytometric analysis was performed on Attune NxT acoustic focusing cytometer (Thermo Fisher) and data were analyzed using the Attune NxT software. Results were expressed as median fluorescence intensity (MFI).

Statistical analysis

SPSS software (IBM, NY, USA) version 16.0 was used to perform statistical analysis. All values are expressed as mean \pm standard deviation (SD). One-way ANOVA followed by Tukey's multiple comparison tests were used to evaluate differences between means, and $P < 0.05$ were considered as statistically significant.

Results

ADMSC characterization

Passage 4 cells subjected to MSC characterization were found to be positive for CD90 (98.78%), CD73 (95.86%), CD (92.62%), and negative for CD45 (1.61%), CD34 (2.15%), HLA-DR (1.06%) as depicted in Fig. 1A. The tri-lineage potential of AD-MSCs was also confirmed as presented in Fig. 1B.

Levels of TNF- α , IL-6, IL-12, IL-17, and IFN- γ in normal serum and DS

Inflammation plays a pivotal role in the development of IR.⁵ To confirm the pro-inflammatory status of DS, we performed cytokine analysis (TNF- α , IL-6, IL-12, IL-17, and IFN- γ) in normal serum and DS. We observed a significant increase in the levels of TNF- α (2.28 ± 0.03 pg/mL vs. 2.38 ± 0.03 pg/mL, $P < 0.01$), IL-6 (1.94 ± 0.02 pg/mL vs. 2.17 ± 0.04 pg/mL, $P < 0.01$), IL-17 (2.16 ± 0.02 pg/mL vs. 2.22 ± 0.002 pg/mL, $P < 0.05$), and IFN- γ (2.07 ± 0.02 pg/mL vs. 2.15 ± 0.04 pg/mL, $P < 0.05$) in DS as compared to that in N-DS as depicted in Fig. 2.

Restoration of DS-induced decrease in 2-NBDG uptake by ADMSC-CM

3T3-L1 cells are commonly used to study IR in adipocytes²¹ and dexamethasone, TNF- α , and palmitate (DTP) are known inducers of IR in 3T3-L1 cells.^{13,16,17} As depicted in Fig. 3, treatment with these factors significantly reduced glucose uptake (9.33 ± 0.73 vs. 6.56 ± 0.21 , $P < 0.01$), while treatment with metformin significantly reversed DTP-induced decrease in glucose uptake (6.56 ± 0.21 vs. 11.18 ± 0.20 , $P < 0.01$). Interestingly, we found that treatment with 5% DS significantly reduced glucose uptake similar to the treatment with DTP (9.33 ± 0.73 vs. 7.20 ± 0.30 , $P < 0.01$). Treatment with ADMSC-CM significantly reversed DS-induced reduction in glucose uptake (11.01 ± 0.50 vs. 7.20 ± 0.30 ,

Table 1

Primers used in examining the ability of DS to induce IR and the potential of ADMSC-CM.

Gene	Forward Primer	Reverse Primer	Temperature ($^{\circ}\text{C}$)
ACTB	TGGTGGGAATGGGTCAGAA	TCTGGGTCATCTTTCACGG	60
GLUT4	TTCCTGGTTCATTGTGGC	AAGATGAAGAAGCCAAGCAGG	60
PPAR γ	AGACCACTCGCATTCCTTTG	ATCGCACTTTGGTATTCTTGG	60
IKK- β	AGCCAAGAGACCAAAGGACAG	TCCTTCTGCCGCTTCTCC	58

DS: diabetic serum; IR: insulin resistance; ADMSC-CM: adipose-derived mesenchymal stem cell conditioned medium.

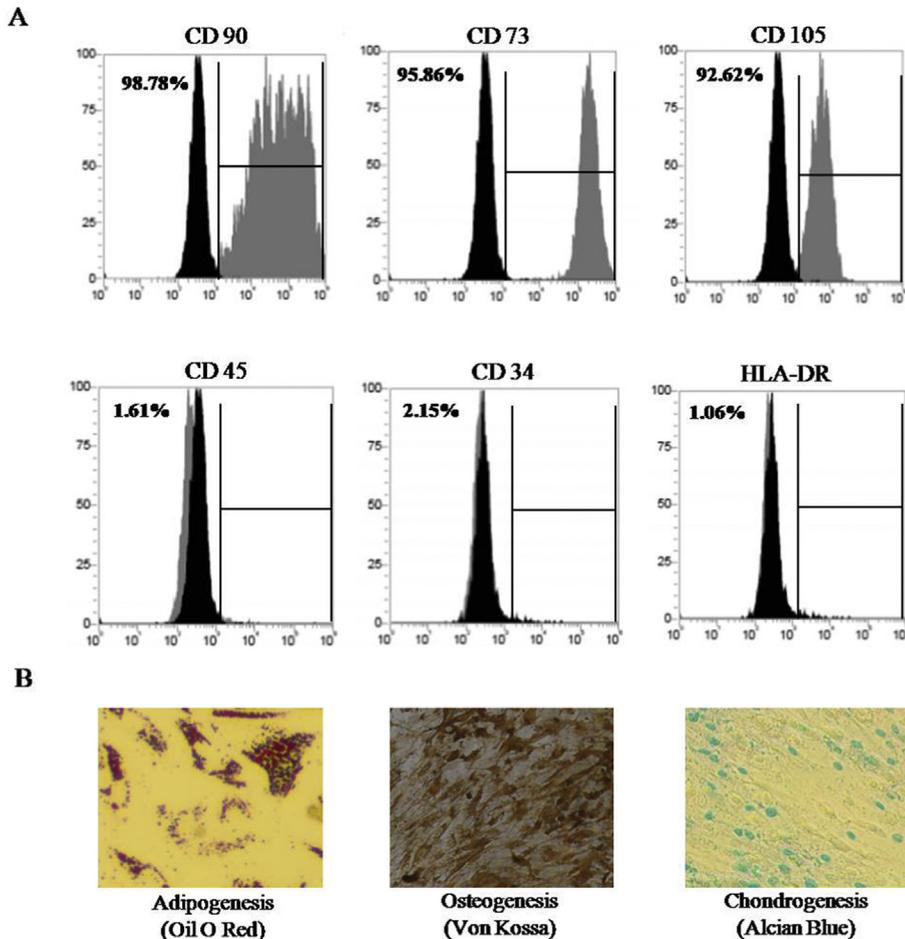


Fig. 1. Characterization of adipose derived mesenchymal stem cells (ADMSC): (A) ADMSC were found to be positive for CD90 (98.78%), CD73 (95.86%), CD105 (92.62%) and negative for CD45 (1.61%), CD34 (2.15%), HLA-DR (1.06%). (B) Tri-lineage potential of ADMSC (Adipogenesis, Oil O Red Staining, original magnification 10 × ; Osteogenesis, Von Kossa Staining, original magnification 4 × ; Chondrogenesis, Alcian Blue Staining, original magnification 4 ×).

$P < 0.01$). The N-DS did not result in a significant change in 2-NBDG uptake.

Protective effect of ADMSC-CM on DS-induced ROS and O^{2-} generation

Intracellular generation of ROS and O^{2-} cause damage to tissue contributing to the development of IR.⁷ We observed that treatment of differentiated 3T3-L1 cells with DS significantly enhanced the production of ROS (Green fluorescence) (fluorescence count, 7.50 ± 0.18 vs. 9.80 ± 0.10 , $P < 0.01$) and O^{2-} (Orange fluorescence) (fluorescence count, 3.83 ± 0.14 vs. 4.60 ± 0.09 , $P < 0.05$), which were comparable to the DTP-induced production of ROS and O^{2-} as depicted in Fig. 4A. Interestingly, ADMSC-CM treatment

significantly reduced DS-induced production of ROS (fluorescence count, 6.35 ± 0.46 vs. 9.80 ± 0.10 , $P < 0.01$) and O^{2-} (fluorescence count, 3.00 ± 0.10 vs. 4.60 ± 0.09 , $P < 0.01$). Microscopic findings were also validated by fluorometric measurements, which showed similar trends as depicted in Fig. 4B.

Restoration of the DS-induced gene expression by the treatment of ADMSC-CM

Impaired GLUT4, PPAR γ , and IKK β mRNA expression is linked to the abnormal insulin signal transduction, adipogenesis, and inflammation in 3T3-L1 cells respectively.^{10,11,23} As depicted in Fig. 5A, treatment with DTP significantly reduced the expression of GLUT4 expression (0.30-fold, $P < 0.01$)

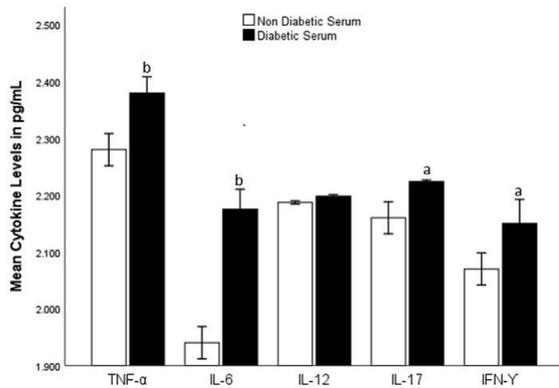


Fig. 2. Cytokine analysis (Tumour Necrosis Factor alpha (TNF- α), Interleukin (IL)-6, IL-12, IL-17 and Interferon gamma (IFN- γ)) by using flow cytometry: Pro-inflammatory cytokines such as TNF- α , IL-6, IL-17, and IFN- γ were significantly higher in diabetic serum (DS) as compared to the non-diabetic serum (N-DS). All the experiments were performed in triplicates (^a $P < 0.05$, ^b $P < 0.01$). Cytokine levels are expressed in pg/mL. Cytokine levels were normalized to the protein present per well.

whereas metformin significantly reversed the effect of DTP (2.00-fold, $P < 0.05$). Also, DTP treatment up-regulated PPAR γ (1.40-fold, $P < 0.05$) and IKK β expression (1.43-fold, $P < 0.05$) whereas metformin reversed the DTP-induced increase in PPAR γ (0.50-

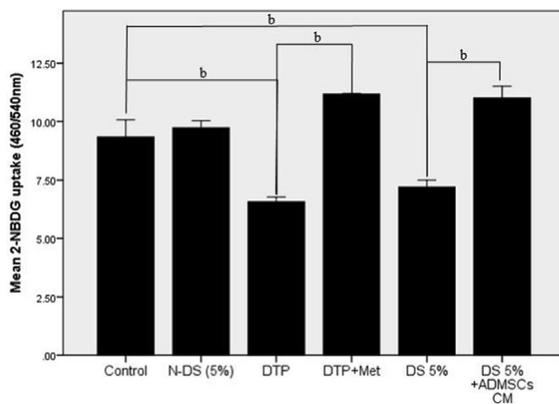


Fig. 3. 2-NBDG uptake in 3T3-L1 cells: Treatment of dexamethasone, TNF- α , and palmitate (DTP) significantly lowered 2-NBDG uptake ($P < 0.01$), where treatment of metformin rescue DTP induced lowering of 2-NBDG uptake ($P < 0.01$). 5% diabetic serum (DS) significantly reduced the 2-NBDG uptake where adipose derived mesenchymal stem cell conditioned medium (ADMSC-CM) significantly improved DS induced lowering of 2-NBDG uptake ($P < 0.01$). Treatment of non-diabetic serum (N-DS) did not affect 2-NBDG uptake significantly. 2-NBDG uptake was normalized to the mg of protein/well. All the experiments were performed in triplicates (^a $P < 0.05$, ^b $P < 0.01$). Data are shown as mean \pm SD.

fold, $P < 0.01$) and IKK β expression (0.53-fold, $P < 0.01$).

Interestingly, DS significantly down regulated GLUT4 expression (3.90-fold, $P < 0.01$) and ADMSC-CM significantly reversed DS-mediated decrease in GLUT4 expression (1.61-fold, $P < 0.05$). DS treatment upregulated expression of PPAR γ (1.41-fold, $P < 0.01$) and IKK β (1.37-fold, $P < 0.01$) whereas ADMSC-CM reversed DS-induced increase in PPAR γ (0.37-fold, $P < 0.01$) and IKK β expression (0.35-fold, $P < 0.01$) as shown in Fig. 5A.

ADMSC-CM decreased DS-induced increase in phosphor Ser (307) expression

Abnormal phosphorylation of IRS- at serine 307 negates normal insulin signaling which promotes IR.²⁴ We observed that DS treatment significantly up-regulated MFI of phospho-IRS (307) protein (75,617 \pm 2747 vs. 88,192 \pm 2720, $P < 0.05$). Interestingly, ADMSC-CM treatment reversed DS-associated increase in MFI of phospho-IRS (307) (median fluorescence intensity, 88,192 \pm 2720 vs. 65,450 \pm 3111, $P < 0.01$) as shown in Fig. 5B.

Discussion

IR is the forerunner of T2DM and associated secondary complications.⁶ Recent studies have shown that the prevention of IR, which halts the consequent onset of diabetes is the best strategy for future T2DM therapies.¹ Several *in vitro* models have been identified and thoroughly investigated to study novel therapeutic targets to reverse IR.²³ 3T3-L1 cells have been extensively studied for identifying key molecular pathways and transcription factors for the screening of novel therapeutic agents. To induce IR in 3T3-L1 cells, numerous pharmacological inducers have been employed.²¹ As these factors are used alone or in combination, the extent to which these models mimic the *in vivo* conditions of IR remains doubtful.²¹

In this study, we investigated a novel strategy that includes the use of DS as an inducer of IR in 3T3-L1 cells. Our data unequivocally demonstrated that 5% DS significantly reduced 2-NBDG uptake, which was comparable to the IR induced by the combination of dexamethasone, TNF- α , and palmitate. GLUT4 gene expression is influenced by the metabolic, nutritional, or hormonal status of adipocytes.²⁵ In the state of IR, expression of GLUT4 is down regulated negating

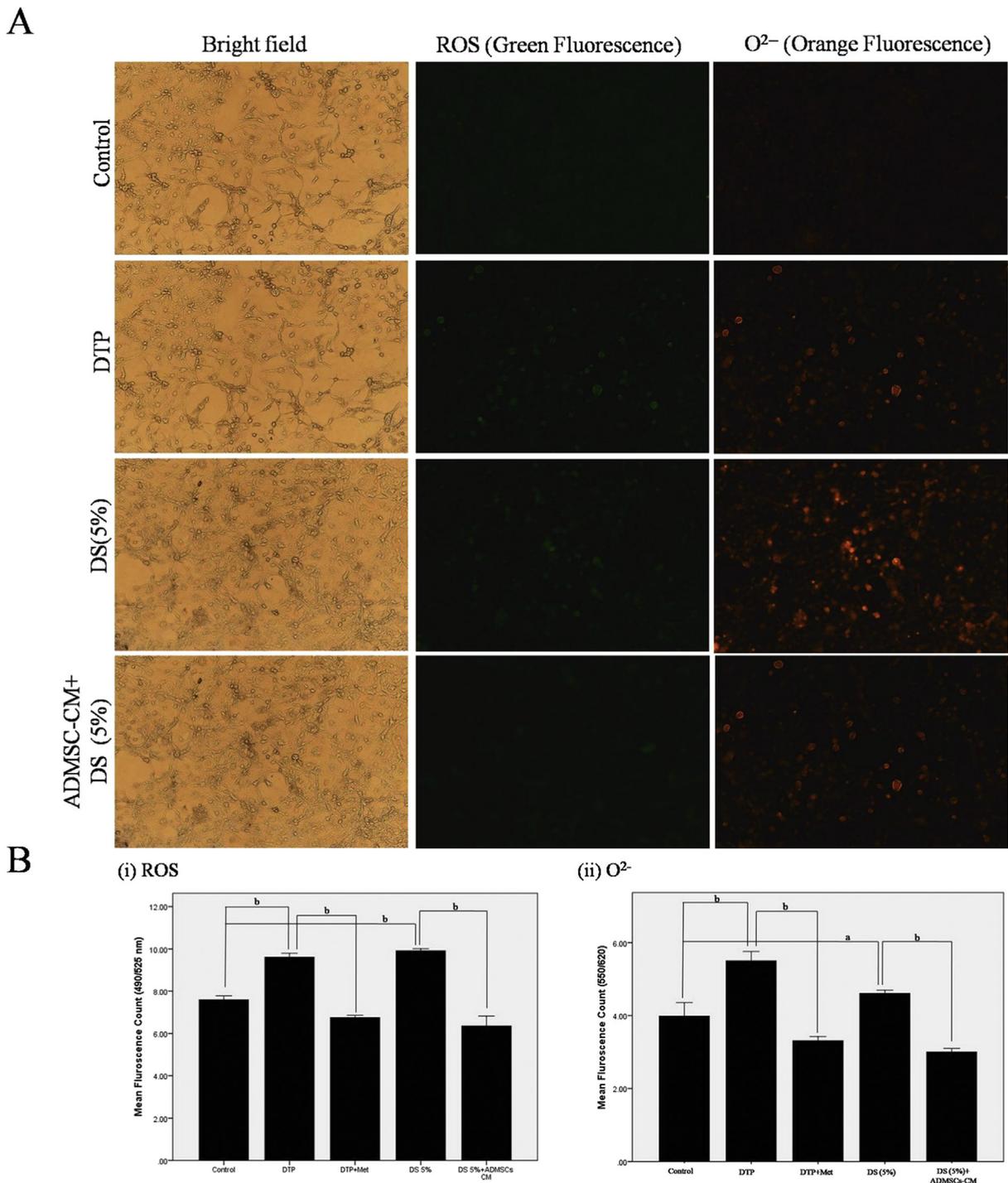


Fig. 4. Intracellular reactive oxygen species (ROS) and superoxide (O²⁻) measurement using fluorescence microscopy in 3T3-L1 cells by using EnzoLife ROS-ID® ROS/RNS Detection Kit: Treatment of dexamethasone, TNF- α , and palmitate (DTP) enhances generation of ROS (Green fluorescence) and O²⁻ radicals (Orange fluorescence); similarly diabetic serum (DS) enhances the generation of ROS & O²⁻ radicals. Adipose derived mesenchymal stem cell conditioned medium (ADMSC-CM) effectively reduced DS induced increase of ROS & O²⁻ radicals (original magnification 4 \times) (A). ROS and O²⁻ measurement using fluorometry in 3T3-L1 cells: Microscopic findings were validated by using fluorometry measurement for ROS (Ex/Em: 490/525 nm) and O²⁻ (Ex/Em: 550/620 nm). DTP and DS show a significant increase in the generation of ROS & O²⁻ radicals ($P < 0.01$) where ADMSC-CM treatment reduces DS induced increase in ROS & O²⁻ radicals ($P < 0.01$). Fluorescence intensity was normalized to the mg of protein/well. All the experiments were performed in triplicates (^a $P < 0.05$, ^b $P < 0.01$) (B). Data are shown as mean \pm SD.

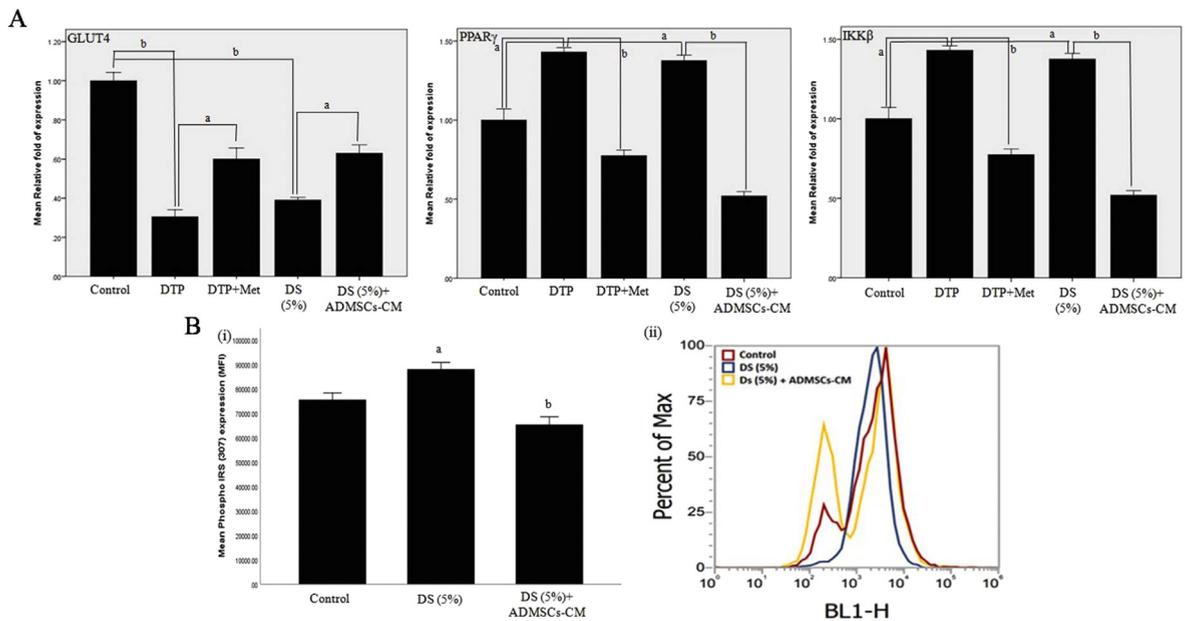


Fig. 5. mRNA expression of GLUT4, PPAR γ , and IKK β in 3T3-L1 cells by using reverse transcription polymerase chain reaction (RT-PCR) (A): Adipose derived mesenchymal stem cells conditioned medium (ADMSCs-CM) restored diabetic serum (DS) induced decrease in GLUT4 expression ($P < 0.05$); ADMSCs-CM treatment also restored DS induced increase in PPAR γ and IKK β expression. Data shown are mean \pm SD. All the experiments were performed in triplicates (^a $P < 0.05$, ^b $P < 0.01$). The expression of phosphorylated insulin receptor substrate 1 (Phospho IRS-1307) in 3T3-L1 cells by using fluorescence activated cell sorting (FACS) (B): (i) DS (5%) upregulated phospho-IRS (307) protein levels (Median Fluorescence Intensity) where ADMSCs-CM reversed DS mediated change in phospho-IRS (307) protein expression. Data represent mean \pm SD. All the experiments were performed in triplicates (^a $P < 0.05$, ^b $P < 0.01$). (ii) Histogram representing percentage of cells treated with Control, DS (5%), and DS (5%) + ADMSCs-CM. Data shown are from one of the three experiments performed.

intracellular influx of glucose.³ Our results showed that DS significantly down-regulated expression of GLUT4 mRNA followed by decrease in 2-NBDG uptake, affirming our hypothesis.

ROS and O²⁻ radicals are known to contribute to IR, and the concurrent apoptosis through activation of the fork head boxO subfamily of transcription factors in 3T3-L1 cells.²⁶ Excess glucose and saturated fatty acids are known to cause ROS and O²⁻ generation through the pentose phosphate pathway, and mitochondrial oxidative phosphorylation.²⁷ Our data indicate that DS significantly enhanced ROS and O²⁻ generation contributing to IR in 3T3-L1 cells.

The IKK- β -NF- κ B pathway is a master regulator of inflammation in obesity and inflammation that induces IR.⁵ TNF- α treatment of 3T3-L1 cells activates the IKK- β -NF- κ B pathway and downstream expression of pro-inflammatory genes.¹³ In the present study, DS significantly up-regulated IKK- β expression contributing to the inflammation linked to IR. DS contains a combination of pro-inflammatory cytokines and fatty acid derivatives such as ceramides and diacylglycerol

which can trigger metabolic and inflammatory stress. Stress-induced serine kinases (IKK- β , c-Jun N-terminal kinases, extracellular signal regulated kinases, and p70S6 kinase) can negate normal insulin signaling by promoting inhibitory serine phosphorylation at IRS1-307 with concomitant increase in IR.²⁸ DS can be a better alternative as compared to the pharmacological factors for inducing IR *in vitro*. Signaling pathways influenced by these pharmacological factors are limited, which can be overcome by the use of DS, which is an appropriate diabetic environment as it is economical to procure and easily accessible.

One of the key problems in designing T2DM therapy is to develop cost-effective methods to predict and diagnose the onset of IR at an early stage, monitor its progression, and risk stratify patients in terms of subsequent cardiovascular and diabetes complications.²⁹ Hyperinsulinemic euglycemic clamp test is considered as the gold standard whereas the intravenous glucose tolerance test and the insulin tolerance test (ITT)/insulin suppression test are routinely used for the assessment of IR. However, these tests are invasive,

expensive, and complex to use in daily clinical practice and also difficult to perform in population-based research studies.³⁰ To overcome these limitations, several surrogate markers such as fasting plasma insulin, the homeostasis model assessment IR, and the quantitative insulin sensitivity check index have been used in clinical settings.³¹ However, the unavailability of reliable normal ranges and the changing β -cell function over time limits their predictive potential.³⁰ Our findings regarding DS-induced IR in 3T3-L1 cells can lead to the development of a cell culture model where a onetime blood sample can be tested for the occurrence of IR. This model with further standardization uses large scale DS samples, which will be an accurate measurement of the *in vivo* IR state of the patient. This model can also be used for testing novel insulin sensitizers.

Recently, our findings from another study have suggested that ADMSC-CM reverses TNF- α - and Palmitate-induced IR in 3T3-L1 cells.¹⁰ In the present study, we tested the ability of ADMSC-CM to reverse DS-induced IR. Our data indicate that ADMSC-CM reversed DS-induced decrease of 2-NBDG uptake, reduced DS-induced ROS and O²⁻ generation, and effectively restored DS-driven down regulation of GLUT4 expression, and down-regulated PPAR γ and IKK- β expression. It is a reaffirmation of our earlier findings on the all-round protective effect of ADMSC-CM against inflammation and oxidative stress-induced IR.

Our data show that DS can be used to induce IR as an alternative to pharmacological factors in 3T3-L1 cells. Reversal of DS-induced IR by ADMSC-CM reaffirms its prospective use as a novel insulin sensitizer for T2DM therapy. In the future, ADMSC-CM could be of therapeutic value to treat T2DM as it is a cell free product, which is patient friendly.

Conflict of interest

None.

Acknowledgements

First author would like to thank Council of Scientific and Industrial Research (CSIR), Government of India for providing research fellowship. We thank Ms. Himangi Lubree for her guidance and cooperation during the study. We are thankful to Department of Biotechnology, Sinhgad College of Engineering, Pune

(India) for the extended support provided to carry out the work. Thanks are also due to Dr. D.Y. Patil Vidyapeeth for permitting to undertake these studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cdtm.2020.09.001>.

References

- Marín-Peñalver JJ, Martín-Timón I, Sevillano-Collantes C, del Cañizo-Gómez FJ. Update on the treatment of type 2 diabetes mellitus. *World J Diabetes*. 2016;7:354. <https://doi.org/10.4239/wjd.v7.i17.354>.
- World Health Organization. Global report on diabetes. *Isbn*. 2016;978:88. ISBN 978 92 4 156525 7.
- Samuel VT, Shulman GI. Mechanisms for insulin resistance: common threads and missing links. *Cell*. 2012;148:852–871. <https://doi.org/10.1016/j.cell.2012.02.017>.
- Muoio DM, Newgard CB. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and β -cell failure in type 2 diabetes. *Nat Rev Mol Cell Biol*. 2008;9:193–205. <https://doi.org/10.1038/nrm2327>.
- Arkan MC, Hevener AL, Greten FR, et al. IKK- β links inflammation to obesity-induced insulin resistance. *Nat Med*. 2005;11:191–198. <https://doi.org/10.1038/nm1185>.
- Czech MP. Insulin action and resistance in obesity and type 2 diabetes. *Nat Med*. 2017;23:804–814. <https://doi.org/10.1038/nm.4350>.
- Yuzefovych LV, Musiyenko SI, Wilson GL, Rachek LI. Mitochondrial DNA damage and dysfunction, and oxidative stress are associated with endoplasmic reticulum stress, protein degradation and apoptosis in high fat diet-induced insulin resistance mice. *PLoS One*. 2013;8. <https://doi.org/10.1371/journal.pone.0054059>.
- Herschkovitz A, Liu Y, Ilan E, Ronen D, Boura-halfon S, Zick Y. Common inhibitory serine sites phosphorylated by IRS-1 kinases, triggered by insulin and inducers of insulin resistance. *J Biol Chem*. 2007;282:18018–18027. <https://doi.org/10.1074/jbc.M610949200>.
- Matsushita K. Mesenchymal stem cells and metabolic syndrome: current understanding and potential clinical implications. *Stem Cell Int*. 2016;2016. <https://doi.org/10.1155/2016/2892840>.
- Shree N, Bhonde RR. Conditioned media from adipose tissue derived mesenchymal stem cells reverse insulin resistance in cellular models. *J Cell Biochem*. 2017;118:2037–2043. <https://doi.org/10.1002/jcb.25777>.
- Stafeev IS, Michurina SS, Podkuychenko NV, Menshikov MY, Parfyonova YV, Vorotnikov AV. Chemical inducers of obesity-associated metabolic stress activate inflammation and reduce insulin sensitivity in 3T3-L1 adipocytes. *Biochem*. 2019;84:553–561. <https://doi.org/10.1134/S0006297919050092>.
- Thomson MJ, Williams MG, Frost SC. Development of insulin resistance in 3T3-L1 adipocytes. *J Biol Chem*. 1997;272:7759–7764. <https://doi.org/10.1074/jbc.272.12.7759>.
- Ruan H, Hacoen N, Golub TR, Van Parijs L, Lodish HF. Tumor necrosis factor- α suppresses adipocyte-specific genes

- and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor- κ B activation by TNF- α is obligatory. *Diabetes*. 2002;51:1319–1336. <https://doi.org/10.2337/diabetes.51.5.1319>.
14. Jager J, Grémeaux T, Cormont M, Le Marchand-Brustel Y, Tanti JF. Interleukin-1 β -induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. *Endocrinology*. 2007;148:241–251. <https://doi.org/10.1210/en.2006-0692>.
 15. Rotter V, Nagaev I, Smith U. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor- α , overexpressed in human fat cells from insulin-resistant subjects. *J Biol Chem*. 2003;278:45777–45784. <https://doi.org/10.1074/jbc.M301977200>.
 16. Nguyen MTA, Satoh H, Favelyukis S, et al. JNK and tumor necrosis factor- α mediate free fatty acid-induced insulin resistance in 3T3-L1 adipocytes. *J Biol Chem*. 2005;280:35361–35371. <https://doi.org/10.1074/jbc.M504611200>.
 17. Sakoda H, Ogihara T, Anai M, et al. Dexamethasone-induced insulin resistance in 3T3-L1 adipocytes is due to inhibition of glucose transport rather than insulin signal transduction. *Diabetes*. 2000;49:1700–1708. <https://doi.org/10.2337/diabetes.49.10.1700>.
 18. Nelson BA, Robinson KA, Buse MG. High glucose and glucosamine induce insulin resistance via different mechanisms in 3T3-L1 adipocytes. *Diabetes*. 2000;49:981–991. <https://doi.org/10.2337/diabetes.49.6.981>.
 19. Smith TR, Elmendorf JS, David TS, Turinsky J. Growth hormone-induced insulin resistance: role of the insulin receptor, IRS-1, GLUT-1 and GLUT-4. *Faseb J*. 1997;11. <https://doi.org/10.1152/ajpendo.1997.272.6.E1071>.
 20. Regazzetti C, Peraldi P, Grémeaux T, et al. Hypoxia decreases insulin signaling pathways in adipocytes. *Diabetes*. 2009;58:95–103. <https://doi.org/10.2337/db08-0457>.
 21. Lo KA, Labadorf A, Kennedy NJ, et al. Analysis of in vitro insulin resistance models and their physiological relevance to in vivo diet-induced adipose insulin resistance. *Cell Rep*. 2014;5:1–20. <https://doi.org/10.1016/j.celrep.2013.08.039>.
 22. Sanap A, Chandravanshi B, Shah T, et al. Herbal pre-conditioning induces proliferation and delays senescence in Wharton's Jelly Mesenchymal Stem Cells. *Biomed Pharmacother*. 2017;93:772–778. <https://doi.org/10.1016/j.biopha.2017.06.107>.
 23. Ruiz-Ojeda FJ, Rupérez AI, Gomez-Llorente C, Gil A, Aguilera CM. Cell models and their application for studying adipogenic differentiation in relation to obesity: a review. *Int J Mol Sci*. 2016;17:1–26. <https://doi.org/10.3390/ijms17071040>.
 24. Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF. Phosphorylation of ser 307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action*. *J Biol Chem*. 2002;277:1531–1537. <https://doi.org/10.1074/jbc.M101521200>.
 25. Im SS, Kwon SK, Kim TH, Kim HIL, Ahn YH. Regulation of glucose transporter type 4 isoform gene expression in muscle and adipocytes. *IUBMB Life*. 2007;59:134–145. <https://doi.org/10.1080/15216540701313788>.
 26. Kojima T, Norose T, Tsuchiya K, Sakamoto K. Mouse 3T3-L1 cells acquire resistance against oxidative stress as the adipocytes differentiate via the transcription factor FoxO. *Apoptosis*. 2010;15:83–93. <https://doi.org/10.1007/s10495-009-0415-x>.
 27. Han CY. Roles of reactive oxygen species on insulin resistance in adipose tissue. *Diabetes Metab J*. 2016;40:272–279. <https://doi.org/10.4093/dmj.2016.40.4.272>.
 28. Sanap A, Bhonde R, Joshi K. Conditioned medium of adipose derived Mesenchymal Stem Cells reverse insulin resistance through downregulation of stress induced serine kinases. *Eur J Pharmacol*. 2020;173215. <https://doi.org/10.1016/j.ejphar.2020.173215>.
 29. Scirica BM. Use of biomarkers in predicting the onset, monitoring the progression, and risk stratification for patients with type 2 diabetes mellitus. *Clin Chem*. 2017;63:186–195. <https://doi.org/10.1373/clinchem.2016.255539>.
 30. Stapor N, Beń-Skowronek I. Insulin resistance in children. *Pediatr Endocrinol Diabetes Metab*. 2014;20:107–115. <https://doi.org/10.18544/PEDM-20.03.0010>.
 31. Buchanan TA, Watanabe RM, Xiang AH. Limitations in surrogate measures of insulin resistance. *J Clin Endocrinol Metab*. 2010;95:4874–4876. <https://doi.org/10.1210/jc.2010-2167>.

Edited by Yi Cui