JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2023, 74, 2, 159-168
www.jpp.krakow.pl | DOI: 10.26402/jpp.2023.2.04

R. MA[1], L. QUAN[1], Q.-Q.-G. ALETENG[1], L. LI[1], J. ZHU[2], S. JIANG[1]

THE IMPACT OF SITAGLIPTIN IN PALMITIC ACID-INDUCED INSULIN RESISTANCE IN HUMAN HEPG2 CELLS THROUGH THE SUPPRESSOR OF CYTOKINE SIGNALING 3/PHOSPHOINOSITIDE 3-KINASE/PROTEIN KINASE B PATHWAY

[1]Department of Endocrinology, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, China;
[2]Department of Endocrinology, People's Hospital of Shenzhen Baoan District, Shenzhen, Guangdong, China

Patients with type 2 diabetes respond differently to sitagliptin, an oral anti-hyperglycemic medication. Patients whose blood sugar levels were effectively managed while using sitagliptin had significantly lower levels of a protein called suppressor of cytokine signaling 3 (SOCS3), according to our earlier research. In this study, we established an *in vitro* insulin resistance cell model for human HepG2 cells to investigate the possible mechanism of the effect of sitagliptin on glucose metabolism *via* the SOCS3/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway. Since insulin resistance first develops in the liver, palmitic acid was used to generate an insulin resistance cell model in human HepG2 cells, after which small interfering ribonucleic acid (siRNA)-SOCS3 and sitagliptin were used to intervene. We then examined the changes in cell viability and biochemical indices in the insulin resistance cell model. SOCS3, Akt, and glycogen synthase kinase 3beta (GSK-3β) gene expression levels were quantified using reverse transcription-polymerase chain reaction, and the protein expression levels of SOCS3, Akt, phosphorylated Akt (p-Akt), GSK-3β, and phosphorylated GSK-3β (p-GSK-3β) were quantified using Western blot. In results: the expression of the SOCS3 gene was considerably raised in both the insulin resistance model group and the insulin resistance model + siRNA-negative control group, but decreased following treatment with sitagliptin. After sitagliptin intervention, the protein expression of Akt, p-Akt, and p-GSK-3β were dramatically decreased in the model group, while SOCS3 was significantly decreased. We conclude that sitagliptin can reduce insulin resistance by downregulating SOCS3 and regulating glucose metabolism in a hypoglycemic manner.

Key words: *HepG2 cells, insulin resistance, siRNA, sitagliptin, SOCS3/PI3K/Akt pathway*

INTRODUCTION

Sitagliptin, an oral dipeptidyl peptidase-4 inhibitor, is the first drug of its class to be approved for the monotherapy or combination treatment of type 2 diabetes in adults (1). Sitagliptin has the benefits of not raising the risk of hypoglycemia or weight gain, not aggravating the risk of heart failure, and having few gastrointestinal adverse effects (2). Individual differences in the efficacy of sitagliptin in the treatment of type 2 diabetes have been reported during therapeutic administration. For instance, some individuals experienced a significant hypoglycemic impact, yet a substantial percentage of patients had inadequate glycemic control after taking the medicine (3, 4). Using transcriptome analysis, our previous research revealed that suppressor of cytokine signaling 3 (SOCS3) was significantly lower in individuals with effective glycemic control compared to those with inadequate glycemic control (4).

SOCS3 is located in the insulin resistance signaling pathway (5), which acts as a negative modulator of insulin signaling. Many studies have reported that the PI3K/AKT pathway is the main pathway of insulin action (6, 7). SOCS3 can compete with STAT5b for insulin receptor binding and block IRS-2 tyrosine phosphorylation, resulting in insulin resistance. In addition, SOCS3 inhibits insulin resistance by the downregulation of insulin receptor substrate proteins. It can also impact insulin resistance by influencing signal transduction factors such as the substrates of insulin receptors. SOCS3 suppresses the insulin signaling pathway *via* insulin receptors. Low SOCS3 expression enhances glycogen synthesis and glucose homeostasis *via* the PI3K/Akt-glycogen synthase kinase 3 beta (GSK-3β) pathway (8).

The liver is the metabolic hub of the body and a crucial organ for insulin action, as it regulates glucose metabolism (9). Insulin resistance is a condition in which the cells of the body become resistant to insulin; this occurs in organs such as the liver, as well as muscle and adipose tissue (10). In the present study, we built an insulin resistance cell model induced by palmitic acid in human HepG2 cells *in vitro* to examine the effect of sitagliptin on the SOCS3/PI3K/Akt pathway.

MATERIALS AND METHODS

Ethics approval

This study was conducted with approval from the Ethics Committee of The First Affiliated Hospital of Xinjiang Medical University (K-202106-05). Consent for publication was obtained from every individual whose data are included in this manuscript.

Experiment reagents

Fetal bovine serum was purchased from ExCell Bio (Shanghai, China); 0.25% trypsin- ethylenediaminetetraacetic was sourced from Gibco (New York, NJ, USA); a Cell Counting Kit-8 (CCK-8) cell proliferation/toxicity assay kit, an Easy II Protein Quantitative Kit (BCA), TransScript One-Step gDNA Removal, and cDNA Synthesis SuperMix Trans2K DNA Marker TransZol Up were purchased from Full Gold Bio (Beijing, China). A glucose detection kit was purchased from Shanghai Rongsheng Biology Pharmaceutical Co., Ltd. (Shanghai, China). A glycogen detection kit was purchased from Nanjing Jicheng (Nanjing, China). A lipofectamine RNAiMAX transfection kit Block-ITTM Alexa Fluor@ Red Positive Control was purchased from Invitrogen (Carlsbad, CA, USA), the small interfering ribonucleic acid (siRNA) negative control siRNA SOCS3-HOMO-993, siRNA SOCS3-HOMO-617, and siRNA SOCS3-HOMO-908 were purchased from Gima Gene (Suzhou, China). Palmitic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). The SuperSignal West Pico PLUS chemiluminescent substrate was purchased from Thermo Fisher (Waltham, MA, USA). SOCS3 (D6E1T) Rabbit mAb, Akt (Pan) (C67E7) Rabbit mAb, Phospho-Akt (Ser473) Antibody, GSK-3β (D5C5Z) XP Rabbit mAb, phospho-GSK-3 α/β (Ser21/9) (37F11) Rabbit MAb (GSK-3α Preferred) Preferred Cell Signaling Technology (Danvers, MA, USA), Anti-ACTb Rabbit Polyclonal Antibody Preferred Shanghai (Shanghai, China), Goat Anti-Rabbit IgG H&L; monoclonal Antibody Preferred Cell Signaling Technology (Danvers, MA, USA), Anti-ACTb Rabbit Polyclona Antibody Preferred Shanghai, China; L (HRP) from Abcam (Cambridge, UK), QuantiNova SYBR Green Kit from Capgemini (Hilden, Germany).

Cell culture

The human liver cancer HepG2 cell line was cultured in Dulbecco's Modified Eagle Medium (high glucose), 10% fetal bovine serum, and 1% penicillin-streptomycin at 37°C, with 5% carbon dioxide, and in saturated humidity.

Establishing the insulin resistance model

The HepG2 cells were diluted to the desired concentration using a serum-free medium and prepared as a cell suspension with a density of 5×10^4/ml. The cells were then inoculated into 96-well plates. After being cultured to the wall under standard conditions, the medium was discarded and the plates washed with phosphate-buffered saline (PBS), and the culture medium containing different concentrations of palmitic acid (0.25 mM, 0.5 mM) was serum-free for 24 h, which was then replaced with a serum-free medium containing 100 nM insulin for 30 min, before being replaced with a complete medium for 48 h. The CCK-8 kit was used to detect cell viability, and the modeling situation was evaluated by detecting glucose consumption.

Sitagliptin treatment

The cells were treated with 0.5 nM palmitic acid and 100 nM insulin. Then, 100 μL of a sitagliptin solution (0, 1, 10, 20, 50, 100, and 150 μM) was added to the complete medium, and CCK-8 glucose consumption was detected 24 h and 48 h later. The intervention time of sitagliptin concentration (10 μM, 48 h) that had no significant inhibition on HepG2 cell proliferation and significantly increased glucose consumption, was selected for the follow-up experiment.

siRNA transfection

Three SOCS3 siRNAs were designed and synthesized - SOCS3 Homo-993, SOCS3 Homo-908, and SOCS3 Homo-617. Each siRNA sequence and negtive control sequences are presented in *Table 1*. The HepG2 cells that showed good growth status and a 90% convergence rate were selected and digested by trypsinase and centrifuged to prepare a cell suspension with a density of 5×10^4 /mL. The suspension was inoculated into a 24-well plate with a density of 500 μL. Transfection began after the convergence rate of cells reached 60–70%. Two Eppendorf (EP) tubes (labeled A and B) were used and a 75 μL serum-free medium and BLOCK-iT™ Alexa Fluor@Red positive control reagent (0.01 and 0.1 μM) were added to each, respectively, in different concentrations, and the solutions were gently mixed. One EP tube was labeled C, and a 150 μL serum-free medium was added to it, as well as a 9 μL lipofectamine RNAiMAX reagent. After gently mixing the solution, 75 μL was absorbed into tubes A and B, respectively. After mixing the solutions, placing it at room temperature for 5 minutes to obtain siRNA/lipofectamine RNAiMAX compounds. They were added into the well containing the cell plates and slotted appropriately. Meanwhile, blank controls were cultured in an incubator at 37°C with 5% carbon dioxide (CO_2) for 24 h and 48 h, respectively, and fluorescence imaging was conducted to determine the best transfection conditions. Reverse transcription-polymerase chain reaction (RT-PCR) was used to evaluate the knockdown efficiency of the siRNA.

Experimental grouping

Based on the previously established model, drug treatment, and siRNA transfection conditions, the experiment was divided into six groups. The treatment conditions of each group were as follows. The glucose consumption and glycogen level of cells in each group were detected. Western blot was used to detect the protein expressions of SOCS3, Akt, p-Akt, GSK-3β, and p-GSK-3β in each group.

Control group (control): model group: 0.5 mM palmitic acid intervention for 24 h, 100 nM insulin intervention for 30 min, replaced with a complete medium for 48 h (FA).

Model + siRNA-SOCS3 group: 0.5 mM palmitic acid intervention for 24 h, 100 nM insulin intervention for 30 min, replaced with a complete medium, and siRNA SOCS3-HOM-617 transfection at a concentration of 0.1 μM for 48 h (siRNA).

Model + siRNA negative control (NC) group: 0.5 mM palmitic acid intervention for 24 h, 100 nM insulin intervention for 30 min; then, the complete medium was replaced. For the transfection siRNA NC, the transfection condition was 0.1 μM for 48 h (siRNA negative).

Model + sitagliptin group: 0.5 mM palmitic acid for 24 h, 100 nM insulin for 30 min, and then changed to a complete medium containing 10 μM sitagliptin for 48 h (sitagliptin).

Model + siRNA-SOCS3 + sitagliptin group: after 0.5 mM palmitic acid intervention for 24 h and 100 nM insulin intervention for 30 min, the medium was replaced with a complete medium containing 10 μM sitagliptin, and siRNA SOCS3-HOM-617 was transfected for 48 h (sitagliptin + siRNA).

Table 1. Design and synthesis of siRNA sequences.

Group	**Sequence (5' to 3')**
Negative control	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT
SOCS3-homo-617	ACCUUUCUGAUCCGCGACATT UGUCGCGGAUCAGAAAGGUTT
SOCS3-homo-908	GCCUAUUACAUCUACUCCGTT CGGAGUAGAUGUAAUAGGCTT
SOCS3-homo-993	UCUGUCGGAAGACCGUCAATT UUGACGGUCUUCCGACAGATT

Table 2. Real-time PCR primer information table for fluorescence quantitative detection.

Substance index name	**Sequence (5' to 3')**	**Primer size**
SOCS3-F	CCTGCGCCTCAAGACCTTC	99 bp
SOCS3-R	GTCACTGCGCTCCAGTAGAA	
AKT-F	TCCTCCTCAAGAATGATGGCA	181 bp
AKT-R	GTGCGTTCGATGACAGTGGT	
GSK-3B-F	TGGTCGCCATCAAGAAAGTATTG	216 bp
GSK-3B-R	GCGTCTGTTTGGCTCGACTAT	
β-Actin-F	CATGTACGTTGCTATCCAGGC	250 bp
β-Actin-R	CTCCTTAATGTCACGCACGAT	

Cell viability measured by cell counting kit-8 (CCK-8)

The HepG2 cells with a good growth status and a convergence rate of 90% were taken and prepared into a suspension with a density of 1×10^5/mL after trypsin digestion. The suspension was inoculated into 96-well plates. After 24 h culture under standard environmental conditions and adherence, the medium was discarded, and the plates washed with PBS. Next, the medium was collected for glucose consumption detection. The volume of 100 μL of the medium was taken and added to each well with 100 μL of a 10% CCK-8 solution, then the medium was incubated in an incubator. After 1 h, the OD value at 450 nm was determined with a microplate reader for 5 replicates in each group.

Glucose consumption and glycogen levels

The HepG2 cells with good growth status and a convergence rate of 90% were taken, digested by trypsin, and centrifuged, and then prepared to form a suspension with a density of 5×10^4/mL. The suspension was inoculated into a 25 cm^2 culture flask with a density of 25,000/bottle and cultured at 37°C, with 5% CO_2 for 24 h adherence, and the medium was abandoned. Following the intervention, the cell culture medium supernatant was collected for glucose consumption detection. Cells were collected and the total protein was extracted. After protein quantification, glycogen levels were detected using a glycogen detection kit for the 5 replicates in each group.

Real-time PCR

After 24 h adherence, the culture medium was discarded. Following the intervention, the culture medium was discarded, and the cells were collected. Then, 1 mL Trizol digestion cells were added, RNA was extracted according to the Trizol instructions, and 2 μg RNA was extracted according to the instructions of the TransScript one-step deoxyribonucleic acid (gDNA) full-gold complementary (c)DNA synthesis kit. The cDNA was synthesized by SuperMix and amplified by quantitative (q)PCR using Capgemini's QuantiNova SYBR Green Kit. Data were analyzed using the $2^{-\Delta\Delta CT}$ method; beta (β)-actin was used as an internal reference; the primers used in the experiment are given in *Table 2*. All experiments were repeated five times.

Western blot

The culture medium was discarded after adherence for 24 h. Following the intervention, the cells were digested by trypsin, and the supernatant was discarded after centrifugation to leave the cell precipitate. SOCS3, Akt, p-Akt, GSK-3β, and p-GSK-3β were detected by the standard operation of a Western blot assay. The following antibodies were used: SOCS3 (D6E1T) Rabbit mAb, Akt (Pan) (C67E7) Rabbit mAb, Phospho-Akt (Ser473) Antibody, GSK-3β (D5C5Z) XP Rabbit mAb, Phospho-gsk-3 α/β (Ser21/9) (37F11) Rabbit mAb (GSK-3α Preferred), β-actin as Preferred, goat Anti-Rabbit IgG H&L; phospho-GSK-3 α/β (Ser21/9) (37F11) Rabbit mAb (GSK-3α Preferred), β-actin as Preferred; L(HRP). All experiments were repeated three times.

Statistical analysis

The measurement data are described as the mean ± standard deviation (mean±s) and were statistically processed using the SPSS Statistics 19.0 software. One-way analysis of variance (ANOVA) was used for comparison between the groups, and an LSD test was used for pairwise comparison. Multi-factor ANOVA was used to determine the optimal intervention concentration and time of sitagliptin, and the significant difference level was set as $P<0.05$.

RESULTS

Screening stable cell lines using an insulin resistance model of HepG2 cells and sitagliptin intervention according to concentration and time

Glucose consumption of HepG2 cells was detected using the methods described below, which required using palmitic acid as well as other interventions. The HepG2 cells were selected as the experimental cells, with a palmitic acid concentration of 0.5 mM and an intervention time of 48 h. The effects of different concentrations of sitagliptin on proliferation and glucose consumption in the HepG2 insulin resistance cell model were then examined. Sitagliptin concentration (10 μM) and intervention time (48 h), which significantly increased glucose consumption but did not significantly inhibit the proliferation of HepG2 cells, were selected for follow-up experiments.

Exploration of siRNA transfection conditions

The siRNA concentration of 0.1 μM and transfection time of 48 h were selected as the optimal transfection conditions for subsequent experiments (*Fig. 1*). Lipofectamine® RNAiMAX transfection reagent was used to transfect three siRNA-SOCS3s (concentration 0.1 μM, transfection time 48 h). Concurrently, a negative control and a blank control group were set, and the expression levels of SOCS3 mRNA in all groups were detected using qRT-PCR. The results revealed that siRNA-SOCS3-HOMO-617 significantly inhibited the expression of SOCS3 mRNA; therefore, siRNA-SOCS3-HOMO-617 was selected for subsequent experiments (*Fig. 2*). SOCS3 homo-617 showed significant reduction of the gene, hence it was selected as the optimal option.

Cell viability measured by cell counting kit-8 (CCK-8)

CCK-8 showed no significant statistical difference in cell viability among any of the groups, indicating that the different treatment methods in each group had no effect on cell proliferation and cell activity (*Fig. 3*).

Effects of different interventions on biochemical indices in the HepG2 insulin resistance cell model.

As shown in *Fig. 4*:

1) Glucose consumption and cell glycogen content in the model group were significantly lower than in the control group, indicating that the model group produced insulin resistance.

2) After the knockdown of the SOCS3 gene, glucose consumption and glycogen content were significantly higher than in the model group and negative control group (no-load group), indicating that the SOCS3 gene could promote the effect of insulin resistance.

3) After sitagliptin intervention, glucose consumption and glycogen content were significantly higher in the model group, indicating that sitagliptin could significantly improve the effect of insulin resistance.

Analysis of the mRNA expression levels of various genes in HepG2 cells in different intervention groups

The effects of different treatments on the expression of various genes in the HepG2 insulin resistance cell model are shown in *Fig. 1* (mean±s , n=5).

1) Compared with the blank group, expression of the SOCS3 gene in the model and model + siRNA-NC groups were significantly increased.

2) Compared with the model group, the expression of the SOCS3 gene in the model + siRNA-SOCS3, model + sitagliptin, and the model + siRNA-SOCS3 + sitagliptin groups was significantly decreased.

3) Compared with the model + siRNA-NC group, expression of the SOCS3 gene in the model + si-SOCS3, model + sitagliptin, and the model + si-SOCS3 + sitagliptin groups was significantly decreased.

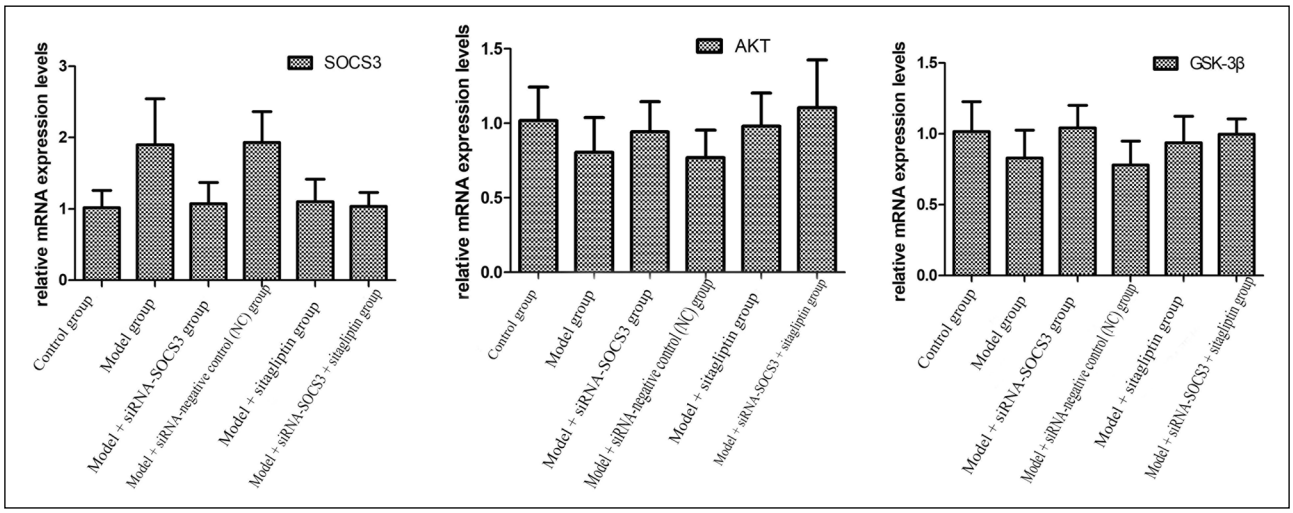

Fig. 1. The mRNA expression analysis of the verified silenced gene (mean±s, n=3). SOCS3 homo-617 showed significant reduction of the gene, hence it was selected as the optimal option. After 24 h adherence, the culture medium was discarded. Following the intervention, the culture medium was discarded, and the cells were collected. Then, 1 mL Trizol digestion cells were added, RNA was extracted according to the Trizol instructions, and 2 μg RNA was extracted according to the instructions of the TransScript one-step deoxyribonucleic acid (gDNA) full-gold complementary (c)DNA synthesis kit. The cDNA was synthesized by SuperMix and amplified by qPCR using Capgemini's QuantiNova SYBR Green Kit. Data were analyzed using the $2^{-\Delta\Delta CT}$ method; β-actin was used as an internal reference; the primers used in the experiment are given in *Table 2*. All experiments were repeated five times.

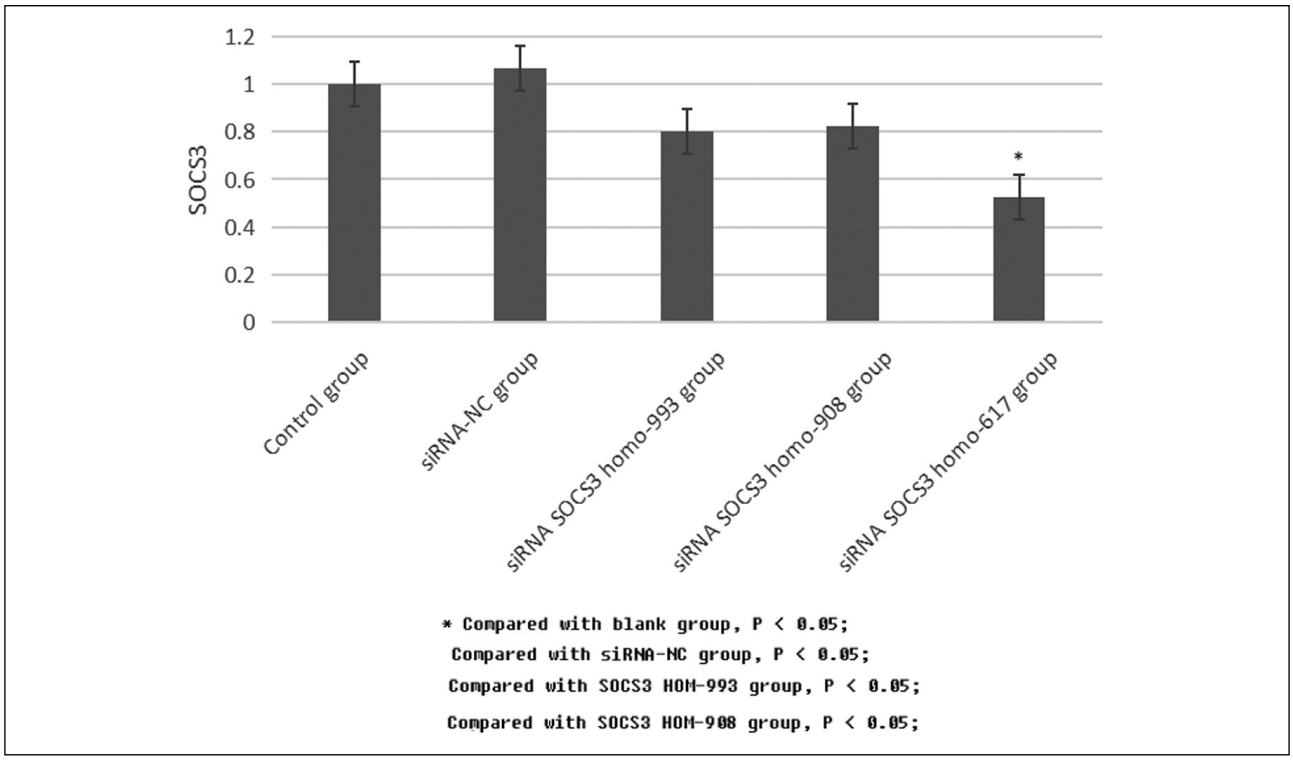

Fig. 2. An image collation of siRNA optimal transfection conditions; a siRNA concentration of 0.1 μM and a transfection time of 48 h were selected as the best transfection conditions for subsequent experiments. Control group (control). Model group: 0.5 mM palmitic acid intervention for 24 h, 100 nM insulin intervention for 30 min, replaced with a complete medium for 48 h (FA). Model + siRNA-SOCS3 group: 0.5 mM palmitic acid intervention for 24 h, 100 nM insulin intervention for 30 min, replaced with a complete medium, and siRNA SOCS3-HOM-617 transfection at a concentration of 0.1 μM for 48h) (siRNA). The model + siRNA negative control (NC) group: 0.5 mM palmitic acid intervention for 24 h, 100 nM insulin intervention for 30 min; then, the complete medium was replaced. For the transfection siRNA NC, the transfection condition was 0.1 μM for 48 h (siRNA negative). The model + sitagliptin group: 0.5 mM palmitic acid for 24 h, 100 nM insulin for 30 min, and then changed to a complete medium containing 10 μM sitagliptin for 48 h (sitagliptin). The model + siRNA-SOCS3 + sitagliptin group: after 0.5 mm palmitic acid intervention for 24 h and 100 nM insulin intervention for 30 min, the medium was replaced with a complete medium containing 10 μM sitagliptin, and siRNA SOCS3-HOM-617 was transfected for 48 h (sitagliptin + siRNA).

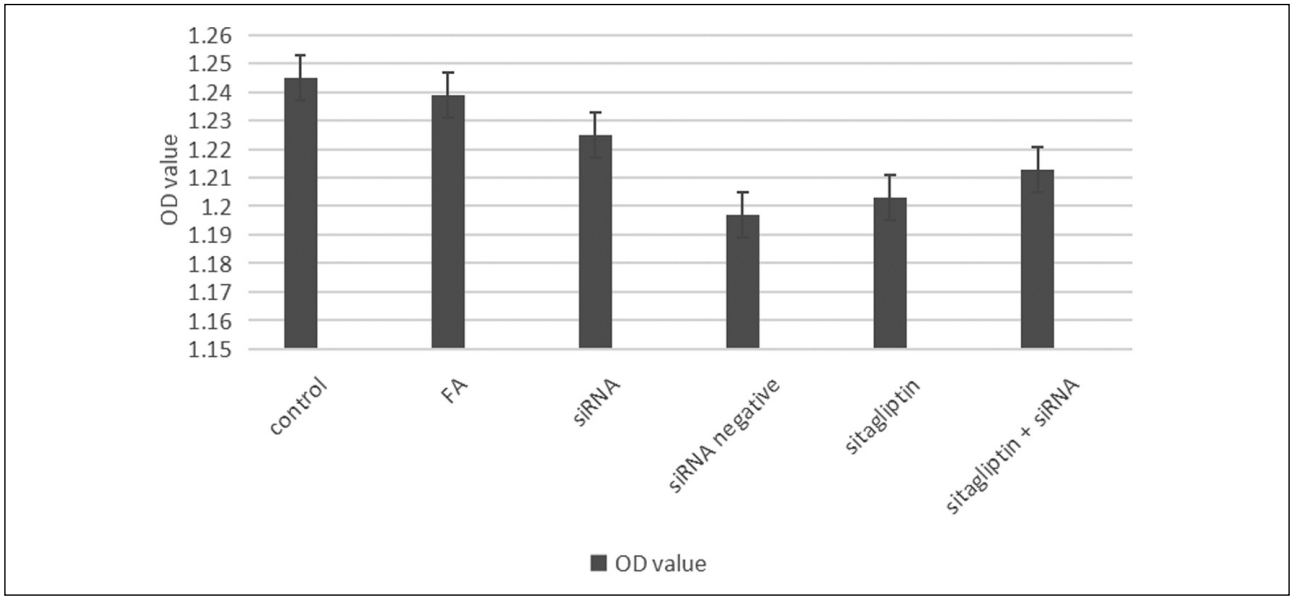

Fig. 3. The effects of different treatments on the proliferation of insulin-resistant HepG2 cells (mean±s, n=5). The HepG2 cells with a good growth status and a convergence rate of 90% were taken and prepared into a suspension with a density of 1×10^5/mL after trypsin digestion. The suspension was inoculated into 96-well plates. After 24 h culture under standard environmental conditions and adherence, the medium was discarded, and the plates were washed with PBS. Next, the medium was collected for glucose consumption detection. Then, 100 μL of the medium was taken and added to each well with 100 μL of a 10% CCK-8 solution, and the medium was incubated in an incubator. After 1 h, the OD value at 450 nm was determined with a microplate reader for 5 replicates in each group.

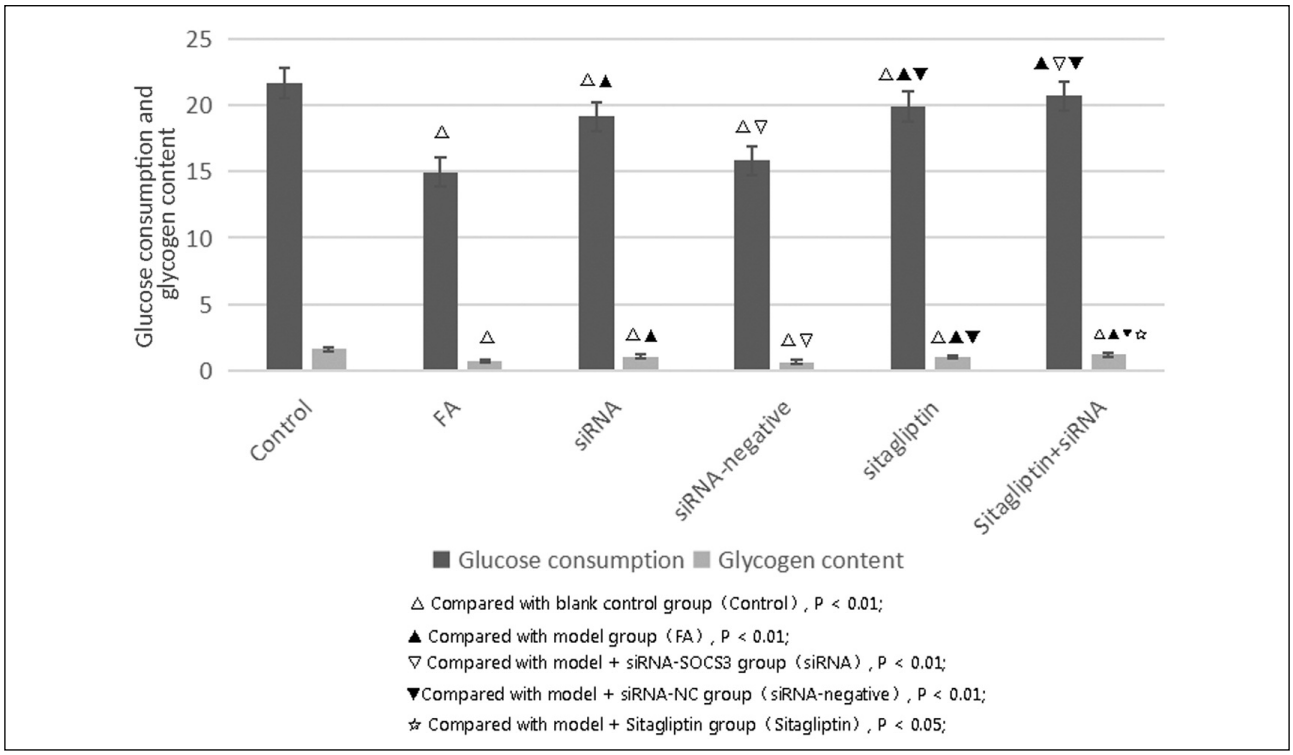

Fig. 4. The effects of different treatments on the glucose consumption and glycogen content of insulin-resistant HepG2 cells (mean±s, n=5). The HepG2 cells with good growth status and a convergence rate of 90% were taken, digested, and centrifuged by trypsin, and then prepared to form a suspension with a density of 5×10^4/mL. The suspension was inoculated into a 25 cm^2 culture flask with a density of 25,000/bottle and cultured at 37°C, with 5% CO_2 for 24 h adherence, and the medium was discarded. Following the intervention, the cell culture medium supernatant was collected for glucose consumption detection. Cells were collected and the total protein was extracted. After protein quantification, glycogen levels were detected using a glycogen detection kit for the 5 replicates in each group.

Analysis of the effect of different interventions on the expression of various proteins in the HepG2 cells.

As shown in *Fig. 6* the protein expression levels of SOCS3, Akt, p-Akt, GSK-3β, and p-GSK-3β were detected by Western blot (means ± s, n=3).

1) Compared with the control group, the SOCS3 proteins in the model group were significantly increased, while the protein expressions of Akt, p-Akt, and p-GSK-3β were significantly decreased.

2) Compared with the model group, the SOCS3 proteins in the model + siRNA-SOCS3 and the model + siRNA-SOCS3 + sitagliptin groups were significantly decreased.

3) Compared with the model group, the protein expressions of p-Akt and p-GSK-3β in the model + sitagliptin and the model + siRNA-SOCS3 + sitagliptin groups were significantly increased.

DISCUSSION

Insulin has a significant effect on the liver, which regulates glucose metabolism *via* glycolysis, hepatic glycogen synthesis and breakdown, and gluconeogenesis, among other processes (12). The HepG2 cell line is frequently employed in drug metabolism and hepatotoxicity research because its appearance and activity are more similar to that of human liver tissue. It can be infinitely subcultured according to experimental needs and it has high application value in the screening of hypoglycemic drugs (13-18). In the present study, we established a palmitic acid-induced insulin resistance cell model using HepG2 cells to investigate the expression of the SOCS3/PI3K/Akt pathway in an insulin resistance cell model, as well as the regulatory effect of sitagliptin on the SOCS3/PI3K/Akt pathway in the same model. Additionally, the hypoglycemic mechanism of sitagliptin was analyzed.

Glucose molecules in hepatocytes can be catalyzed by GS to synthesize liver glycogen, which can trigger insulin resistance if glycogen synthesis is insufficient (19, 20). In the present study, we found that the glycogen content in the HepG2 insulin resistance model cells was significantly reduced, suggesting the presence of insulin resistance in the cell model. During liver glycogen synthesis, insulin binds to insulin receptors on the cell surface and activates insulin receptor substrate 2 (IRS-2), PI3K, GSK-3β, and others. The phosphorylation of GSK-3β leads to its inactivation, which means it no longer inhibits GS activity (21-23) and there is increased liver glycogen synthesis under the influence of this factor. GSK-3α and GSK-3β are two subunits of GSK-3 (24). Related studies have identified a close relationship between GSK-3β and the pathology of type 2 diabetes mellitus (T2DM) - the excessive expression of GSK-3β can lead to T2DM (25-27).

Existing studies indicate that the mechanism of SOCS3 mediating insulin resistance is as follows (28): 1) SOCS3 competitively binds to insulin receptors with a signal transducer and activator of transcription 5B, which inhibits insulin IRS-2 tyrosine phosphorylation, and triggers insulin resistance; 2) abnormal expression of IRS-2 can reduce IRS-2 phosphorylation and promote IRS-2 degradation; 3) it can also negatively regulate the Janus kinase 2/signal transducer and

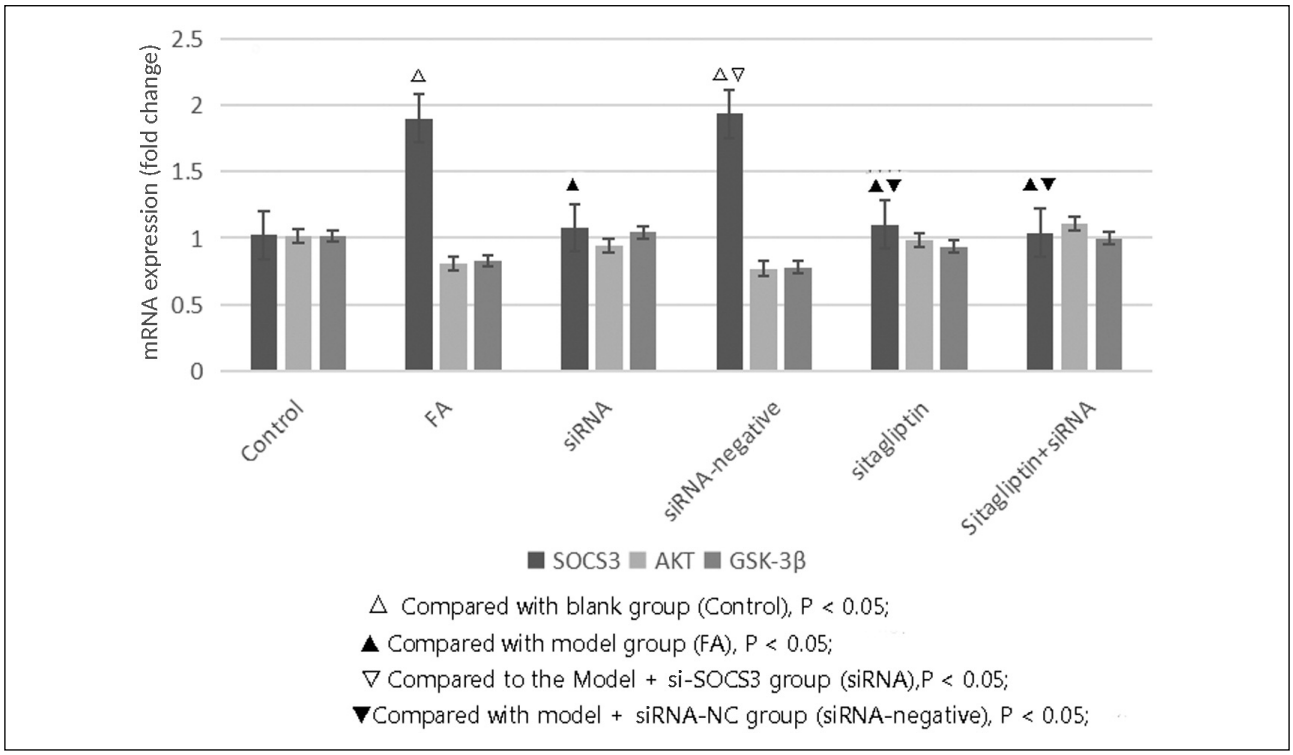

Fig. 5. Analysis of the mRNA expression levels of various genes in the HepG2 cells in different intervention groups (mean±s, n=5). After 24 h adherence, the culture medium was discarded. Following the intervention, the culture medium was discarded, and the cells were collected. Then, 1 mL Trizol digestion cells were added, RNA was extracted according to the Trizol instructions, and 2 μg RNA was extracted according to the instructions of the TransScript one-step deoxyribonucleic acid (gDNA) full-gold complementary (c) DNA synthesis kit. The cDNA was synthesized by SuperMix and amplified by quantitative PCR using Capgemini's QuantiNova SYBR Green Kit. Data were analyzed using the $2^{-\Delta\Delta CT}$ method; β-actin was used as an internal reference; the primers used in the experiment are given in *Table 2*. All experiments were repeated five times.
The effect of different interventions on the expression of various genes in the HepG2 insulin-resistance cell model; mRNA expression of SOCS3 (A), Akt (B), and GSK-3β (C); P<0.05 was considered statistically significant.

activator of the transcription 3 pathway; 4) the expression of SOCS3 is correlated with leptin signal transduction and induced insulin resistance. The expression of SOCS3 has been found to be elevated in both insulin resistance diabetic patients and insulin resistance mouse models. The blockage of the PI3K/Akt signaling pathway has also been reported as a characteristic of insulin resistance. Both PI3K and Akt can promote the expression of glucose transporter type 4, hinder the activation of phosphoenolpyruvate carboxykinase, and inhibit gluconeogenesis (29). The results of the present study revealed that the expression of the SOCS3 gene in the insulin resistance model group was significantly increased. After sitagliptin intervention, the expression of this gene decreased but remained elevated relative to the control group. This suggested that sitagliptin may inhibit the expression of the SOCS3 gene in an insulin resistance model, hence reducing the effect of insulin resistance.

The PI3Ks are downstream molecules of G-protein-coupled receptors (30) which can regulate the corresponding glucose transport process. Studies have revealed that the PI3K/Akt pathway is closely related to insulin regulation and plays an important role in the regulation of cell metabolism by insulin (31) which is closely related to cell proliferation and movement. PI3K can effectively catalyze phosphatidylinositol 4,5-diphosphate (PIP2), and the resulting product can activate Akt, which accelerates the generation of glycogen through the regulation of GSK-3β-related molecules, inhibits gluconeogenesis, and reduces blood glucose (30, 32, 33). In the present study, after sitagliptin intervention, the SOCS3 gene was downregulated and the expression of SOCS3 proteins decreased. The low expression of SOCS3 can increase the phosphorylation level of IRS-2 and reduce its degradation, thus promoting PI3K to catalyze PIP2. The regulation of GSK-3β by activated Akt increases glycogen production, thus inhibiting gluconeogenesis and serving a hypoglycemic purpose.

In the present study, there was no difference in Akt protein expression between silencing the SOCS3 gene and sitagliptin intervention, although p-Akt expression increased. Protein kinase B is a bioactive Akt, which promotes glycogen synthesis and maintains glucose homeostasis by inhibiting GSK-3β in the insulin resistance signaling pathway. There was also no difference in total protein levels for GSK-3β, while p-GSK-3β levels increased after sitagliptin intervention. After phosphorylation of GSK-3β, it could not effectively inhibit GS activity, leading to increased glycogen synthesis levels in the liver and, ultimately, lower blood glucose.

In this study, although the expression of p-Akt was elevated, it promoted glycogen synthesis in the insulin resistance signaling pathway by inhibiting the downstream GSK-3β. However, no difference was found in the total protein level of GSK-3β. Therefore, the hypoglycemic effect of sitagliptin was not only realized through the PI3K/Akt/GSK-3β pathway but also *via* a multitude of other factors that warrant further investigation.

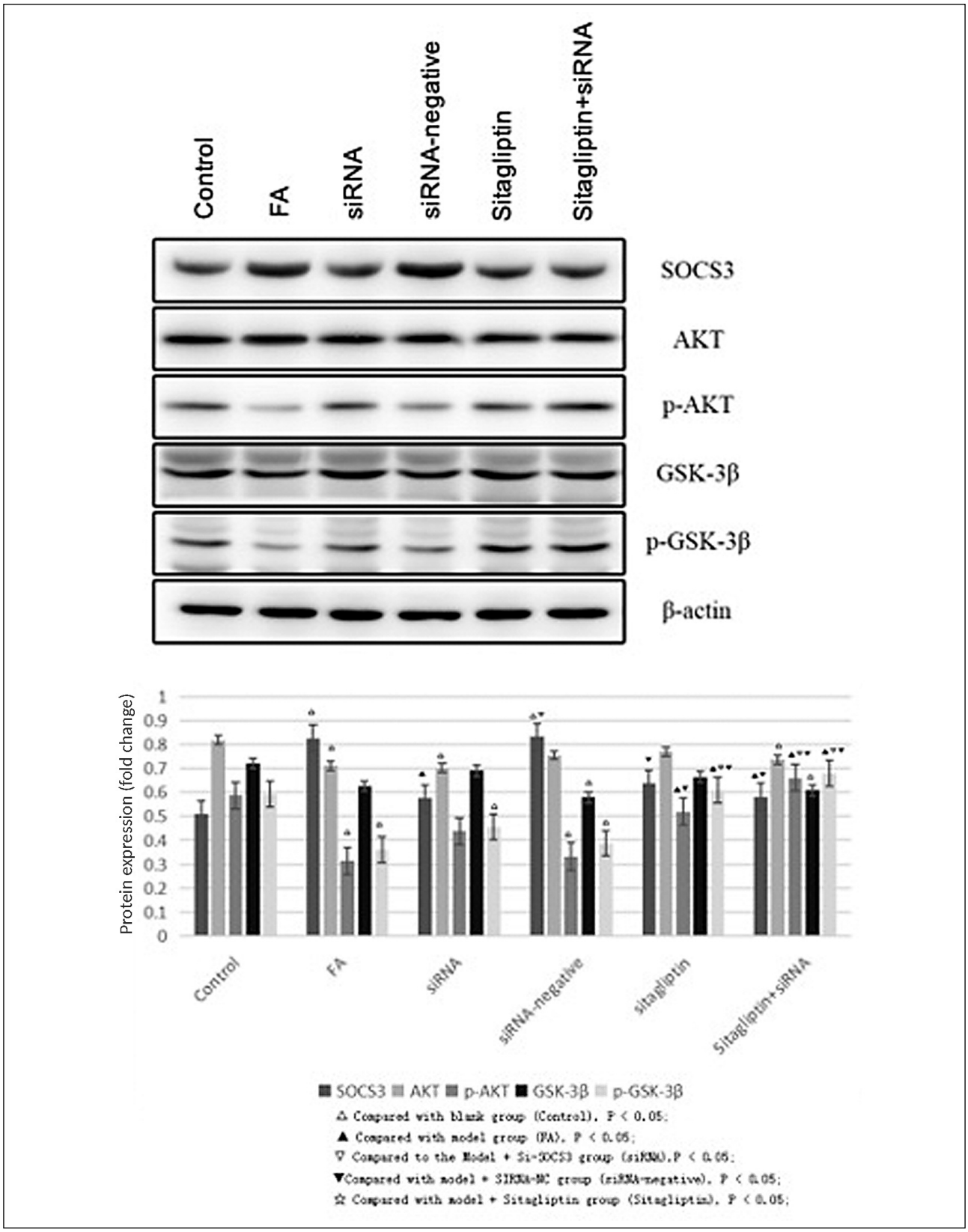

Fig. 6. Western blot analysis showing the protein bands (A), gray values of SOCS3 (B), Akt (C), p-Akt (D), GSK-3β (E), and p-GSK-3β (F); β-actin was used as an internal standard, and P<0.05 was considered statistically significant. The culture medium was discarded after adherence for 24 h. Following the intervention, the cells were digested by trypsin, and the supernatant was discarded after centrifugation to leave the cell precipitate. SOCS3, Akt, p-Akt, GSK-3β, and p-GSK-3β were detected by the standard operation of a Western blot assay. The SOCS3 (D6E1T) Rabbit mAb, Akt (Pan) (C67E7) Rabbit mAb, Phospho-Akt (Ser473) Antibody, GSK-3β (D5C5Z) XP Rabbit mAb, Phospho-gsk-3 α/β (Ser21/9) (37F11) Rabbit mAb (GSK-3α Preferred), β-actin as Preferred, goat Anti-Rabbit IgG H phospho-GSK-3 α/β (Ser21/9) (37F11) Rabbit mAb (GSK-3α Preferred), β-actin as Preferred; L (HRP). All experiments were repeated three times.

In conclusion: the expression of the SOCS3 gene was considerably increased in the insulin resistance model group, but decreased after sitagliptin intervention. In the model group, the protein levels of Akt, p-Akt, and p-GSK-3β were considerably reduced. After intervention with sitagliptin, SOCS3 was dramatically decreased, showing that sitagliptin could ameliorate insulin resistance by downregulating SOCS3 and regulating glucose metabolism in a hypoglycemic manner.

Funding: Autonomous Region Health Young Medical Science And Technology Talents Special Research Project,WJWY-202208,2021.6.1-2023.5.31.

Conflict of interests: None declared.

REFERENCES

1. Scott JL. Sitagliptin: a review in type 2 diabetes. *Drugs* 2017; 77: 209-224.
2. Mohan V, Yang W, Son HY, *et al*. Efficacy and safety of sitagliptin in the treatment of patients with type 2 diabetesin China, India, and Korea. *Diabetes Res Clin Pract* 2009; 83: 106-116.
3. Kim YG, Hahn S, Oh TJ, Kwak SH, Park KS, Cho YM. Differences in the glucose lowering efficacy of dipeptidyl peptidase-4 inhibitors between Asians and non-Asians: a systematic review and meta-analysis. *Diabetologia* 2013; 56: 696-708.
4. Ma R, Deng XL, Aleteng QQ, Li L, Zhu J. Genome-wide transcriptome analysis in type 2 diabetes patients treated by sitagliptin. *Diabetes Metab Syndr Obes* 2022; 15: 1761-1770.
5. Zaheri Z, Fahremand F, Rezvani ME, Karimollah A, Moradi A. Curcumin exerts beneficial role on insulin resistance through modulation of SOCS3 and Rac-1 pathways in type 2 diabetic rats. *J Funct Foods* 2019; 60: 103430. doi: 10.1016/j.jff2019.103430
6. Anitha M, Gondha C, Sutliff RR, *et al*. GDNF rescues hyperglycemia-induced diabetic enteric neuropathy through activation of the PI3K/Akt pathway. *J Clinical Invest* 2006; 116: 344-356.
7. Padiya R, Chowdhury D, Borkar R, Srinivas R, Pal Bhadra M, Banerjee SK. Garlic attenuates cardiac oxidative stress via activation of PI3K/AKT/Nrf2-Keap1 pathway in fructose-fed diabetic rat. *PLoS One* 2014; 9: e94228. doi: 10.1371/journal.pone.0094228
8. Zhang B, Fang L, Wu HM, Ding PS, Xu K, Liu RY. Mer receptor tyrosine kinase negatively regulates lipoteichoic acid-induced inflammatory response via PI3K/Akt and SOCS3. *Mol Immunol* 2016; 8: 98-107.
9. Jia GQ, Xu YH, Dong RQ. Advances in hepatogenic diabetes. *Clin Focus* 2009; 24: 1193-1194.
10. Gariani K, Philippe J, Jornayvaz FR. Non-alcoholic fatty liver disease and insulin resistance: from bench to bedside. *Diabetes Metab* 2013; 39: 16-26.
11. Ma H, Yan J, Zhang KB, *et al*. Mibefradil improves insulin-resistance HepG2 cells by down-regulating FoxO1 expression [in Chinese]. *J Third Military Med Univ* 2017; 39: 1973-1979.
12. Sun YJ, Xie Xm, Zhang J, *et al*. Research progress of the mechanisms on hepatic insulin resistance [in Chinese]. *Chin J Exp Surg* 2020; 37: 776-780.
13. Li B. Synthesis Of Humpanin A, Formononetin And Genistein Derivatives From Chickpea And Screening Of Their Hypoglycemic Activity Against Insulin-Resistant HEPG2 Cells. Beijing, Beijing University of Chinese Medicine, 2017.
14. Qi G, Guo R, Tian H, *et al*. Nobiletin protects against insulin resistance and disorders of lipid metabolism by reprogramming of circadian clock in hepatocytes. *Biochim Biophys Acta Mol Cell Biol Lipids* 2018; 1863: 549-562.
15. Siwiec E, Szynkowska A, Milkiewicz M, Marlicz W, Stachowska E. Diverse inhibition of forkhead box O1 activity by linoleic acid isomers - potential role in lipid metabolism in HepG2 cells and livers of C57BL/6J mice. *J Physiol Pharmacol* 2019; 70: 389-398.
16. Sakr HF, Abbas AM, Khalil K, Shata AM. Modulatory effect of concomitant administration of sitagliptin and vitamin E on inflammatory biomarkers in rats fed with high fat diet: role of adiponectin. *J Physiol Pharmacol* 2019; 70: 955-967.
17. Huang J, Li M, Zhou WJ, *et al*. Integrated miRNAand mRNAanalysisidentified potential mechanisms and targets of qianggan extracts in preventing nonalcoholic steatohepatitis. *World J Tradit Chin Med* 2022; 8: 77-86.
18. Heo JI, Yoon DW, Yu JH, *et al*. Melatonin improves insulin resistance and hepatic steatosis through attenuation of alpha-2-HS-glycoprotein. *J Pineal Res* 2018; 65: e12493. doi: 10.1111/jpi.12493
19. Cohen P. The Croonian Lecture 1998. Identification of a protein kinase cascade of major importance in insulin signal transduction. *Philos Trans R Soc Lond B Biol Sci* 1999; 354: 485-495.
20. Kim HS, Joo SS, Yoo YM. The correlation between amylin and insulin by treatment with 2-deoxy-D-glucose and/or mannose in rat insulinoma ins-1E cells. *J Physiol Pharmacol* 2021; 72: 517-528.
21. Turnbull J, Tiberia E, Pereira S, *et al*. Deficiency of a glycogen synthase-associated protein, Epm2aip1, causes decreased glycogen synthesis and hepatic insulin resistance. *J Biol Chem* 2013; 288: 34627-34637.
22. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glyeogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*1995; 378: 785-789.
23. Liu HF, Lu JM, Mu YM, *et al*. Effects of n-3 polyunsaturated free fatty acids on liver and muscle insulin receptor and GluT4 in the insulin resistance rats induced by high fatfeeding. *Chin J Diabet* 2005; 13: 192-195.
24. Woodgett JR. Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO J* 1990; 9: 2431-2438.
25. Lappas M. GSK3β is increased in adipose tissue and skelet al muscle from women with gestational diabetes where it regulates the inflammatory response. *PLoS One* 2014; 9: e115854. doi: 10.1371/journal.pone.0115854
26. Zhang T, Fang M, Fu ZM, *et al*. Expression of PI3K, PKB and GSK-3 β in the skelet al muscle tissue of gestational diabetes mellitus. *Asian Pac J Trop Med* 2014; 7: 309-312.
27. Motawi TM, Bustanji Y, El-Maraghy SA, Taha MO, Al Ghussein MA. Naproxen and cromolyn as new glycogen synthase kinase 3β inhibitors for amelioration of diabetes and obesity:an investigation by docking simulation and subsequent in vitro/in vivo biochemical evaluation. *J Biochem Mol Toxicol* 2013; 27: 425-436.
28. Li YX, Hong YZ. The mechanism and research progress of SOCS-3 in insulin resistance. *China Modern Doctor* 2006; 12: 163-168.
29. Wang J, Wu T, Fang L, *et al*. Anti-diabetic effect by walnut (Juglans mandshurica Maxim.)-derived peptide LPLLR through inhibiting α-glucosidase and α-amylase, and alleviating insulin resistance of hepatic HepG2 cells. *J Funct Foods* 2020; 69: 103944. doi: 10.1016/j.jff.2020. 103944
30. Chi YJ, Li J, Guan YF, *et al*. PI3K/Akt Signaling Axis in Regulation of Glucose Homeostasis. *Chin J Biochem Mol Biol* 2010; 26: 879-885.

31. Vanhaesebroeck B, Leevers SJ, Ahmadi K, *et al*. Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem* 2001; 70: 535-602.
32. Wang S, Jin L, Hai CX, *et al*. Research progress on the role of PI3K/Akt signaling pathway in insulin resistance. *J Toxicol* 2015; 029: 313-316.
33. Dai YX, Li YC, Liang FR. Research progress of PI3K/AKT/GSK3β pathway affecting glucose metabolism and cognitive process. *Cardiovasc Dis J Integr Tradit Chin Western Med* (electronic) 2018; 006: 28-29.

Received: October 31,2022
Accepted: April 30, 2023

Author's address: Dr. Jun Zhu; Department of Endocrinology, People's Hospital of Shenzhen Baoan District, No.118, Baocheng Longjing 2nd Road, Bao 'an District, Shenzhen, Guangdong 518101, China.
E-mail:zhujundr@163.com

Dr. Sheng Jiang, Department of Endocrinology, The First Affiliated Hospital of Xinjiang Medical University, No. 137, South Li Yu Shan Road, Urumqi, Xinjiang 830054, China.
E-mail: jiangshengdr_88@163.com