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ORIGINAL CONTRIBUTIONS

Gastric Sleeve Surgery Alleviates Obesity-Associated Insulin Resistance and Suppresses Endoplasmic Reticulum Stress in Adipose Tissue of *db/db* Mice

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Abstract

Objective The aims of this study were to evaluate the effects of gastric sleeve surgery on diabetes remission in *db/db* mice as well as to determine the underlying mechanisms.

Methods Thirty spontaneously obese, diabetic mice (C57BL/Ksj-*db/db*) were randomly divided into three groups: sleeve gastrectomy group, sham-operated group, and control *db/db* group. Ten *db/m* lean mice were used as nondiabetic littermate controls. All mice were sacrificed on day 28. The fasting plasma glucose, serum insulin, lipid profile, and oral glucose tolerance were measured pre- and postoperatively. Inflammatory cytokines (TNF-α and IL-6), endoplasmic reticulum (ER) stress–related markers (GRP78, PERK, IRE-1, and ATF6), and glucose transporter 4 (GLUT4) in the adipose tissue were assayed.

Results Sleeve gastrectomy significantly reduced the body weight and food intake in the *db/db* mice. This surgery improved glucose and lipid metabolism, as manifested by the decrease in the fasting plasma glucose level and partial restoration of lipid abnormalities. Also, the surgery improved glucose tolerance and alleviated insulin resistance in *db/db* mice. Sleeve gastrectomy surgery induced downregulation of the inflammatory adipocytokines TNF-α and IL-6; suppressed expression of the ER stress–related markers GRP78, PERK, IRE-1, and ATF-6; and increased the expression and distribution of GLUT4 in adipose tissue of *db/db* mice.

Conclusion The improvement in glucose tolerance following sleeve gastrectomy is associated with alleviation of insulin resistance, reduction of inflammatory adipocytokine levels, and suppression of ER stress. Further studies are needed to assess whether these effects have a causal role.

Keywords Gastric sleeve surgery · Type 2 diabetes mellitus · Obesity · Endoplasmic reticulum stress

Introduction

Morbid obesity and its related disorders, including type 2 diabetes mellitus (T2DM), have become a serious health problem worldwide. Obesity is typically characterized by an excessive amount of adipose tissue and marked changes in its secretory functions, together with chronic inflammation and a significantly increased risk of developing insulin resistance and diabetes [1–3].

Weight reduction is one of the most important therapeutic strategies to achieve glycemic control for obese patients with T2DM [4]. Diet, physical activity, and pharmacotherapy are common means assisting patients to lose weight; however, bariatric surgery has been shown to be the most effective way to maintain long-lasting weight loss, especially for diabetic patients with morbid obesity [5]. Sleeve gastrectomy is a type of bariatric surgery; it produces a resection of approximately 80% of the stomach, leading to a significant reduction of gastric capacity. To date, sleeve gastrectomy has gradually become a widely accepted surgical procedure, although its effectiveness and safety are still controversial [6]. Sleeve gastrectomy has been found to reduce body mass and improve

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glucose metabolism in both experimental models as well as clinical settings [7, 8]. As reported by some nonrandomized clinical studies, this technique displays an almost similar efficacy in remission of T2DM and normalization of insulin resistance in obese individuals as compared with the standard Roux-en-Y gastric bypass procedure [9, 10]. However, the mechanisms by which sleeve gastrectomy improves glucose metabolism and insulin resistance are not fully understood.

Endoplasmic reticulum (ER) stress has been implicated in obesity-associated insulin resistance in peripheral tissues, such as adipose tissue and skeletal muscle [11, 12]. Obesity leads to changes of the energy metabolic status, characterized by the increase of anabolism and the decrease of catabolism, which may result in the accumulation of unfolded proteins in the ER lumen and eventually ER stress [12]. ER stress activates the unfolded protein response (UPR), which is mediated via three major ER transmembrane proteins, PKR-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE1), and activating transcription factor-6 (ATF6), transmitting signals from the ER to the cytoplasm or nucleus [13]. Under unstressed conditions, immunoglobulin protein/glucose-regulated protein 78 (GRP78), as a central regulator of ER homeostasis, binds to these proteins to inhibit their activation. In adipose tissue and skeletal muscle, obesity-induced ER stress leads to chronic inflammation and increased cytokine production [14]. On the other hand, reduction in insulin-stimulated phosphorylation of insulin receptors, translocation of glucose transporters, and defective insulin signaling may be, at least in part, responsible for insulin resistance [15]. However, there has been no research focusing on the postoperative changes in ER stress or the insulin-responsive glucose transporter 4 (GLUT4) in adipose tissues of obese diabetic individuals.

In this study, we assessed the role of sleeve gastrectomy in diabetes remission by using an obese diabetic *db/db* mouse model. In addition, the underlying mechanisms, in terms of ER stress, the inflammatory response, and GLUT4 in the adipose tissue, were also elucidated.

Materials and Methods

The experimental protocols were approved by the Ethics Committee of Shengjing Hospital of China Medical University.

Animals

Male obese diabetic mice (C57BL/Ksj-*db/db*, 12–18 weeks old, $n = 30$) and lean wild-type littermate controls (*db/m*, $n = 10$) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). The *db/db* mice were randomly allocated into three groups: the *db/db*-control group ($n = 10$), the *db/db*-sham group ($n = 10$) that received a sham operation, and the *db/db*-SG group ($n = 10$) that received sleeve gastrectomy. Another ten age-matched *db/m* mice were used as nondiabetic controls. All animals were maintained on a normal rodent chow diet and housed in individual cages in a constant environment with a natural light/dark cycle, room temperature of 26 ± 1 °C, and relative humidity of 50 ± 2%. They were allowed to acclimatize to their surroundings for 10 days before the operation.

Surgical Procedures

The mice in the *db/db*-SG group underwent gastric sleeve surgery after a 10-day adaptation period. The mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and placed on a surgical board in the supine position with their extremities immobilized. An epigastric incision of approximately 1.5 cm in length was made and was kept open during surgery via the use of a retractor. A gastric tube was placed from the distal antrum (1.5–2 mm from the pylorus) to the Hiss angle. Nearly 80% of the stomach, including the fundus and the great curvature, was completely removed. The gastric tube was closed with 9-0 nonabsorbable sutures. Then, the abdomen was lavaged with saline and closed with 6-0 silk sutures. Sham-operated control mice were subjected to an abdominal incision without removal of the stomach. All mice were given an intraperitoneal infusion of penicillin (80,000 U/100 g body weight) to prevent infection before closing the peritoneal cavity. Moreover, 5 mL of 0.9% saline was administered subcutaneously to avoid dehydration. The mice were returned to their respective home cages and fed with liquid food for the first day following the surgery and thenceforth with regular chow.

Measurement of Body Weight and Food Intake

Body weight and food intake were measured before the surgery as well as 7, 14, 21, and 28 days postoperatively.

Oral Glucose Tolerance Test

The oral glucose tolerance test (OGTT) was performed before and 28 days after the surgery. All mice were fasted for 12–14 h, and then, they were intragastrically administered with glucose solution (0.15 g/100 g body weight). The blood glucose concentrations in the tail-vein samples were measured with a Breeze portable glucose meter (Bayer, Germany) at the following time points: 0, 30, 60, and 120 min.

Sample Collection

All mice were sacrificed using CO_2 and cervical dislocation on postoperative day 28. Blood samples were collected from the inferior vena cava and centrifuged at 4000*g* and 4 °C for

10 min to obtain the plasma samples, which were stored at − 20 °C for further use. The epididymal adipose tissue was collected and frozen in liquid nitrogen.

Biochemical Analysis

After fasting for 12–14 h, the blood glucose levels of mice were measured every week with a Breeze portable glucose meter (Bayer, Germany) at the same time point (08:00 A.M.).

Plasma concentrations of insulin, total cholesterol (TC), triglycerides (TG), and low-density lipoprotein (LDL) at day 28 were measured using enzyme-linked immunosorbent assay (ELISA) commercial kits (R&D system, Minneapolis, MN, USA), according to the manufacturer's instructions.

Insulin resistance was assessed according to the homeostatic model assessment–estimated insulin resistance (HOMA-IR) with the formula HOMA-IR = fasting glucose (mmol/L) × fasting insulin (mIU/L)/22.5.

Quantitative Real-time Polymerase Chain Reaction (PCR)

Total RNA was extracted from epididymal adipose tissues with TRIzol (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed into complementary DNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed on a 7500 real-time PCR system (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems). The primers were obtained from Takara (Takara Bio Shiga, Japan). The sequences used for analysis were as follows: interleukin (IL)-6, 5′-CCTC TGGTCTTCTGGAGTACC-3′ (forward), 5′-ACTC CTTCTGTGACTCCAGC-3′ (reverse); tumor necrosis factor (TNF)-α, 5′-TACTGAACTTCGGGGTGATTGGTCC-3′ (forward), 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′ (reverse); β-actin, 5′-CGCCACCAGTTCGCCATGGA-3′ (forward), 5′-TACAGCCCGGGGAGCATCGT-3′ (reverse). The thermal conditions were as follows: 2 min at 50 °C and 10 min at 94 °C (initial denaturation), and then 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. Each PCR amplification was performed in triplicate.

Western Blotting Analysis

Total protein of epididymal adipose tissues was extracted and then quantified with a BCA protein assay kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, equal amounts of protein (15 μg) were loaded onto a sodium dodecylsulfate-polyacrylamide gel, separated by electrophoresis, transferred to a polyvinylidene fluoride membrane (Thermo Scientific, Rockford, IL, USA), and blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween 20 (TBST) solution, followed by incubation with the following primary rabbit polyclonal antibodies: IL-6 (Invitrogen; dilution 1:400), TNF-α (Invitrogen; dilution 1:400), GRP78 (Cell Signaling Technology, Danvers, MA, USA; dilution 1:200), PERK (Abcam, Cambridge, UK; dilution 1:400), IRE1 (Abcam; dilution 1:400), ATF6 (Abcam; dilution 1:400), and β-actin (Santa Cruz; dilution 1:1000) overnight at 4 °C, respectively. After washing three times with TBST, the membranes were incubated with horseradish peroxidase–conjugated anti-rabbit IgG secondary antibody at 1:2000 dilution for 1 h at room temperature. Bands were visualized with an enhanced chemiluminescence detection kit (Amersham Biosciences, Little Chalfont, UK) and quantified with Image J software.

Immunohistochemistry

Epididymal adipose tissues were fixed with 4% paraformaldehyde for 24 h, followed by routine paraffin embedding and sectioning (4 μm) procedures. Immunohistochemical staining was performed using a SABC kit (BosterBio, Wuhan, China). The sections were subjected to gradient ethanol hydration and dewaxing with xylene, and then, they were boiled for 2 min in citrate buffer in an autoclave for antigen retrieval. The sections were treated with 3% H_2O_2 to quench endogenous peroxidase, and then, they were incubated with primary anti-GLUT4 antibody (Abcam) at 1:400 dilution overnight at 4 °C. After washing, the sections were incubated with secondary biotinylated antibody (Dako Inc., Denmark) for 30 min at 37 °C. After adequate washing with phosphate-buffered saline, horseradish peroxidase–conjugated streptavidin was added and the sections were incubated for 30 min at 37 °C. Immunoreactivity was visualized using a 3,3′-diaminobenzidine kit (Abcam) and counterstained with Gill's hematoxylin. Each slide was scored by observing five random microscopic visual fields by two independent pathologists in a blind manner. The positive immunostaining presented as brown granules in the cytoplasm and/or cell membrane. The intensity of GLUT4 expression was graded as follows: 0, absent; 1, weak; and 2, strong. Percentage scores were assigned as follows: 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%. Total scores were acquired by multiplying the intensity score and the percentage score. The expression level of GLUT4 was judged as negative if the total score was < 4 or positive if the total score was > 4.

Statistical Analysis

Statistical analyses were performed using SPSS statistical software (version 19.0, USA). Data were expressed as the mean ± standard deviation (SD). Differences between groups were assessed using the nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U* test. *P* values less than 0.05 denoted significance.

Results

General Condition of the Animals

Only one mouse, which was in the *db/db*-SG group, died of incision infection during the experimental period. The remaining mice survived and recovered from the surgery. In general, the *db/db* diabetic mice had a greater body weight and food consumption than those in the *db/m* group. The body weight and food intake were not significantly different among the three *db/db* subgroups preoperatively. However, sleeve gastrectomy resulted in a deceased body weight as well as food intake in the *db/db*-SG group, especially at the first week postoperatively. Of note, the body weight and food intake in the *db/db*-SG group were still higher than those of the *db/m* group. No differences were observed in body weight or food intake between the *db/db*-sham and *db/db*-control groups (Fig. 1).

Fasting Blood Glucose and Glucose Tolerance

The *db/db* diabetic mice exhibited a higher fasting blood glucose (FBG) level than their nondiabetic controls (*db/m*) preoperatively. However, sleeve gastrectomy induced a decrease in the blood glucose concentration to a nearly normal level at days 7 and 14 (Fig. 2a), which was significantly lower than that in the *db/db*-control or *db/db*-sham group throughout the 28-day follow-up period.

The OGTT was performed before and 28 days after the surgery to evaluate the efficacy of sleeve gastrectomy on glucose tolerance (Fig. 2b, c). The mice treated with sleeve gastrectomy had significantly lower blood glucose levels at all time points tested (0, 30, 60, and 120 min), compared with those of the *db/db*-control and *db/db*-sham mice at postoperative day 28. However, the blood glucose levels in the *db/db*-SG group were still higher than those of their lean controls (*db/m* mice), $P < 0.05$ at all time points. These results were further confirmed by the area under the curve (AUC) of glucose in the OGTT (Fig. 2d), suggesting an improvement in glucose tolerance by sleeve gastrectomy in obese diabetic mice.

Insulin Level and HOMA-IR

Fasting circulating insulin and FBG were used to calculate the HOMA-IR on postoperative day 28 for assessing insulin resistance. The mice in the *db/db*-SG group exhibited a significantly lower HOMA-IR, compared with the *db/db*-control and *db/db*-sham groups, suggesting that sleeve gastrectomy contributed to the alleviation of insulin resistance in obese diabetic mice (Table 1).

Lipid Metabolism

Compared with the *db/db*-control and *db/db*-sham groups, the plasma concentrations of TC, TG, and LDL in the *db/db*-SG group were significantly lower after the surgery (Fig. 3). However, only the plasma concentrations of TC in the *db/db*-SG group decreased to a level comparable with that of the *db/m* group ($P > 0.05$).

Expression of IL-6 and TNF-α in Epididymal Adipose Tissues

Based on the quantitative PCR and Western blotting analysis results, sleeve gastrectomy was associated with significantly decreased expression levels of IL-6 and TNF-α, at both the mRNA and protein levels, in epididymal adipose tissues (Supplementary Fig. 1).

Expression of ER Stress-Related Proteins

To evaluate the effects of sleeve gastrectomy on ER stress, we detected the expression levels of ER stress–related marker proteins in epididymal adipose tissue by Western blotting analysis. As shown in Fig. 4, the expression levels of

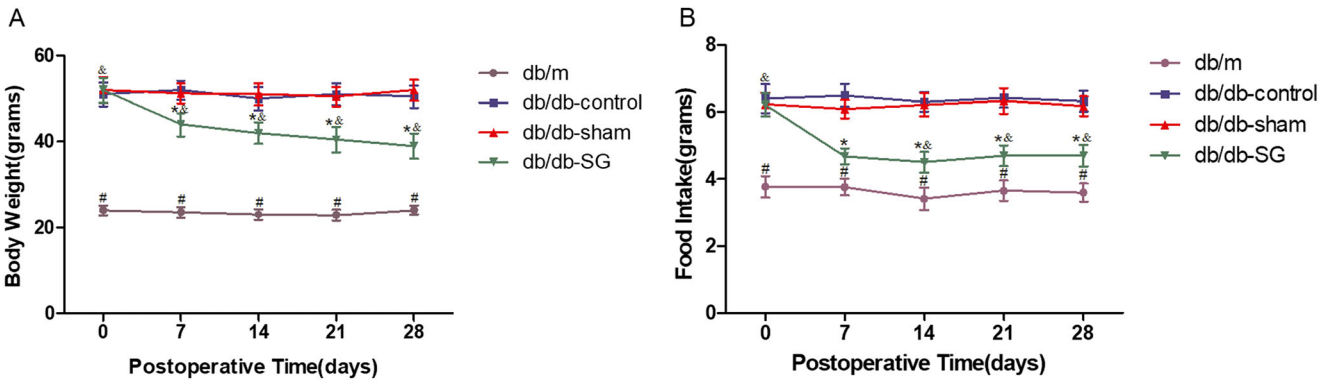

Fig. 1 Changes in body weight and food intake before and after gastric sleeve surgery. **a** Body weight and **b** food intake were measured weekly. $^{*}P < 0.01$ compared with the *db/db*-control or *db/db*-sham group; $^{\#}P < 0.01$ compared with all the *db/db* subgroups; $^{\&}P < 0.05$ compared with the *db/m* subgroup

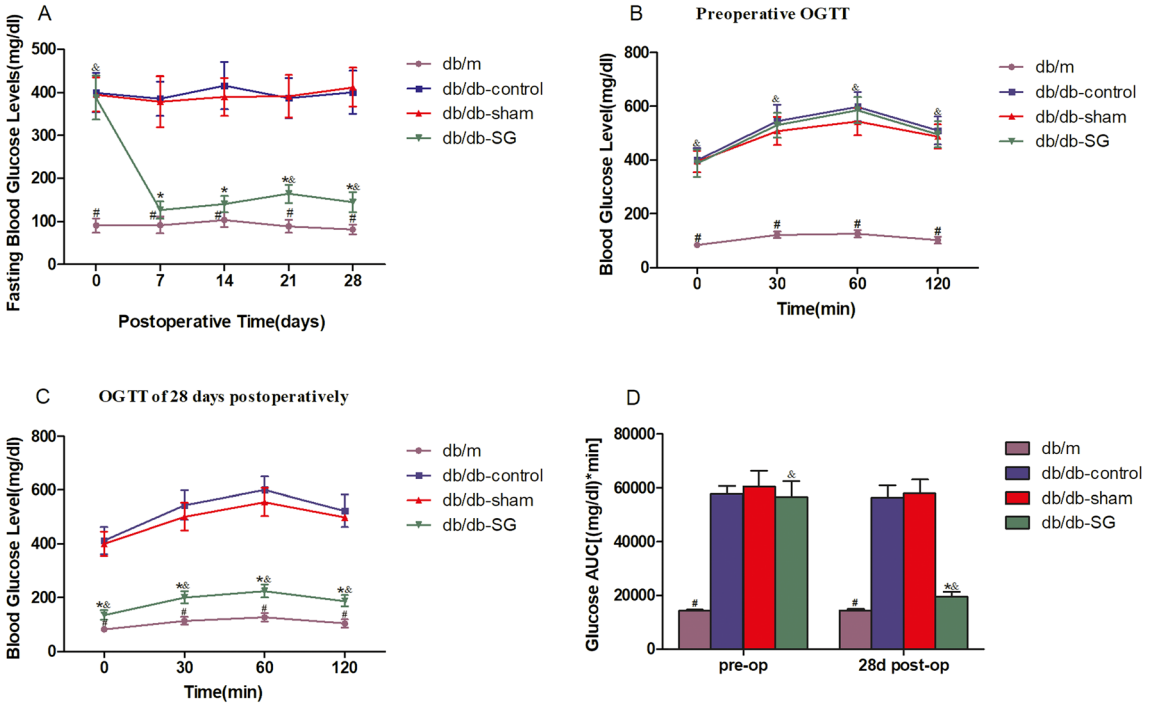

Fig. 2 Postoperative FBG and OGTT tests. **a** FBG concentrations were measured weekly. The OGTT test was conducted preoperatively (**b**) and at postoperative day 28 (**c**). **d** The OGTT results were analyzed by the area under the curve (AUC) of the blood glucose level. $^{*}P < 0.01$ compared with the *db/db*-control or *db/db*-sham group; $^{\#}P < 0.01$ compared with all the *db/db* subgroups; $^{\&}P < 0.05$ compared with the *db/m* subgroup. FBG, fast blood glucose; OGTT, oral glucose tolerance test

GRP78, PERK, IRE1, and ATF in the *db/db*-control group were approximately 4.05-fold, 2.60-fold, 5.23-fold, and 4.77-fold of those of the *db/m* group, respectively. However, the *db/db*-SG group showed significantly decreased expression levels of these proteins postoperatively, compared with the *db/db*-control and *db/db*-sham groups, even to a level similar to that of the *db/m* group (GRP78, PERK, and ATF6), suggesting that sleeve gastrectomy facilitated the alleviation of ER stress in adipose tissue.

GLUT4 Detection in Adipose Tissue by Immunohistochemical Analysis

Based on the improvement of ER stress, we also examined the effect of sleeve gastrectomy on GLUT4 expression by immunohistochemical staining. Compared with the *db/db*-control and *db/db*-sham groups, the expression and distribution of GLUT4 on the cell membrane were significantly increased in the epididymal adipose tissue of the *db/db*-SG group (Fig. 5).

Table 1 Homeostatic model assessment–estimated insulin resistance (HOMA-IR) at postoperative day 28

Group	Fasting insulin (mIU/L)	HOMA-IR
db/m	$1.81 \pm 0.25^{\#}$	$0.52 \pm 0.18^{\#}$
db/db-control	9.82 ± 0.42	9.28 ± 1.95
db/db-sham	10.55 ± 0.47	10.16 ± 2.33
db/db-SG	$5.66 \pm 0.58^{*}$	$2.24 \pm 0.27^{*}$

$^{*}P < 0.01$ compared with the *db/db*-control or *db/db*-sham group; $^{\#}P < 0.01$ compared with all the *db/db* subgroups

Discussion

In this study, we used an obese diabetic *db/db* mouse model to examine the role of sleeve gastrectomy in diabetes remission and the underlying molecular mechanisms. The results showed that sleeve gastrectomy significantly decreased the body weight and food intake, improved hyperglycemia and insulin resistance, and restored glucose and lipid metabolism to almost normal levels. The beneficial effects of sleeve gastrectomy may be associated with its capacity to alleviate ER stress and downregulate inflammatory adipocytokine levels, although this is probably a simple effect due to weight loss. These data add to our knowledge of the mechanism by which sleeve gastrectomy improves insulin resistance.

Sleeve gastrectomy has been reported to induce remission of diabetes by mechanisms, in part, involving weight loss as well as reduction of insulin resistance and improvement of β cell function [16, 17]. Numerous studies have demonstrated that sleeve gastrectomy contributes to overall glycemic control and lipid metabolism [18, 19]. The beneficial effects of

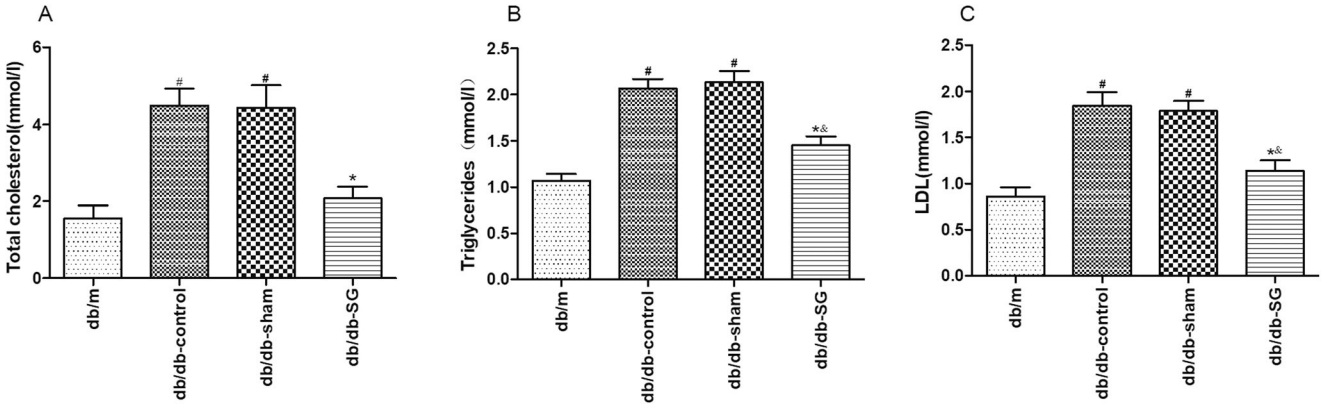

Fig. 3 Measurements of lipid metabolism parameters. The total cholesterol (**a**), triglyceride (**b**), and LDL (**c**) levels were measured at postoperative day 28. $^{*}P < 0.01$ compared with the *db/db*-control or *db/db*-sham group; $^{\#}P < 0.01$ compared with all the *db/db* subgroups; $^{\&}P < 0.05$ compared with the *db/m* subgroup. LDL, low-density lipoprotein

sleeve gastrectomy on obese diabetics were further confirmed in our study. Adipocytes secrete a number of proinflammatory factors; among them, TNF-α and IL-6 have been proposed to be linked with insulin resistance, obesity, and T2DM [20]. Consistent with our findings, sleeve gastrectomy has been demonstrated to produce robust improvement in inflammation and oxidative stress, with the decreased expression of several adipokines, including TNF-α, IL-6, monocyte chemoattractant protein (MCP)-1, C-reactive protein, leptin, and adiponectin, in obese patients [21, 22], suggesting the benefits of this technique in the alleviation of obesity-induced inflammation.

Persistence of obesity is likely to induce ER stress, followed by activation of UPR signaling in adipocytes, leading to metabolic disorders, inhibition of insulin signal transduction, the inflammatory response, and cytokine production [23]. In diabetic rats, sleeve gastrectomy significantly decreased the expression levels of GRP78, PERK, and CHOP, but not IRE1α or ATF6 in myocardial tissue [24]. In this study, we found that diabetic *db/db* mice that underwent sleeve gastrectomy had lower levels of PERK, IRE1, and ATF6 as well as the chaperone protein GRP78, compared with those of the controls. The possible reasons behind the beneficial effect of sleeve gastrectomy on ER stress are as follows. First, sleeve gastrectomy may reduce food intake and delay gastric emptying, resulting in less calorie consumption, which decreases the ER load and alleviates ER stress. Second, sleeve gastrectomy exerts a positive effect on the secretion of various gastrointestinal hormones, such as glucagon-like peptide 1, glucose-dependent insulinotropic peptide [25], and adipocytokines

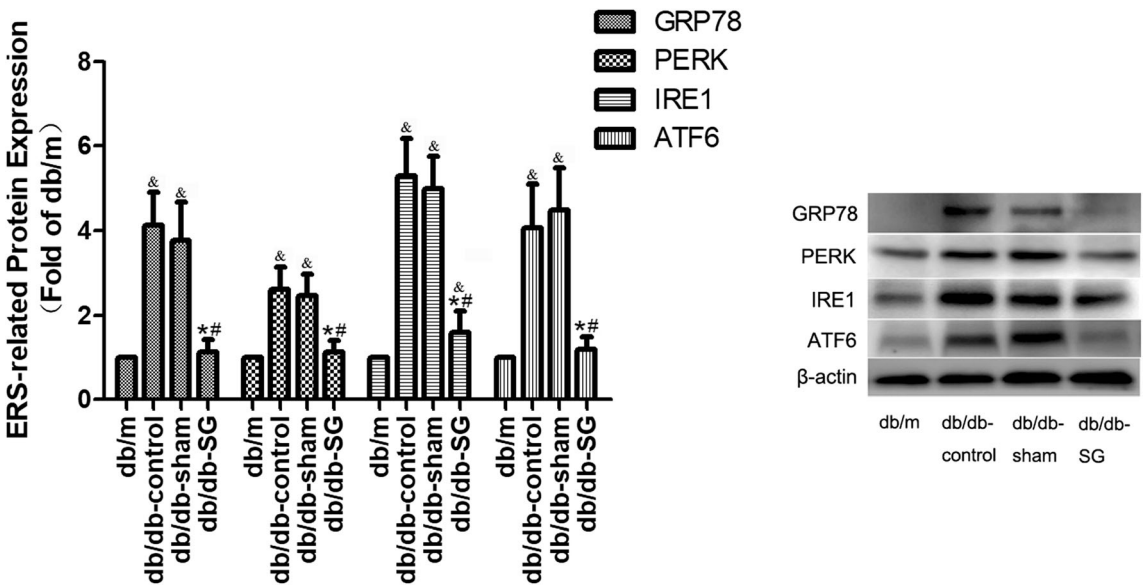

Fig. 4 Expression levels of the ER stress–related proteins GRP78, PERK, IRE1, and ATF6 in epididymal adipose tissues. Data are presented as the fold increase relative to those of *db/m* mice after normalization to β-actin. $^{*,\#}P < 0.01$ as compared with the *db/db*-control and *db/db*-sham groups, respectively; $^{\&}P < 0.01$ compared with the *db/m* group

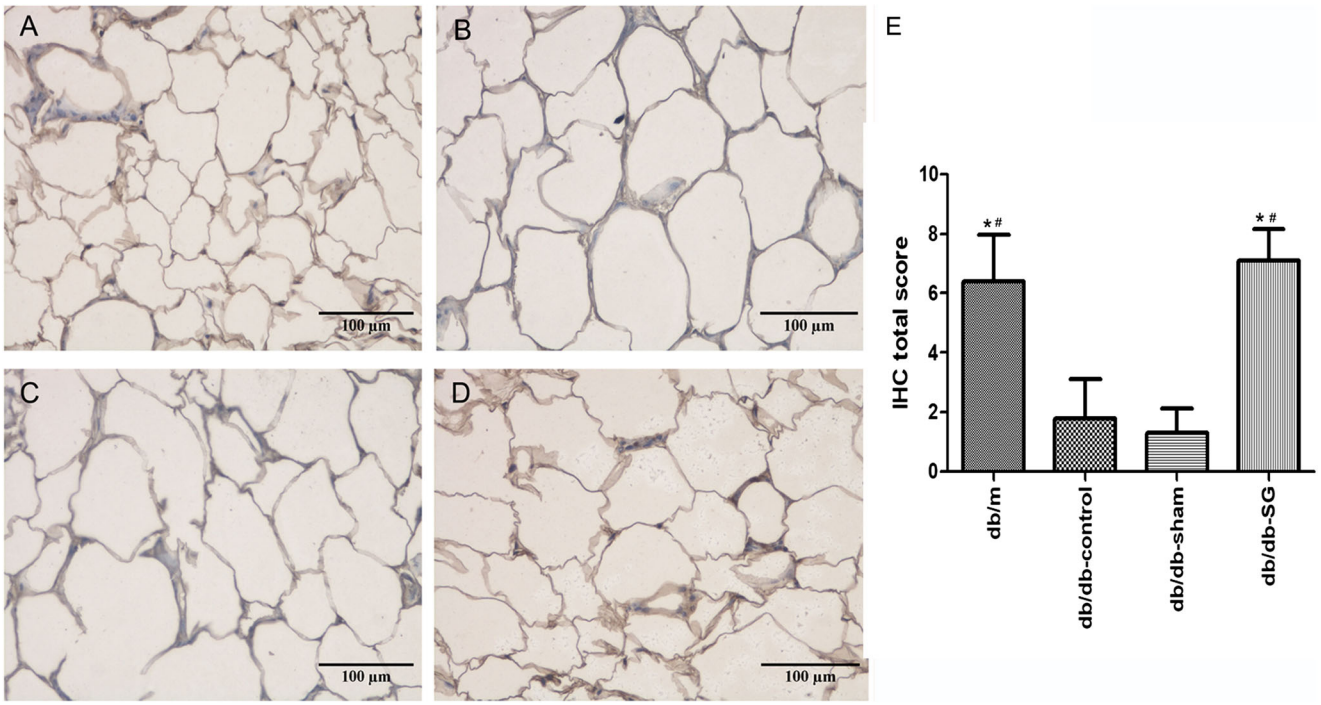

Fig. 5 Immunochemical staining of GLUT4 in epididymal adipose tissue. **a** *db/m* group (6 points, positive); (**b**) *db/db*-control group (2 points, negative); (**c**) *db/db*-sham group (1 point, negative); (**d**) *db/db*-SG group (8 points, positive). Magnification, × 200. **e** The quantitative analysis of GLUT4 expression. *, $^{\#}P < 0.01$ as compared with the *db/db*-control and *db/db*-sham groups, respectively

[26], which may promote homeostasis in vivo and consequently optimize the interaction between ER stress and inflammatory conditions in insulin-sensitive tissues.

Glucose uptake in adipose cells is considered to be mediated by glucose transporter proteins. GLUT4 is an insulin-stimulated glucose transporter that facilitates insulin-stimulated glucose uptake into skeletal muscle and adipose tissue [27]. Defects of GLUT4 in adipose tissue, including decreases of protein content and mislocalization, are early signs of metabolic diseases involving insulin resistance [28, 29], while overexpression of GLUT4 improves insulin sensitivity and lipid metabolism [30]. In this study, we found that sleeve gastrectomy induced an increase in the expression and distribution of GLUT4 on the cell membrane of epididymal adipose cells. Consistently, Nausheen et al. also found that the protein abundance of GLUT4 in muscle and adipose tissue was increased following sleeve gastrectomy surgery, which was accompanied by glycemic improvement [31]. These data suggest the beneficial role of sleeve gastrectomy on insulin-stimulated glucose transport and postoperative glucose metabolism.

Obesity-related metabolic disorders are attributed to a variety of factors, including ER stress, mitochondrial dysfunction, insulin resistance, inflammation, and impaired glucose transport and uptake, among which each is interconnected with others, leading to the exacerbation of disease. As we found in this study, sleeve gastrectomy induced weight loss, alleviation of insulin resistance, reduction of inflammatory cytokines, promotion of glucose transport, as well as attenuation of ER stress. These findings suggest that sleeve gastrectomy is effective for diabetes remission, and the mechanisms are multifactorial. However, in-depth analysis of the mechanisms, for instance, how sleeve gastrectomy contributes to the alleviation of insulin resistance, is needed.

This study explored the mechanism underlying the improvement of insulin resistance following sleeve gastrectomy from the perspective of regulation of ER stress in adipose tissue, which has not been reported previously. However, this study had several limitations. Firstly, this study lacked a control group of sham-operated mice that was pair-fed to the SG group. Thus, it is difficult to tease apart the relative contribution of the surgery versus that of decreased food intake as compared with the genetically similar (*db/db*) studied mice. Secondly, the effects of SG on glucose and lipid metabolism as well as ER stress were only detected in adipose tissue. Further investigation is needed to verify the effects of SG on other target organs or tissues such as skeletal muscle and the liver.

In conclusion, by using an obese, diabetic mouse model, the improvement in glucose tolerance following sleeve gastrectomy was associated with alleviation of insulin resistance, reduction of inflammatory adipocytokine levels, and suppression of ER stress. The data in this study provide experimental evidence elucidating the potential mechanisms of a beneficial effect of SG on diabetes remission.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical Approval The experimental protocols were approved by the Ethics Committee of Shengjing Hospital of China Medical University. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Informed Consent Not applicable.

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