

Received: 2019.08.19

Accepted: 2019.10.29

Published: 2019.11.13

Gegen Qinlian Decoction Ameliorates Hepatic Insulin Resistance by Silent Information Regulator1 (SIRT1)-Dependent Deacetylation of Forkhead Box O1 (FOXO1)

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Source of support: This study was supported by the National Natural Science Foundation of China (No. 81603585)

Background: Gegen qinlian decoction (GGQLD) is a form of traditional Chinese medicine used for hundreds of years for its efficacy in treating diabetes. However, the mechanisms underlying the therapeutic effects of GGQLD on diabetes are still not clear. We aimed to evaluate the effect of GGQLD on hepatic insulin resistance (IR) through silent information regulator1 (SIRT1)/forkhead box O1 (FOXO1) in an IR mouse model.

Material/Methods: A high-fat diet (HFD) mouse model was established and GGQLD was administrated by oral gavage. Metabolic parameters were detected, including body weights, triglyceride, fasting glucose, fasting insulin and HOMA-IR index, glucose intolerance, and insulin resistance. HE-stained sections were used to observe the histopathology of liver tissue. For *in vitro* study, GGQLD-medicated serum was used to treat palmitic acid-stimulated HepG2 cells. The glycogen synthesis and downstream SIRT1/FOXO1 signaling pathways were examined. Specific siRNAs were used to knock down SIRT1 in HepG2 cells.

Results: GGQLD administration significantly decreased body weights, triglyceride level, fasting glucose level, fasting insulin level, and HOMA-IR index, and improved IR in HFD mice. GGQLD enhanced SIRT1 expression and suppressed the expression of Ac-FOXO1 in liver tissues. Further, GGQLD-medicated serum promoted SIRT1 upregulation and suppressed Ac-FOXO1 levels in palmitate-stimulated HepG2 cells. GGQLD-medicated serum also increased the protein expression of PPAR γ and reduced the expression of FABP4 in palmitate-stimulated HepG2 cells.

Conclusions: We found that GGQLD alleviates insulin resistance through SIRT1-dependent deacetylation of FOXO1.

MeSH Keywords: **Diabetes Mellitus • Forkhead Transcription Factors • Hep G2 Cells • Insulin Resistance • Sirtuin 1**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/919498>

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Background

Diabetes and its chronic complications have become a global public health problem that seriously affects human health. A recent study has shown that there are 425 million diabetic patients worldwide [1]. Clinical statistics show that over 90% of diabetes cases are type 2 diabetes mellitus (T2DM). Insulin resistance is responsible for the pathogenesis of T2DM and can be found in muscle, adipose tissue, and liver, among which hepatic IR is a key mediator of the progression of T2DM [2,3].

Traditional Chinese medicine has been commonly used in China for millennia and is a subject of increasing global interest. Herbal extracts contain various chemical components that can treat diabetes by targeting various pathological pathways, controlling complex disease systems. Gegen Qinlian Decoction (GGQLD) is a famous prescription in traditional Chinese medicine, which was originally described in “*Shanghan Lun*” compiled by Zhong-Jing Zhang. GGQLD is formulated from 4 herbs: *Radix puerariae*, *Radix scutellariae*, *Rhizoma coptidis*, and *Radix glycyrrhizae*. In previous studies, it has been found that various parts of GGQLD and their isolated components were effective in treating diabetes [4–6].

SIRT1, an NAD⁺-dependent deacetylase, is involved in the improvement of several metabolic diseases. SIRT1 can modulate hepatic glucose metabolism by interacting with PGC1 α [7] and promotes fat mobilization [8]. SIRT1 can directly increase insulin sensitivity in the liver [9] and has been closely linked to insulin resistance in many different pathological conditions. SIRT1 can regulate the insulin pathway by enhancing the phosphorylation of insulin receptor [10]. FOXO1 is an important downstream factor of insulin resistance in the progression of T2DM. Under the condition of insulin resistance, the phosphorylation level of FOXO1 decreases, and the inhibition of FOXO1 expression can improve insulin sensitivity of the liver [11]. Multiple studies have suggested a link between FOXO1 and SIRT1 [12]. SIRT1 was shown to regulate the activity of FOXO1 by deacetylating of FOXO1, which promotes the nuclear retention of FOXO1 and maintains the FOXO1-induced signaling pathway [12–15].

GGQLD can treat lipid metabolism and inflammation by stimulating the SIRT1 pathway [16]. The evidence presented above suggests that GGQLD acts through the SIRT1/FOXO1 pathway to regulate liver insulin sensitivity in T2DM. To achieve this, GGQLD was used to treat T2DM in C57BL/6J mice. We used pioglitazone, which can reduce insulin resistance by stimulating PPAR γ , as a positive control treatment. Based on multiple parameters such as body weight, triglyceride, fasting glucose, fasting insulin, and HOMA-IR index we found that IR in high-fat diet (HFD) mice was significantly improved by oral GGQLD administration. We also studied the molecular mechanisms using

HepG2 cells and found that GGQLD acts on the SIRT1/FOXO1 pathway to improve hepatic insulin resistance.

Material and Methods

Preparation of GGQLD and pioglitazone

All herbs used in the study were purchased from the Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine. GGQLD was composed of 4 herbs – *Radix puerariae* (batch number: 20170301), *Radix scutellariae* (batch number: 1610009), *Rhizoma coptidis* (batch number: 1703015), and *Radix glycyrrhizae* (batch number: 170109) – at a ratio of 8: 3: 3: 2 (w/w/w/w). The preparation of GGQLD was made according to the method described in the literature [17]. Pioglitazone was purchased from MedChem Express, China (Cat. No: HY-14601) and configured to a suspension with a final concentration of 2 mg/ml. Each medicine was stored at 4°C until use.

Animals

Forty-eight 12-week-old SPF male C57BL/6J mice weighing 18–20 g were obtained from Shanghai Slack Laboratory Animal Co. (Shanghai, China). The guidelines for the use of laboratory animals were followed for all experiments. Animals were maintained in a climate-controlled facility (temperature 22±2°C) with a 12-h light/dark cycle. C57BL/6J mice were split into a normal fat diet (NFD) group (n=12) and an HFD group (n=36). Mice in the NFD group were fed a standard chow diet, while mice in the HFD group were fed a diet containing 60% fat, 14.1% protein, and 25.9% carbohydrate for 6 weeks. HFD group mice were equally divided into 3 groups: an HFD group (n=12) that received saline (10 ml/kg/day), a GGQLD group (n=12) that received GGQLD (10 g/kg/day), and a PIO group (n=12) that received pioglitazone (20 mg/kg/day). The NFD group (n=12) were also administrated with saline (10 ml/kg/day). All these doses were given via oral gavage daily over 8 weeks. At the end of the experiment, 10 mice in each group survived because of gastric administration. Body weight was measured every 2 weeks. Food was removed 2 h before measuring the weight. The Animal Ethics Committee of Jiangsu Province Hospital on Integration of Chinese and Western Medicine approved this study (license No. SCXK (Shanghai) 2012-0002).

Preparation of drug-containing serum

We obtained 8-week-old male Sprague-Dawley rats (200±20 g) from the Comparative Medicine Centre of Yangzhou University (license number: SCXK (Su) 2017-0007) and housed them in an environment with temperature 22±2°C, humidity 55±5%, and a 12-h light/dark cycle. Animals had free access to water and food.

Healthy SD rats were randomly divided into 2 groups: a GGQLD-treated group and a normal saline group (n=25 each). The rats were intragastrically administered GGQLD or saline at 8 a.m. and 8 p.m., respectively, for 4 consecutive days. The administered dosage of GGQLD was 6 ml/100 g/d body weight, at the decoction concentration of 1 g/ml. The administered dosage of normal saline was 10 ml/kg/d.

Abdominal aortic blood samples were taken after the last dose. The serum samples were inactivated using a water bath and sterilized, and then stored at -80°C until use. The serum containing drug was named GGQLD-medicated serum.

Determination of metabolic parameters

IPGTT and IPITT were tested at the 8th week of treatment. We performed IPGTT and IPITT according to methods described in the literature [18]. After mice were sacrificed, blood samples were taken and centrifuged. The serum was stored at -20°C . The levels of serum triglycerides (TG) and plasma insulin were measured by ELISA Assay Kit (ALPCO, USA). Glucose concentrations were determined from the tail vein by glucose monitor.

Histological analysis

Fresh liver tissues were collected upon experiment termination, fixed in 10% formaldehyde, and subjected to paraffin embedding. Paraffin sections then underwent HE staining (Zeping, Beijing, China) to investigate the architecture. An Olympus microscope and digital camera (BX20, Beijing, China) were used to visualize samples with the NIS Element SF 4.00.06 software (Beijing, China). Per group, liver samples from 3–5 mice were prepared, stained, and analyzed.

HPLC-MS analysis

The authentic standards were purchased from Chengdu Biopurity Phytochemicals (Chengdu, China). The contents of puerarin, tables berberine, coptisine hydrochloride, drug root base, daidzein, bar martin, berberine, scutellarin, baicalein, and gutellarin in GGQLD- and GGQLD-medicated serum samples were assessed via LTQ Orbitrap-MS (Thermo Fisher Scientific, CA, USA) coupled with an HPLC model U3000 device (Dionex, CA, USA). Negative ion mode in the 100–1000 m/z mass range was used for full-scan mass spectra acquisition.

Cell culture and treatment

The human hepatocellular carcinoma (HepG-2) cells were from the ATCC and were grown using DMEM containing 10% FBS (Gibco, Carlsbad, CA, USA), penicillin (100 units/mL), and streptomycin (100ug/mL) at 37°C under 5% CO_2 , in a 95% humidified atmosphere. HepG2 cells were seeded in 6-well

Table 1. Primers used in qRT-PCR.

Gene	Primers (5'→3')
SIRT1	Forward: GAAGTATGACAAAGATGA
	Reverse: AGAGCTTCTTGAGACTG
FOXO1	Forward: ACAATCTGTCCCTACACAG
	Reverse: AAATTTGCTAAGAGCCGAGAPDH
GAPDH	Forward: CAAGATTGTGACCAATGCAT
	Reverse: TCACTGCCACTCAGAAGA C

plates (3×10^5 cells/well). After HepG2 cells grew to 60% confluence, they were subjected to 6-h starvation, then treated with or without 0.25 mM palmitic acid (Sigma, USA) or palmitic acid together with GGQLD-medicated serum or palmitate together with pioglitazone for 24 h.

HepG2 cells were transfected with specific siRNA using HiPerFect Transfection Reagent (QIAGEN, Germany). Cells transfected with scrambled siRNA were used as negative control. The sequence of siRNA targeting SIRT1 was 5'-CAGGATTATTGTATTACGTT-3'. At 48 h after transfection, the cells were treated as detailed above.

RT-PCR assay

After treatment, RNA from mice liver or HepG2 cells were extracted. Absorbance at 260 and 280 nm was used to assess RNA purity and concentration. A ReverTra Ace qPCR RT Kit (Toyobo, Japan) was then used for reverse transcription, followed by RT-PCR reactions based on provided directions. Triplicate samples were analyzed, with GAPDH mRNA used for normalization and relative expression calculated via the $2^{-\Delta\Delta\text{CT}}$ method. Primers used in these analyses are shown in Table 1.

Western blot

Proteins were prepared according to the method described in the literature [19]. The supernatants were collected and then quantitated for protein determination using the PIERCE BCA protein assay kit (Thermo Fisher Scientific). Denatured protein samples were separated on 12% SDS-polyacrylamide gels (SDS-PAGE), and transferred to nitrocellulose membranes (0.45 μm ; Millipore, MA, USA). The membranes were blocked with 5% skimmed milk and incubated with the following primary antibodies overnight at 4°C : anti-SIRT1, anti-FOXO1, anti-Acetylated-Lysine, anti-FABP4, anti-PPAR γ , and anti- β -actin. All antibodies were from CST (USA) and were used at a 1: 1000 dilution. Blots were subsequently washed and incubated with the corresponding peroxidase-conjugated secondary antibodies (1: 3000; CST, USA) at room temperature. The target protein bands were scanned and quantified via Gel Doc2000 (Bio-Rad, USA).

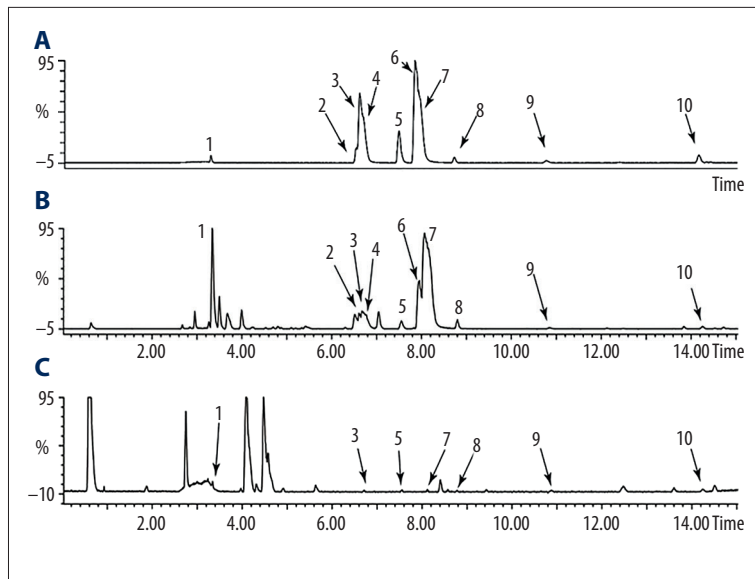


Figure 1. HPLC-MS chromatograms of GGQLD extracts. **(A)** The HPLC-MS chromatogram of the standards. **(B)** The HPLC-MS chromatogram of GGQLD extract. **(C)** The HPLC-MS chromatogram of GGQLD-medicated serum. Each number indicates a specific component: 1 puerarin; 2 tables berberine; 3 coptisine hydrochloride; 4 drug root base; 5 daidzein; 6 bar martin; 7 berberine; 8 scutellarin; 9 baicalein; 10 gutellarin.

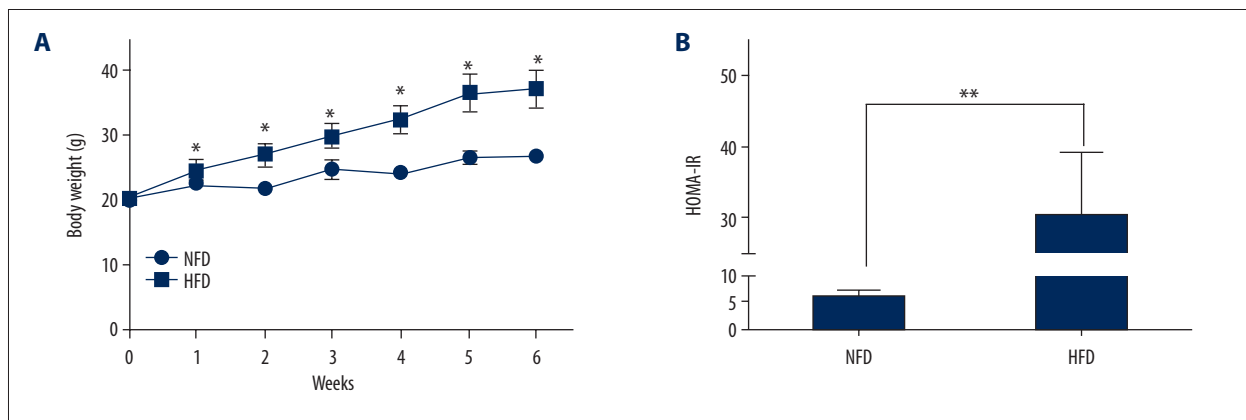


Figure 2. HFD increased body weight and HOMA-IR. C57BL/6J mice were assigned to either the NFD group (n=12) or the HFD group (n=36) fed with standard chow or HFD, as detailed in Methods. The 2 groups had similar average body weights at the beginning of the experiment. **(A)** Body weight was measured weekly during the experiment. **(B)** HOMA-IR was measured in the mice 6 weeks after HFD treatment. * $P < 0.05$ vs. NFD; ** $P < 0.01$ vs. NFD.

Statistical analysis

All data are presented as means \pm standard deviation (mean \pm SD). Samples were analyzed via t tests and one-way analyses of variance (ANOVA) for 2 and more than 2 groups, respectively. $P < 0.05$ was the significance threshold.

Results

HPLC Analysis of GGQLD

The major components of GGQLD extract and GGQLD-medicated serum were identified and quantified by HPLC (Figure 1) with standard reference compounds (Figure 1A). The contents of puerarin (RT=3.37 min), tables berberine (RT=6.59 min), coptisine

hydrochloride (RT=6.64 min), drug root base (RT=6.78 min), daidzein (RT=7.56 min), bar martin (RT=7.97 min), berberine (RT=8.08 min), scutellarin (RT=8.81 min), baicalein (RT=10.82 min), and gutellarin (RT=14.23 min) in GGQLD extract were identified (Figure 1B). The contents of puerarin (RT=3.37 min), coptisine hydrochloride (RT=6.64 min), daidzein (RT=7.56 min), berberine (RT=8.08 min), scutellarin (RT=8.81 min), baicalein (RT=10.82 min), and gutellarin (RT=14.23 min) were found in GGQLD-medicated serum (Figure 1C), which indicates these contents are the bioactive compounds responsible for the therapeutic effects of GGQLD.

Effect of GGQLD on glycolipid metabolism indexes

Body weight was monitored once a week during the experimental period. Six weeks later, body weight and HOMA-IR in

HFD group were significantly higher than those in the NFD group (Figure 2A, 2B). After further high-fat diet feeding and medication for 8 weeks, GGQLD treatment significantly slowed the weight gain compared with the HFD group (Figure 3A), which was similar to the effect of pioglitazone. The serum levels of triglyceride (TG) and insulin were much higher than those in the NFD group but were decreased in the GGQLD treatment and pioglitazone treatment groups (Figure 3B, 3C). In addition, HOMA-IR, which was markedly increased in the HFD group, was significantly reduced by GGQLD or pioglitazone treatment (Figure 3D). The area under the curve (AUC) of IPGTT and IPITT in the HFD group were significantly increased compared with the NFD group, and GGQLD significantly decreased the AUC compared with that of the HFD group (Figure 3E–3H).

GGQLD attenuated the pathological changes in HFD mouse liver tissues and affected the SIRT1 and Ac-FOXO1 protein expression

A large number of hepatocytes with steatosis and ballooning degeneration were found in the HFD group, while GGQLD or pioglitazone treatment reduced cytoplasmic vacuolation (Figure 4A). The expression of SIRT1 was significantly lower in the HFD group ($P < 0.01$) and the expression of Ac-FOXO1 was greatly increased ($P < 0.05$) compared with the NFD group (Figure 4B). The expression of SIRT1 in the HFD-GGQLD group was higher ($P < 0.01$), and the expression of Ac-FOXO1 was lower (Figure 4B) compared with the HFD group. The expression of PPAR γ in the HFD-GGQLD group was much higher ($P < 0.01$), and the expression of FABP4 was lower.

GGQLD-mediated serum promotes glycogen synthesis and inhibits Ac-FOXO1 protein expression

After being treated with palmitic acid, the glycogen synthesis of HepG2 cells decreased significantly, regardless of the addition of insulin. However, the addition of the GGQLD-mediated serum restored the decreased glycogen synthesis of HepG2 cells (Figure 5A). The expression of SIRT1 decreased and the level of Ac-FOXO1 increased significantly after stimulation with palmitic acid (Figure 5B). However, when cotreated with the GGQLD-mediated serum, the expression of SIRT1 was increased and the expression of Ac-FOXO1 was decreased significantly (Figure 5B).

Palmitic acid reduced the expression of PPAR γ in HepG2 cells, while GGQLD, as well as pioglitazone, increased the expression of PPAR γ . The expression of FABP4 was increased after palmitic acid stimulation, and GGQLD or pioglitazone treatment reduced FABP4 expression levels in palmitic acid-treated HepG2 cells (Figure 5C).

GGQLD-mediated serum reduces Ac-FOXO1 by promoting expression of SIRT1 in HepG2 cells

To prove that the increase of SIRT1 mediated the decrease of Ac-FOXO1 in GGQLD-mediated serum-treated HepG2 cells, we silenced SIRT1 by specific siRNAs in HepG2 cells and studied the expression of Ac-FOXO1 and PPAR γ in these cells. After the transfection of HepG2 with SIRT1 siRNA, the relative expressions of SIRT1 mRNA and protein in HepG2 cells were significantly reduced (Figure 6A–6C). The Ac-FOXO1 protein level was increased (Figure 6B, 6E) compared with the scrambled siRNA group. Palmitate alone could no longer affect the expression of SIRT1 (Figure 6B, 6D), Ac-FOXO1 (Figure 6B, 6E) and PPAR γ (Figure 6B, 6F). What's more, GGQLD-mediated serum also showed no effects on FOXO1, Ac-FOXO1 and PPAR γ (Figure 6B, 6D, 6E, 6F) in SIRT1-silenced HepG2 cells.

Discussion

Herein, we investigated the effect of GGQLD on IR in a T2DM mouse model. Animal experiments showed that GGQLD could reduce body weight, decrease triglyceride, and improve glucose metabolism and IR in C57BL/6J mice on HFD. The above results demonstrate that GGQLD indeed has therapeutic effects in treating T2DM.

We also found that the protein expression levels of SIRT1 and PPAR γ in the liver tissues of HFD mice were significantly decreased, while GGQLD could restore them. Consistent with this, the protein expression levels of Ac-FOXO1 in the liver tissues of HFD mice were increased, while GGQLD inhibited the expression of Ac-FOXO1. In addition, GGQLD-mediated serum restored the expression of SIRT1 and PPAR γ , which was decreased in palmitic acid-treated HepG2 cells.

Accordingly, GGQLD-mediated serum inhibited the level of Ac-FOXO1 in palmitic acid-treated HepG2 cells. We also found that GGQLD counteracted the induction of FABP4, an indicator of insulin resistance [20], by palmitic acid. Therefore, our results demonstrated that, in T2DM, GGQLD enhanced the expression of SIRT1 and subsequently reduced the level of Ac-FOXO1, as did pioglitazone, *in vitro* and *in vivo*. PPAR γ has the function of regulating insulin sensitivity, and FABP4 plays a key role in the regulation of PPAR γ transcription [21]. Our study also demonstrated that GGQLD up-regulates PPAR γ by inhibiting FABP4, which further indicated GGQLD relieves insulin resistance and oxidative stress.

A previous study showed that SIRT1 agonist can improve insulin sensitivity in the liver of Zucker fa/fa rats, and the sensitivity of insulin secretion in response to blood glucose was decreased after SIRT1 knockout [22]. To further prove that GGQLD

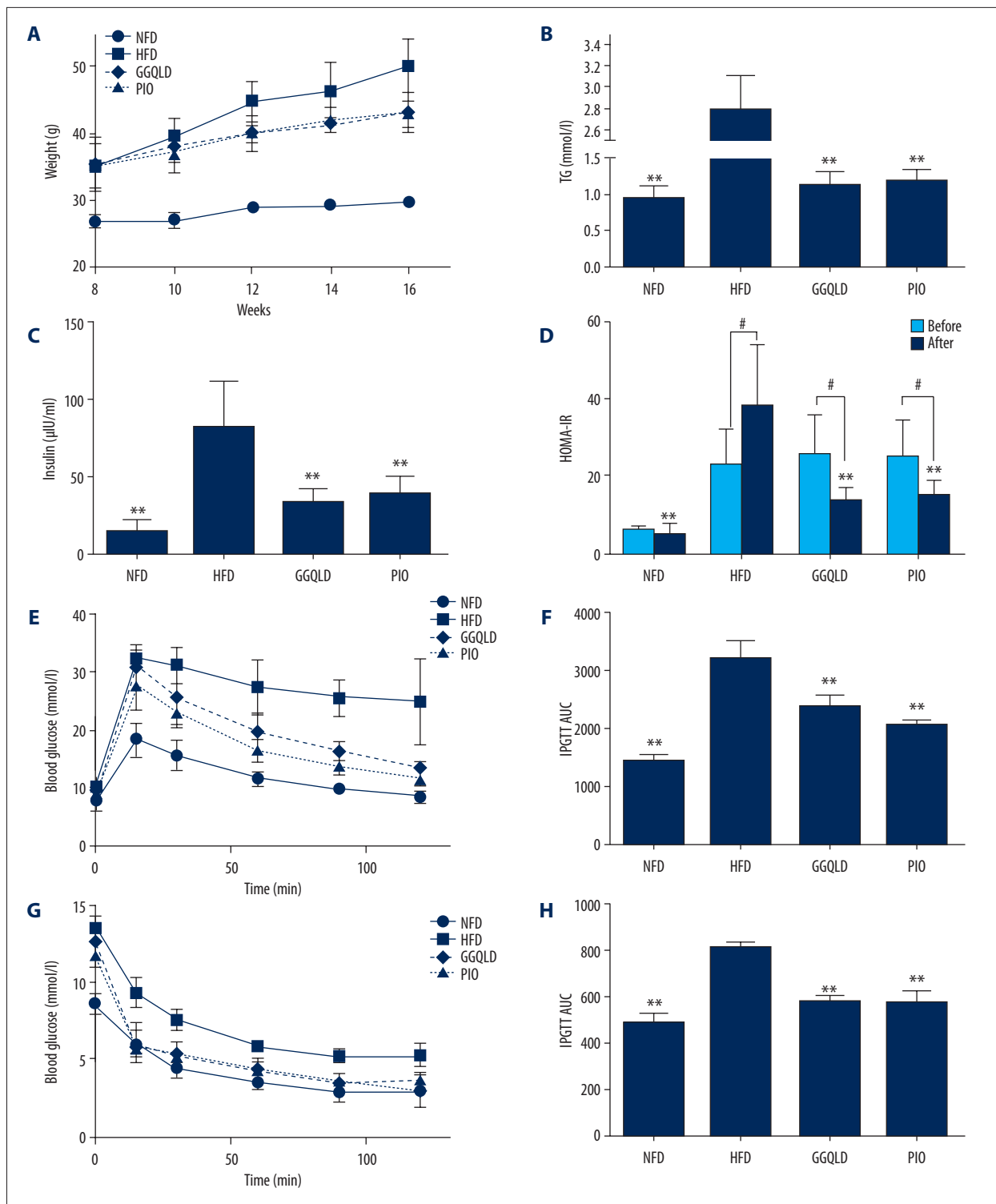


Figure 3. GGQLD improved body weight, glucose and lipid metabolism, and IR in HFD mice. The mice fed a HFD for 6 weeks were subsequently assigned to 3 subgroups (for each subgroup, n=12) with different treatments for an additional 8 weeks (saline-treated, GGQLD-treated, pioglitazone-treated, with pioglitazone used as a positive control). (A) Body weight was measured every 2 weeks during the treatment period. Serum triglyceride (TG) (B), serum insulin (C), and HOMA-IR (D) levels were measured at the end of the experiment. IPGTT (E) and IPITT (G) were determined at the 8th week of the treatment. Area under the curve (AUC) levels of IPGTT (F) and IPITT (H) were calculated. ** $P < 0.01$ vs. HFD, # $P < 0.05$ vs. before.

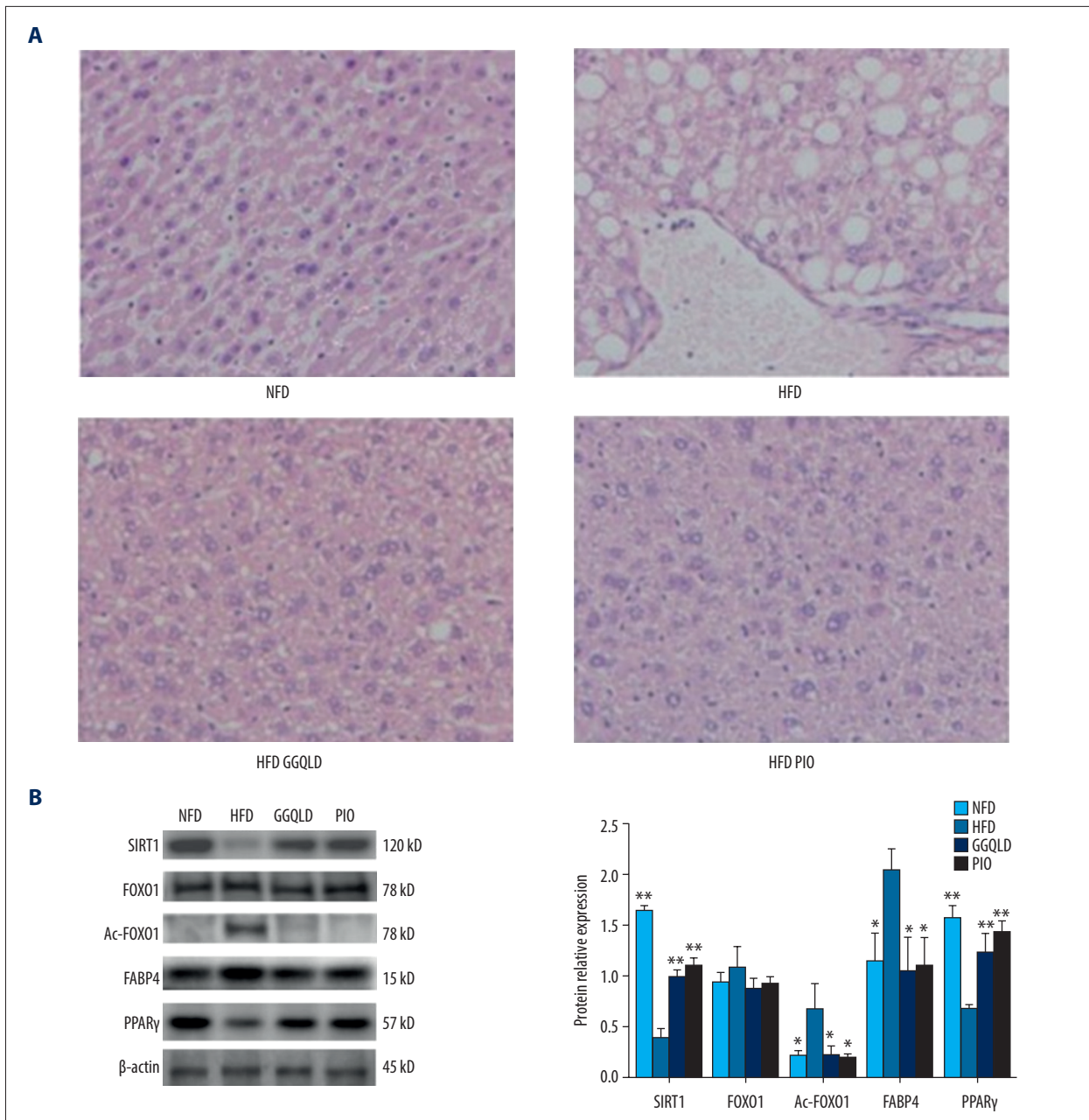


Figure 4. GGQLD improved histopathology and affected the expression levels of key molecules in the SIRT1/FOXO1 signaling pathway in liver. **(A)** Liver tissues were subjected to paraffin sectioning and HE staining. For each group, liver samples from 3–5 mice were used. Representative images are shown ($\times 200$). **(B)** Total proteins of liver tissues were extracted and subjected to Western blot analysis for the detection of SIRT1, FOXO1, Ac-FOXO1, FABP4, and PPAR γ . Representative images are shown (**left**). For quantification, optical densities of the bands were determined. β -actin was used as a loading control. Normalized protein expression levels were calculated. Data from 3 independent experiments were used for statistics, and results are expressed as the mean \pm SEM (**right**). * $P < 0.05$ vs. HFD, ** $P < 0.01$ vs. HFD.

improved hepatic insulin resistance through the SIRT1-FOXO1 signaling pathway, we used siRNA to knock down SIRT1 in HepG2 cells. The results showed that, with SIRT1 being silenced, the expression level of Ac-FOXO1 in HepG2 cells showed no obvious changes when treated with palmitate together with

GGQLD-mediated serum compared to palmitate-treated HepG2 cells, thus suggesting that GGQLD functions through regulating the SIRT1-FOXO1 signaling pathway.

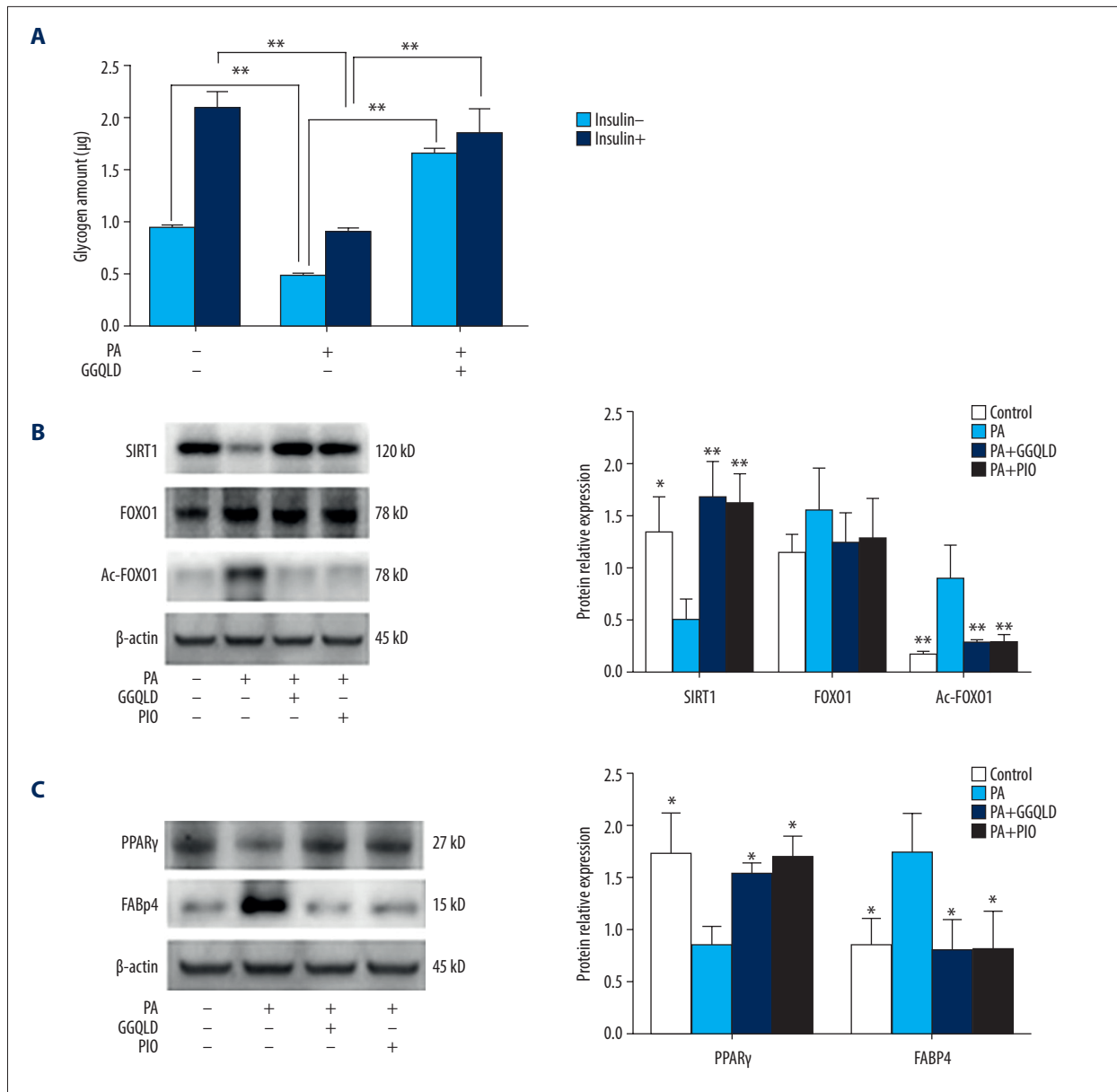


Figure 5. GGQLD-mediated serum promoted glycogen synthesis and affected the key molecules in the SIRT1/FOXO1 signaling pathway. **(A)** HepG2 cells were treated with medium, PA alone, or PA + GGQLD-mediated serum with or without the presence of insulin. Then, glycogen synthesis was measured as indicated in Methods. **(B)** HepG2 cells were treated with medium, PA alone, PA + GGQLD-mediated serum, or PA+PIO. Western blots for SIRT1, FOXO1, and Ac-FOXO1, and **(C)** for the detection of PPAR γ and FABP4. Representative images are shown **(B left, C left)**. For quantification, optical densities of the bands were determined. β -actin was used as loading controls. Normalized protein expression levels were calculated. Data from 3 independent experiments were used for statistics and results were expressed as the mean \pm SEM **(B right, C right)**. PA – palmitic acid. PIO – pioglitazone. * $P < 0.05$ vs. HFD, ** $P < 0.01$ vs. HFD or their respective control indicated by line.

Numerous herbs in GGQLD have been believed to benefit insulin resistance, such as berberine, puerarin, and glycyrrhizin [23,24], which may contribute to the therapeutic effects of GGQLD on insulin resistance. Although in the past, GGQLD was widely used to treat diarrhea [25], and previous modern pharmacology studies of the monomer of GGQLD indicated it

can improve glucose and lipid metabolism disorder. For example, puerarin can improve mitochondria performance in muscle and promote the oxidation of fatty acids, which thus prevents the accumulation of intramyocellular lipids in diabetic rats [26]. Baicalin can treat obesity and IR via the Akt/AS160/GLUT4 and P38MAPK/PGC1 α /GLUT4 pathways [27]. Berberine can also

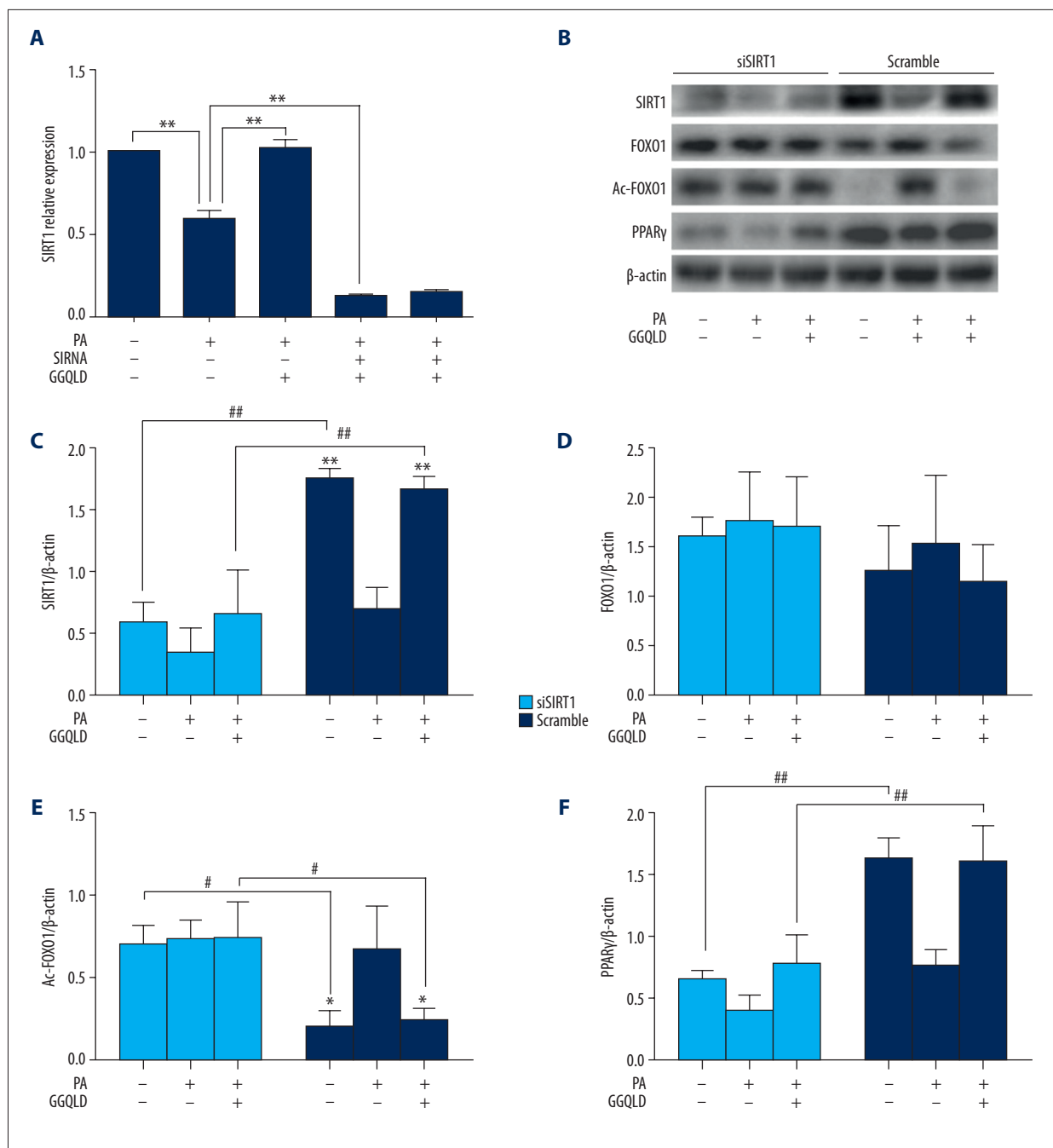


Figure 6. GGQLD-mediated serum function through regulating the expression of SIRT1 in palmitate acid-treated HepG2 cells. HepG2 cells were transfected with SIRT1 siRNA or scrambled control siRNA. Then, the cells were treated with medium, PA alone, or PA+GGQLD medicated serum, as indicated. **(A)** RNAs were extracted and SIRT1 mRNA expression was measured by qRT-PCR. **(B)** Western blots for SIRT1, FOXO1, Ac-FOXO1, and PPAR γ . Representative images are shown. **(C-F)** For quantification, optical densities of the bands were determined. β -actin was used as loading control. Normalized protein expression levels were calculated. Data from 3 independent experiments were used for statistics, and results are expressed as the mean \pm SEM. PA – palmitic acid. # $P < 0.05$ vs, Scramble. ** $P < 0.01$ vs. PA alone. ### $P < 0.01$ vs. Scramble.

antagonize the increase of blood glucose caused by exogenous glucose, significantly reduce the blood sugar of 4 alloxan diabetic mice, improve the blood glucose and IR of spontaneous diabetic mice, and stimulate the regeneration of islet beta cells [28,29]. The above-mentioned studies indicate that GGQLD may be of benefit in T2D therapy.

Conclusions

In conclusion, our results demonstrate that hepatic IR plays a crucial role in the progression of T2DM, and GGQLD can be used to improve insulin resistance in liver tissues by upregulating SIRT1 and downregulating Ac-FOXO1. GGQLD is a potentially useful medicine for treatment of T2DM.

Conflicts of interest

None.

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