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**Original Research Article**

# Combined administration of Gegen Yinlian decoction and metformin ameliorates liver metabolic disorder in rats by regulating miR-195-5p/IRS1/PI3K/AKT axis

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## Abstract

***Purpose:*** *To investigate the effect of Gegen Yinlian decoction (GYD), made from lobed kudzuvine root, baicalensis, huanglian and liquorice, and metformin on liver metabolism dysfunction in type 2 diabetes mellitus (T2DM) rats.*
***Methods:*** *Wistar rats were treated with high-fat diet and a single intraperitoneal streptozotocin (STZ) injection to establish T2DM model. Thereafter, the T2DM-rats were treated with GYD and metformin (GYD/metformin). Serum triglyceride (TG), TC, LDL, and HDL levels, as well as glucose and insulin tolerance of the rats were evaluated. Furthermore, the effects of GYD/metformin treatment on the expressions of the related genes were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunoblot assay.*
***Results:*** *After GYD-metformin treatment, the metabolic indicators in T2DM rats significantly improved ($p < 0.05$). However, there was decrease in t liver miR-1955p expressions. Silencing miR-195-5p improved glucose consumption and triglyceride levels of palmitate (PA)-induced pathological state in ALM12 cells ($p < 0.05$). Furthermore, miR-195-5p targeted insulin receptor substrate 1 (IRS1). Suppression of IRS1 partly annulled the impact of miR-195-5p silencing on ALM12 cell model. Moreover, IRS1 downregulation significantly mitigated the impact of the silencing on PI3K/AKT pathway activity ($p < 0.05$).*
***Conclusion:*** *Combination of GYD with metformin ameliorated dysfunctional liver metabolism via control of miR-195-5p/IRS1/PI3K/AKT axis.*

***Keywords:*** *MiR-195-5p, IRS1, PI3K/AKT, Gegen Yinlian decoction, Metformin, Liver metabolism dysfunction, Diabetes mellitus type 2*



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## INTRODUCTION

Liver metabolic disorder is a chronic metabolic disease involving complex pathological mechanisms. More than 425 million people have been diagnosed with diabetes all over the world, and the incidence of this disease is continually increasing [1]. Generally, diabetic patients have insulin deficiency and insulin resistance, and they exhibit multiple complications in organs including

kidneys, eyes and nervous system [2]. Drug interventions and diet control are major strategies used to restrain and alleviate the metabolic disturbance in patients with diabetes in the early stage. Indeed, in the treatment of diabetes, the first-line drugs are involved in the regulation of blood glucose, while drug resistance limits the therapeutic effects of these drugs [3]. The long-term practice of traditional Chinese medicine has confirmed the therapeutic effectiveness of herbs and herbal decoctions on diabetes [4]. Recently, some prescriptions have been used for treating the aberrant blood glucose levels of diabetic patients [5]. Intervention using traditional Chinese medicines in combination with hypoglycemic drugs could further improve the prognosis of patients with diabetes [6]. According to *Treatise on Exogenous Febrile Disease, Gegen Yinlian* decoction is a traditional strategy for diabetes treatment. However, the pharmacological mechanism involved in this method with respect to liver metabolic disorder is poorly understood.

The aim of this research was to investigate the impact of combination of *Gegen Yinlian* decoction and metformin on diabetes mellitus so as to provide some scientific evidence for its use in the treatment of liver metabolic disorders.

## EXPERIMENTAL

### Establishment of diabetes mellitus type 2 (T2DM) rat model

Approval for this research was received from the ethical body of Tangshan Gongren Hospital (approval no. 20200330). The animal studies were performed in line with the guideline of Guide for the Care and Use of Laboratory Animals [7]. Thirty male Wistar rats (6 weeks, 180 – 210 g) were used. All rats were maintained in an atmosphere under conditions of controlled temperature (25 °C) and humidity (60 %), and normal day/night durations, with sufficient feed and water. Two groups of rats were used: control and T2DM groups. Rats in T2DM group were given a high-fat diet (HFD) comprising fat, sucrose, cholesterol, cholate, bean sprout and chow diet at percentage ratio of 10:20: 2.5: 1: 30:35.5, for I month. Thereafter, the rats were injected once with streptozotocin (STZ) *i.p.* at a dose of 30 mg/kg. The rats with fasting blood glucose concentration > 16.7 mM were selected as T2DM model.

### *Gegen Yinlian* decoction

A mixture comprising liquorice (6 g), *S. scutellaria* (9 g), *kudzuvine* roots (15 g), and Chinese goldthread rhizome (9 g) was immersed in 1600 mL of deionized water for 40 min, and then the herbs were boiled until the decoction was concentrated to 400 mL. The T2DM rats in experiment group were administered the warm decoction in the morning and evening (14 g/kg at a time), while the T2DM rats in control group were fed with normal saline at the same dose. The dose of metformin was 100 mg/kg.

### Cell culture, transfection and model establishment

Alpha mouse liver 12 (AML12) cells were maintained in DMEM and Ham's F12 medium containing FBS (10 %), dexamethasone 40 (μg/L), transferrin (5 mg/L), and Se (5 μg/L). To establish cell model of metabolic disorder, cell culture with 100 μM PA was performed for 24 h in an incubator at 37 $^{o}$C and 5 % $CO_2$.

The cells were transfected at 70 % confluence with MiR-195-5p mimic and inhibitor; si-IRS1 vectors, IRS1 expressed vectors and the related negative controls which were products of Biomics Biotechnologies Co. Ltd. (Jiangsu, China). Incubation of RNA or DNA with 0.25 mL serum-free medium was done for 5 min, and it was used to dilute 10 μl Lipofectamine 2000. The diluted RNAs or DNAs were added to equal volumes of Lipofectamine, followed sequentially by 20-min incubation at 25 $^{o}$C, mixture addition to wells, and 24-h cell culturation.

### Determination of metabolic indexes

The serum contents of TG, TC, LDL and HDL were evaluated with their corresponding kits (Wuhan Saipei Biotechnology Co. Ltd) in line with the kit protocols, while TG level was determined using TG measurement kit (Chaoyan Biotechnologies Co. Ltd (Shanghai, China).

### *In vivo* experiments

The OGTT and insulin tolerance test (ITT) were performed on separate days. For OGTT, the rats in all groups were fasted for 6 h, and then were fed with glucose (2 g/kg). The glucose levels of the rats were determined at 0, 30, 60, 90, and 120 min. For ITT, following 6-h fast, the rats in all groups were administered insulin injection (0.4 IU/kg) *i.p.* After that, blood glucose was measured at 0, 30, 60, 90, and 120 min.

### *In vitro* experiments

The glucose consumption of the cells was measured with glucose and glycogen assay kits. Glucose and glycogen assay kits were

purchased from AmyJet Scientific Co. Ltd (Wuhan, China). Glucose consumption was calculated as difference between initial concentration of glucose and final concentration of glucose.

### Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the tissues of the rats with TRIzol reagent and quantified using ultraviolet spectrophotometry. Thereafter, reverse transcription of the RNAs was executed using PrimeScript® kit, followed by qRT-PCR. The comparative levels of mRNA expression were determined using $2^{-\Delta\Delta Ct}$ procedure, with expression of U6 set as endogenous control. The sequences of primers for miR-195-5p, IRS1 and U6 are indicated in Table 1.

**Table 1:** Sequences of miR-195-5p, IRS1 and U6 primers used

| Primer | Sequence |
|---|---|
| miR-195-5p-F | 5'-GATAGCAGCACAGAAATATTGGC-3' |
| miR-196-5p-R | 5'-CTCAACTGGTGTCGTGGA-3' |
| IRS1-F | 5'-TAAGAGCTTACCACCGCTGC-3' |
| IRS1-R | 5'-GTGGCTGCTCTCCTGACATT-3' |
| U6-F | 5'-CTCGCTTCGGCAGCACA-3' |
| U6-R | 5'-AACGCTTCACGAATTTGCGT-3' |

### Immunoblot assay

Protein extraction from the cells was done with RIPA buffer, and protein quantification was carried out using BCA method. Then, equal amounts of proteins were resolved on SDS-PAGE, followed by electro-transfer to PVDF membranes which were thereafter incubated with 5 % fat-free milk for 60 min to block non-specific binding of the blot. This was followed by membrane incubation at 4 $^{o}$C with appropriate 1$^{o}$ antibodies for 12 h. After that, membrane incubation with 2$^{o}$ antibodies was done for 2 h at laboratory temperature. Protein expression levels were measured by subjecting the bands to ECL.

### Double-luciferase reporter measurement

The mutant sequence of IRS1 in the region of the 3'-UTR was designed in line with predicted binding sites of miR-195-5p and IRS1. Subsequently, the wide type of IRS1 (IRS1-wt) and mutant type of IRS1 (IRS1-mut) were inserted into pGL2-Basic vectors. Then, IRS1-wt or IRS1-mut was transfected into AML12 cells, and after 2 days, luciferase activity in the cells was assayed.

### Statistics

Data analysis was done with SPSS 20.0, while graphic analysis was performed with GraphPad prism 8. Statistical differences were determined by $\chi^2$ test or ANOVA. Values of $p < 0.05$ were considered statistically significant.

## RESULTS

### Effect of *Gegen Yinlian* decoction and metformin on disordered glucose metabolism in rats

Rat model of T2DM was established, and indexes including fasting blood glucose (FBG), insulin level, ALT, TG, TC, LDL, and HDL were applied to evaluate the therapeutic effect of GYD-metformin combination on the rat model. The results showed that *Gegen Yinlian* decoction effectively improved levels of the metabolic indexes (FBG, ALT, AST, TG, TC, LDL and HDL) in the rats ($p < 0.01$; Figure 1).

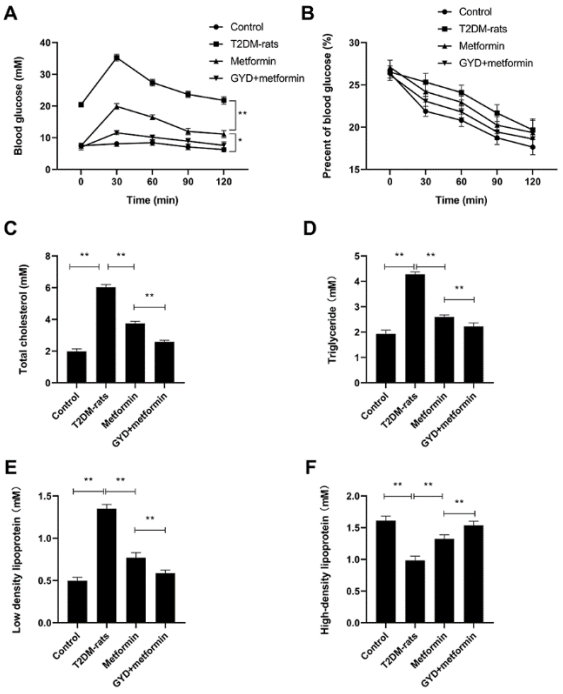


**Figure 1:** *Gegen Yinlian* decoction in combination with metformin mitigated the metabolic disorder in T2DM-rats. A & B: Oral glucose tolerance test and insulin tolerance test results for the rats. C – F: TG, TC, LDL and HDL levels in the rats. * $P< 0.05$, ** p<0.05., vs control

### Effect of *Gegen Yinlian* decoction+ metformin on metabolic disturbance correlated with the abundance of miR-195-5p

To investigate the pharmacological mechanism underlying the effect of *Gegen Yinlian* decoction

and metformin on metabolic disturbance, the serum expression level of IRS1 protein was determined. There was marked up-regulation of serum miR-195-5p protein in the rat model (Figure 2 A, $p < 0.01$). To further verify whether miR-195-5p downregulation played a direct role in the mitigation of glucose and lipid metabolic disorders by combination of *Gegen Yinlian* decoction and metformin, its impact on AML12 cell model was determined. As shown in Figure 2, decreased level of miR-195-5p markedly improved glucose consumption and TG level of AML12 cell model ($p < 0.01$). Thus, *Gegen Yinlian* decoction and metformin suppressed the progression of metabolic disorder via miR-195-5p suppression.

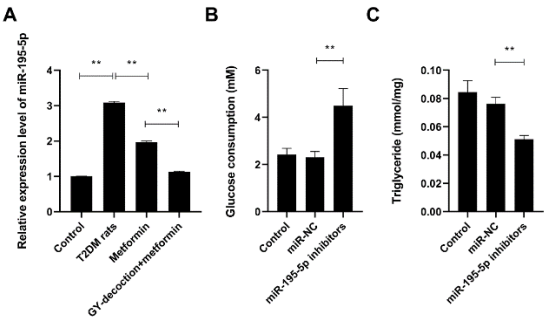


**Figure 2:** *Gegen Yinlian* decoction in combination with metformin mitigated the glycolipid metabolic disorder of T2DM-rats via decreasing miR-195-5p. Expression level of miR-195-5p, as measured with qRT-PCR. B & C: Glucose consumption and triglycerides of the ALM12 cell model. * $P < 0.05$, ** $p < 0.05$, vs control

### 3'-UTR of IRS1 was direct target of MiR-195-5p

The predicted downstream binding site of miR-195-5p was IRS1. Moreover, miR-195-5p was effectively bound to, and acted with IRS1-wt. These observations suggest that miR-195-5p was bound directly to IRS1 (Figure 3 A; $p < 0.01$). Moreover, immunoblot assay revealed that it inhibited the expression of IRS1. Decreased IRS1 levels were also observed in the liver tissues of the rats and ALM12 cell models (Figure 3, $p < 0.01$). These observations suggest that IRS1 is the downstream binding site of miR-195-5p which is implicated in progression of hepatic metabolism dysfunction.

### MiR-195-5p silencing mitigated disturbance in glucose/lipid metabolism *in vitro* by targeting IRS1

To determine whether IRS1 was associated with the control of hepatic metabolism by miR-195-5p, glucose content and TG levels were determined in AML12 cells following co-transfection with si-IRSI and miR-195-5p inhibitors. The results revealed that the cells transfected with miR-195-5p inhibitors expressed higher capacity for glucose consumption than those that received miR-NC. Moreover, those transfected with miR-195-5p inhibitors expressed significant improvement in TG level and glucose consumption in AML12 cell model, and these phenomena were partly reversed by decreasing IRS1. These observations imply that silencing of miR-195-5p markedly inhibited the metabolic disturbance in diabetes via regulating the expression level of IRS1 (Figure 4).

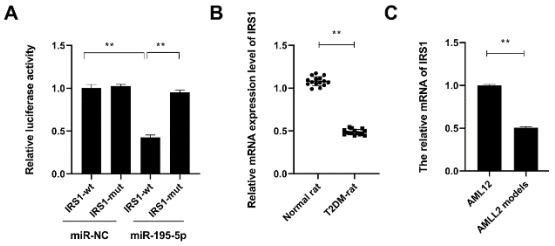


**Figure 3:** MiR-195-5p negatively regulated the abundance of IRS1. A: DR assay. B & C: The mRNA abundance of IRS1 in T2DM rats and AMLL2 cell model. *, ** $P < 0.05$

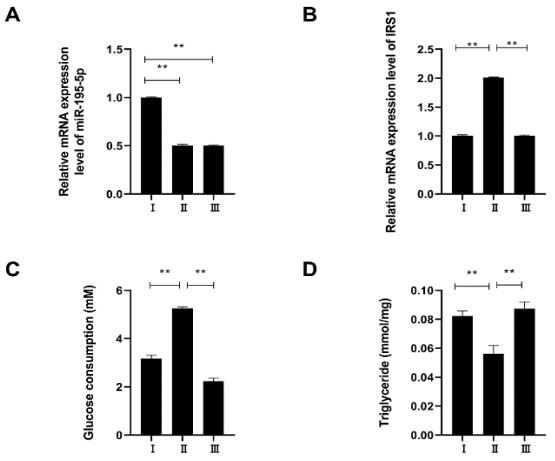


**Figure 4:** IRS1 partly annulled the impact of miR-195-5p in ALM12 model. A & B: Expression levels of miR-195-5p & IRS1, as measured with qRT-PCR. C, D: Glucose content and triglyceride content of the ALM12 cell model. * $P < 0.05$, ** $p < 0.05$. ( Ⅰ = miR-NC+NC; Ⅱ = miR-195-5p inhibitor; Ⅲ = inhibitor + si-IRS1).

### MiR-195-5p inactivated PI3K/AKT pathway by targeting IRS1

The related pivotal genes in the livers of T2DM-rats were identified as confirmation of the related influence of miR-195-5p in the metabolic disturbance induced by T2DM. In the results, decreased levels of p-AKT and p-PI3K were found in the tissues of rats injected with miR-195-5p agonists after treating with GDY/metformin,

which proved that miR-195-5p alleviated the metabolic disturbance of T2DM-rats via targeting IRS1 (Figure 5, $p < 0.01$).

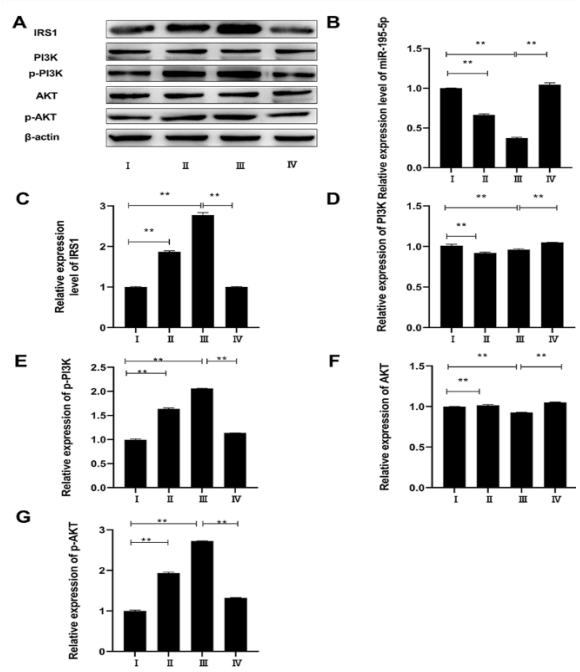


**Figure 5:** PI3K/AKT route was inactivated by MiR-195-5p via targeting IRS1. A-G: Relative levels of miR-195-5p, IRS1, p-PI3K, PI3K, p-AKT and AKT. $^{*}$, $^{**}P < 0.05$. (Ⅰ= T2DM-rats; Ⅱ= Metformin; Ⅲ= GYD/Metformin; Ⅳ= GYD/Metformin+miR-195-5p inhibitors)

## DISCUSSION

Liver metabolism dysfunction is a common symptom of patients with diabetes, and there are limited strategies in clinics for effective treatment of this condition [8]. The use of TCM has increasingly become recognized as efficacious in the treatment of multiple diseases. At present, several studies have confirmed that some TCMs effectively mitigated the symptoms of diabetes, and TCMs are confirmed as promising strategies for diabetes [9]. However, only few studies have revealed the pharmacological mechanisms of those prescriptions. *Gegen Yinlian* decoction has been recorded in *Treatise on cold-attack,* and its clinical efficacy and safety have been tested by countless cases from ancient times to present. In the prescription of *Gegen Yinlian* decoction, lobed kudzuvine root, *S. baicalensis*, huanglian and liquorice are boiled with water to obtain a decoction which is orally administered to patients who assimilate the chemical substances through the digestive system. This study investigated the effect of *Gegen Yinlian* decoction and metformin on diabetes treatment, and revealed the pharmacological mechanism of the decoction in metabolic disturbance.

In this study, it was found that the combined therapy of *Gegen Yinlian* decoction and metformin effectively decreased the blood glucose of diabetic rats. Metformin is a common drug used in clinical treatment of diabetes. It is involved in the regulation of blood glucose. Moreover, the results showed that combined intervention with *Gegen Yinlian* decoction and metformin had better effect on regulation of blood glucose of the diabetes than that of metformin. A study has indicated that puerarin isolated from lobed kudzuvine root, reduced the blood glucose of type II diabetes rats [10]. Waisundara *et al*. have found that baicalensis played an enhancer role by improving the effect of metformin [11]. *Huanglian Jiedu* decoction, one of traditional Chinese prescriptions, improved blood glucose levels of diabetic rats [12]. Besides, licorice has also been found to have anti-diabetic activity recently [13].

In this study, it was also found that *Gegen Yinlian* decoction significantly inhibited miR-195-5p, and increased miR-195-5p was also evident in T2DM rats. Numerous investigations have confirmed the up-regulation of serum miR-195-5p in diabetic patients, which suggests that aberrant abundance of miR-195-5p is related to formation or development of metabolic disorders in carbohydrates and lipids [14]. Moreover, this study showed that miR-195-5p upregulation effectively inhibited glucose consumption and increase in TG level of the cells. Thus, the findings suggest that *Gegen Yinlian* decoction mitigated metabolic disturbance by controlling the level of miR-195-5p.

Dysfunctional miRNA has become a common event in diseases ranging from metabolic to nervous system diseases. Significant differences in the patients with diabetes and healthy people have been reported in many studies. Interaction of miRNAs with 3'-UTRs results in hydrolysis of target mRNAs or to repression of protein synthesis. In this study, IRS1 was identified as downstream binding site of miR-195-5p, and reduced mRNA levels of Insulin receptor substrate 1 (IRS1) were also found in the tissues of T2DM-rats. Indeed, IRS1 is a major regulator of blood glucose level in humans, and IRS1 deficiency induces insulin resistance in patients [15]. Several studies have also indicated that reduced IRS1 level is a common event in the liver tissues of the patients with diabetes. Interestingly, excess IRS1 partly reversed the influence of miR-195-5p on glucose and TG in the AML12 cell diabetes model, which suggests that *Gegen Yinlian* decoction and metformin promoted IRS1 level, thereby mitigating the

symptoms of metabolic disorder via inhibiting the expression of miR-195-5p.

Metabolic disorder involves complex cellular and molecular mechanisms in liver cells. In this study, it was shown that *Gegen Yinlian* decoction and metformin enhanced induction of PI3K/AKT signal route, while miR-195-5p induced inactivation of the same pathway via targeting IRS1. Puerarin has been identified as a protective agent that attenuates the apoptosis of pancreatic β-cell via regulating the activity of PI3K/AKT pathway [16]. Moreover, glycyrrhizic acid isolated from licorice and baicalensis has been reported to activate the PI3K/AKT pathway, thereby reducing inflammation and injury of tissues [17]. Besides, the present research showed that miR-195-5p effectively suppressed activity of PI3K/AKT pathway. A study reported that IRS1 activated the PI3K/AKT pathway [18]. This study has demonstrated that *Gegen Yinlian* decoction and metformin mitigated glucose and lipid metabolic disorders by induction of IRS1/PI3K/AKT signal route via suppressing the expression of tmiR-195-5p.

## CONCLUSION

The findings of this study indicate that *Gegen Yinlian* decoction, when combined with metformin, ameliorates liver metabolic disorder in rats by regulating miR-195-5p/IRS1/PI3K/AKT axis. Thus, the decoction can potentially be developed for the treatment of liver metabolic disorder in type 2 diabetes.

## DECLARATIONS

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### *Funding*

None provided.

### *Ethical approval*

None provided.

### *Availability of data and materials*

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### *Conflict of Interest*

No conflict of interest associated with this work.

### *Contribution of Authors*

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yan Zhao and Jie Gao conceived and designed the study, and drafted the manuscript. Yan Zhao, Jie Chen, Yi Xu, Geling Liu, Jia Cui and Liping Liu collected, analyzed and interpreted the experimental data. Jie Chen, Yi Xu and Jie Gao revised the manuscript for important intellectual content. All authors read and approved the final manuscript.



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