Yunpi Heluo decoction reduces ectopic deposition of lipids by regulating the SIRT1-FoxO1 autophagy pathway in diabetic rats

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\textbf{ABSTRACT}

\textbf{Context:} Yunpi Heluo (YPHL) decoction is a Chinese herbal formula with particular advantages for treating type 2 diabetes. Yet, its exact mechanism of action is not fully understood.

\textbf{Objective:} To examine the therapeutic effect of YPHL on ectopic lipid deposition (EDL) in Zucker diabetic fatty (ZDF) rats and the underlying mechanism.

\textbf{Materials and methods:} The ZDF Rats were randomized into five groups, including model, YPHL (200 mg/kg/d for 10 weeks), SIRT1-overexpression (injected with HBAAV2/9-r-SIRT1-3\textsuperscript{-flag-GFP}), NC (injected with HBAAV2/9-CMV-GFP as blank control) and control group. Pancreatic \(\beta\)-cells obtained from high-lipid-high-glucose fed rats were treated with YPHL (10 mg/mL) for 48 h. Lipid deposition and autophagosomes were analyzed by transmission electron microscopy. Intracellular H\textsubscript{2}O\textsubscript{2} and ROS concentrations were measured by flow cytometry. SIRT1, FOXO1, LC3 and P62 mRNA and protein levels were analyzed using qRT-PCR and Western blots.

\textbf{Results:} Compared with the model group, blood glucose levels in YPHL and si-SIRT1 groups were reduced by 19.3% and 27.9%, respectively. In high-lipid-high-glucose cells treated with YPHL, lipid droplets were reduced and decrease in apoptosis rate (38.6%), H\textsubscript{2}O\textsubscript{2} (31.2%) and ROS (44.5%) levels were observed. After YPHL intervention or SIRT1 overexpression, LC3 and p62 expression increased. Protein expression of SIRT1 and LC3 in model, si-SIRT1, si-NC and si-SIRT1 \textplus{} YPHL groups was lower than those in control group, while FoxO1 expression was increased. All of these protein level alterations were reversed in the si-NC \textplus{} YPHL group.

\textbf{Discussion and conclusions:} YPHL reduced EDL by regulating the SIRT1-FoxO1 autophagy pathway in diabetic rats, which could lead to future perspectives for the treatment of diabetes.

**Introduction**

Diabetes mellitus (DM) is a chronic disease with high incidence. Globally, it has been estimated that approx. 422 million people have diabetes, among whom more the 116 million are from China (Chan 2017). Insulin resistance (IR) and impaired insulin secretion are the main causes of type 2 diabetic mellitus (T2DM), both of which are associated with ectopic deposition of lipids (EDL), a condition characterized by an accumulation of free fatty acids (FFAs) in the body that exceeds the tissues’ oxidative capacity. These FFAs are deposited in non-fat tissues such as the liver, heart, skeletal muscle, pancreas and pancreatic \(\beta\)-cells. To a certain extent, EDL is attributed to the loss of fat cells’ protective effect on non-adipocytes and the effect of lipid toxicity damage (Ost et al. 2010). A long-term high-fat diet can lead to EDL in the pancreas, causing pancreatic steatosis and non-alcoholic fatty pancreas (NAFPD), which in turn leads to hyperglycaemia caused induced by \(\beta\)-cell dysfunction and IR (Komiya et al. 2010). The EDL in pancreatic tissue can also enhance the impairment of \(\beta\)-cells caused by oxidative stress in high-lipid conditions by inhibiting autophagy activity (Ost et al. 2010).

In the past, many medicinal plants were used to treat diabetes and its related complications (Ghorbani 2014). Yunpi Heluo (YPHL) decoction is a traditional dietary supplement composed of four Chinese herbal medicines [\textit{Atractylodes lancea} (Thunb.) DC. (Asteraceae), \textit{Stephania tetrandra} S. Moore. (Menispermaceae)] used for the treatment of diabetes and the regulation of lipid metabolism (Zhang and Chai 2010; Yang et al. 2017), but the mechanism by which the EDL in rat pancreas reduces autophagy activity of cells is still unclear.
In this study, we further examined the regulatory mechanism of YPHL decoction on the pancreas EDL in type 2 diabetes ZDF rats with insulin resistance (T2DM-IR).

Materials and methods

Preparation of YPHL decoction and lyophilized powder

The YPHL decoction was prepared according to the previous report (Mao et al. 2019). Lyophilized YPHL powder included: Astragalus membranaceus (Fisch.) Bunge (Fabaceae) 30 g, A. lancea 30 g, S. tetrandra 20 g and Paeonia lactiflora Pall. (Paeoniaceae) 20 g, which were mixed together and crushed to a total of 100 g, then water was added, and boiled for 1 h, the pH was adjusted to 7, and finally filtered to obtain a concentrated volume of 2 g/mL. Then 10% mannitol was added to the medicinal liquid and the pH of the solution was adjusted to 6–7. The liquid was then pre-frozen at −50°C for 4 h, sublimated at −20°C for 14 h and dried at 30°C for 10 h. The lyophilized powder had a good appearance and was easy to dissolve (Mao et al. 2019). The lyophilized YPHL powder used in this experiment was from the same batch as the above that was used in a previous study (Mao et al. 2019).

Packaging of adeno-associated virus-SIRT1

The adeno-associated virus (AAV)-SIRT1 was obtained from Hanheng Biological Co. Ltd (Shanghai, China) and the AAV was stored at −80°C.

Animals

Forty male Zunker-diabetic-fatty (ZDF) rats (SPF), 8 weeks old, weighing 240–250 g, and 10 male ZL rats, 8 weeks old, weighing 180–200 g were obtained from Beijing Life River Laboratory Animal Technology Co. Ltd., China. All the rats were housed in the environment with a temperature of 22 ± 2°C, a humidity of 50 ± 2%, and a 12 h light/dark cycle, with free access to water. All animal studies were approved by Institutional Animal Care Committee of Zhejiang Chinese Medical University and conducted according to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the Institutional Animal Care and Use Committee (IACUC) guidelines (Animal licence number: SCXK (jing) 2016-0006).

Before the experiment, all animals were acclimatized for one week. The ZDF rats were fed with Purina5008 feed (crude fibre 4.0%, total fat 7.55%, protein 23.75% and digestible energy 14.6 kJ/kg), and the ZL rats were fed with ordinary feed for 4 weeks. The ZDF rats with fasting blood glucose (FBG) ≥11.1 mmol/L were selected as T2DM-IR rats. After successful modelling, the ZDF rats were equally randomized into four groups: model group, YPHL group, SIRT1 overexpression group (SIRT1 group) and negative control group (NC group). YPHL group rats were given oral YPHL decoction for 10 weeks at a dose of 200 mg/kg/d. SIRT1 overexpression group rats were venous injected with HBAAV2/9-r-SIRT1-3′-flag-GFP, while NC group rats were venous injected with HBAAV2/9-CMV-GFP as a blank control. The ZL rats were fed with ordinary feed for 10 weeks as a control group.

Before the end of the experiment, all the rats were fasted for 12 h and then euthanized with pentobarbital sodium (Sigma-Aldrich) (60 mg/kg, i.p.). Blood was collected by heart puncture; the pancreas tissue was immediately removed, part of which was snap-frozen in liquid nitrogen at −80°C for microarray profiling, RT-PCR and Western blotting. The remaining tissue was fixed in 10% buffered formalin for histopathological examination.

Body weight (BW) and oral glucose tolerance test (OGTT)

Each rat was weighed once a week and the blood samples were also collected once a week from the tail vein to test plasma FBG level by the glucose oxidase method (Toecho Super 2, Kagawa, Japan).

After fasting for 12 h, vein blood sampling was used to measure FBG. Each group was given corresponding drugs or distilled water. After 30 min, the blood glucose was measured. Then the rats were given 20% glucose solution according to 10 mL/kg body weight, and the glucose dose was 2 g/kg. Blood glucose was tested at 30, 60, 120 and 180 min after giving glucose, and the changes in the area under the blood glucose curve (AUC) at each time point were analyzed. The AUC was calculated using the following equation:

\[
AUC = 0.5 \times (glucose_{0\text{min}} + glucose_{90\text{min}}) \times 0.5 + 0.5 \times (glucose_{30\text{min}} + glucose_{60\text{min}}) \times 0.5 + 0.5 \times (glucose_{60\text{min}} + glucose_{120\text{min}}) \times 0.5.
\]

Biochemical detection

A biological kit (Erba Diagnostic) and Chem5 Plus-V2 automatic analyzer (Erba Mannhein, Germany) was used to measure Hb1Ac, which was represented the average level of blood glucose value. The insulin ELISA kit (R&D Systems, Abingdon, UK) was used to determine the fasting serum insulin of rats according to the manufacturer’s instructions, and the optical density (OD) of the reaction product at 450 nm was measured using a microplate reader (Europe S.A., Belgium). The IR level was represented by the HOMA-IR index, using the following formula:

\[
\text{HOMA} – \text{IR index} = \frac{\text{fasting glucose (mmol/L)} \times \text{fasting insulin (μU/mL)}}{22.5}
\]

Biochemical methods were applied measure levels of total cholesterol (TC), triglycerides (TG) in serum.

Histopathological examination

The pancreas tissue was fixed in 10% buffered formalin, embedded in paraffin and made into sections with a thickness of 6-μm. HE staining was performed for histological evaluation.

Establishment of the high-lipid-high-glucose (HLHG) cell model

The RIN-m5f cells were purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China). The cells were cultured in DMEM (Hyclone) containing 10% FBS (Hyclone) in a humidified atmosphere containing 5% CO2/95% air at 37°C. After reaching a confluence of 90%, P3 cells (passage for three generations) were counted and plated in culture flasks for subsequent experiments. Serum was removed after 12 h, following which the cells were stimulated with 16.7 mmol/L glucose.
with 500 g for 5 min, after which a supernatant was collected. The glucose concentration was determined using a glucose oxidase method. Briefly, 10 μL of supernatant was mixed with 1000 μL of working solution (a medium volume of R1 reagent and R2 reagent (Jiancheng, Nanjing, China) and placed in a 37 °C water bath for 15 min. The absorbance of each well was measured at a wavelength of 505 nm; blank wells without cells represented the blank control (the sugar content of each group minus the sugar content of the blank group is the glucose consumption of each group of cells).

Determination of glucose consumption rate

RIN-m5f cells were centrifuged at 500 g for 5 min, after which a supernatant was collected. The glucose concentration was determined using a glucose oxidase method. Briefly, 10 μL of supernatant was mixed with 1000 μL of working solution (a medium volume of R1 reagent and R2 reagent (Jiancheng, Nanjing, China) and placed in a 37 °C water bath for 15 min. The absorbance of each well was measured at a wavelength of 505 nm; blank wells without cells represented the blank control (the sugar content of each group minus the sugar content of the blank group is the glucose consumption of each group of cells).

Oil red O staining of RIN-m5f cells

The 24-well plate medium was removed and washed three times with PBS. Then, 500 μL 4% paraformaldehyde was added into each well to fix cells for 20 min at in room temperature, after which a 300 μL filtered oil red dye was added to each well for 5 min. Cells were washed with double distilled water, stained with 500 μL haematoxylin for 10 s and then observed under the microscope (BX43, OLYMPUS).

Transmission electron microscopy (TEM)

The RIN-m5f cells were fixed in 2.5% glutaraldehyde phosphate buffer for 12 h, fixed in 1% osmium acid and stained with 2% uranyl acetate solution. The cells were then dehydrated using gradient alcohol and acetone, fixed with anhydrous acetone and uranyl acetate solution. The cells were then dehydrated using buffer for 12 h, fixed in 1% osmium acid and stained with 2% lead citrate for 5 min, placed on a single-hole TEM grid and observed under a transmission electron microscope (BX43, OLYMPUS).

Detection of ROS and apoptosis of RIN-m5f cells by flow cytometer

RIN-m5f cells were divided into three groups: control group, model group and YPHL group. Cells were cultured as previously described (see above) for 48 h.

ROS probe incubation

After treating the cells with 0.05% trypsin-EDTA solution, the single-cell suspension was placed in a 15 mL centrifuge tube. Medium (5 mL) was added into each tube that was centrifuged at room temperature, at 300 g for 5 min. After removing the supernatant, the cells were resuspended in an equal amount of complete medium and inoculated in a Petri dish (37 °C, 20 min). Then 1 μL of ROS probe (Beyotime, China) was added to the dish at a ratio of 1:1000 and the cells were incubated at 37 °C for 20 min. After additional centrifugation at 500 g for 5 min, the cell pellet was collected and resuspended in 1 mL PBS. A negative control group was set up and treated the same way without adding probes.

Apoptosis probe incubation

Bandung buffer (0.1 mL) was added into the prepared cells pellet, as well as 5 μL of FITC probe and 10 μL of PI probe, which were then mixed and incubated at room temperature for 5 min. The final test was performed after adding band buffer to a total volume of 0.5 mL (Abcam, Waltham, MA, ab14085).

Detection of ROS and apoptosis rate by flow cytometer

Flow cytometer (BIO-RAD, Hercules, CA) records the proportion of ROS-positive cells and the average fluorescence intensity of cells to determine the ROS signal. FL-1A channel was used to detect FITC signal and FL-2A channel was used to detect PI signal. The apoptosis rate of cell was also recorded.

Immunofluorescence staining

The cells were mixed with 0.1% Triton X-100 (room temperature, 20 min) and washed with PBS × 3 (3 min). Next, the cells were mixed with the primary antibody (1:100) (Abcam, Waltham, MA) and incubated in the dark overnight (4 °C). Samples were then washed by PBS × 3 (3 min), mixed with the fluorescent secondary antibody (1:1000) and incubated for 1 h (4 °C). Next, samples were washed with PBS × 3 (3 min), mixed 10 ng/mL DAPI and incubated in the dark for 10 min (4 °C). Finally, samples were washed with PBS × 3 (3 min) again, and observed by inverted fluorescence microscope (Leica, Germany).

Grouping of HLHG model cells and cell transfection

The HLHG model cells were divided into three groups: a model group, an Sh-SIRT1 interference plasmid group (si-SIRT1...
and an Sh-SIRT1 interference negative control group (si-NC group). After 24 h high-lipid and high-glucose stimulation, the cells in si-SIRT1 group and si-NC group were transfected with Sh-SIRT1 interference plasmid and negative control for 72 h, respectively. The cells in si-SIRT1 + YPHL group and si-NC + YPHL group were respectively transfected with the Sh-SIRT1 interference plasmid and negative control for 48 h, after which they were treated with lyophilized YPHL decoction (10 mg/mL) for 24 h. The cells in the control group were routinely cultured without any intervention. 1.25 μL siRNA or NC (20 μmol/L) (Ribobio, Guangzhou, China) per well were mixed with 25 μL opti-MEM dilution medium (Invitrogen, Carlsbad, CA) and stabled for 5 min. Then 1.25 μL transfection reagent and 25 μL opti-MEM dilution medium were mixed. After 5 min, the siRNA/NC–opti-MEM mixture was slowly dropped into the transfection reagent–opti-MEM mixture, and the mixture was kept for 15 min (room temperature). Transfection complex dropwise (50 μL) was then added into the wells and mixed gently.

The mRNA levels of SIRT1 and FoxO1, standardized to GAPDH, were determined by the normalized capacity curve.

### Table 1. Primers for real-time PCR.

| Gene name | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|-----------|------------------------|------------------------|
| SIRT1     | TATCCCTTCAGAACCACCAA   | GGGGACGCAAATATACACATC  |
| FoxO1     | CTCGGAAACCACGTCAAC    | GGATACACCAACGGAGATG    |
| Si-SIRT1  | CCTCAGACGTACGGATG     | GCGTCTTGAGGTATGAA     |
| GAPDH     | AGGGCTCCTCTCCTGAGC    | TGGGTAAGCTGATGGAGTAG   |

### Figure 1. The effect of YPHL decoction on BW (A), fast FBG (B), AUC of blood glucose (C), HbA1C, (D) HOMA-IR index (E), and serum lipid (F). Data are shown by means ± SEM (n = 8). △p < 0.01 and *p < 0.05 vs. control group, **p < 0.01 and ***p < 0.05 vs. model group, ****p < 0.01 and # p < 0.05 vs. NC group. (A) There was no statistically significant difference in BW between YPHL group and model group (p > 0.05). (B) The FBG value of model group was significantly increased than that of control group (p < 0.05), the FBG value of YPHL group was lower than model group by 30.9% (p < 0.01), the FBG value of SIRT1 group was lower than NC group by 40.8% (p < 0.01), while the difference in the FBG value between YPHL group and SIRT1 group was not statistically significant (p > 0.05). (C) Compared with control group, the AUC of blood glucose in model group was increased by 184%, while the AUC in YPHL group was reduced by 19.3% as compared with model group (p < 0.01). Compared with NC group, the AUC in SIRT1 group was reduced by 27.9% (p < 0.01), and the difference of AUC between YPHL group and SIRT1 group was not statistically significant (p > 0.05). (D) Compared with model group, the HbA1C level in YPHL group decreased by 16.1%, the HbA1C level in SIRT1 group decreased by 22.2% compared with the NC group (p < 0.05), while there was no significant difference between YPHL group and SIRT1 group (p > 0.05). (E) Compared with control group, the HOMA-IR index of model group increased significantly (p < 0.01). Compared with model group and NC group, the index of YPHL group and SIRT1 group was reduced by 65.7% and 81.7%, respectively (p < 0.01). Compared with SIRT1 group, the index of YPHL group was increased (p < 0.05). (F) The levels of serum TC in model group were increased by about 1.4 times compared with control group (p < 0.05), while there was no significant difference between model group, YPHL group and SIRT1 group (p > 0.05). Compared with control group, the levels of serum TG in model group and NC group increased by about 17 times and 16 times, respectively (p < 0.01). Compared with model group and NC group, these levels were reduced by 37% and 40% in YPHL group and SIRT1 group, respectively (p < 0.05).

### Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The mRNA levels of SIRT1 and FoxO1 in rat pancreatic tissue and RIN-m5f cells were detected by qRT-PCR, following the instructions of the reverse transcription reaction kit (Thermo Fisher Scientific Inc., Waltham, MA) and the ABI PRISM® 7900 HT Sequence Detection System qPCR kit manual. The primer sequences are listed in Table 1. Total-RNA was extracted by the Trizol method, and RT-PCR was performed after reverse transcription. PCR reaction was performed on 20 μL sample based on the following procedure: pre-denaturation at 95 °C for 1 min, denaturation at 95 °C for 15 s, annealing at 55 °C for 25 s, extension at 72 °C for 25 s, 40 cycles in total. The linked expression levels of SIRT1 and FoxO1 were determined by the normalized capacity curve.
Western blot

The pancreatic tissue and RIN-m5f cells were lysed in NP-40 lysis buffer and centrifuged for 20 min (12,000 g, 4°C). The concentration of protein was then quantitated, and the protein lysate (20 µl g) was analyzed using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method, after which it was electrotransferred onto PVDF membranes (Millipore, Billerica, MA). The membranes were blocked by 5% skimmed milk powder and incubated with anti-SIRT1 (81 kDa; 1:1000) (Abcam, Waltham, MA), anti-FoxO1 (69 kDa; 1:1000) (Abcam), anti-LC3 (15 kDa; 1:2000), anti-p62 (62 kDa; 1:1000) (CST) and anti-GAPDH (36 kDa; 1:2000) (Abcam) at 4°C overnight, and then with the secondary antibodies of horseradish peroxidase (HRP)-conjugated (rabbit) for 2 h at room temperature. Finally, the samples were visualized by enhanced chemiluminescence (ECL) (Millipore, Billerica, MA) on a VersaDoc 4000 MP system (BIO-RAD). The Imagej software (National Institutes of Health, https://imagej.nih.gov/ij/) was used to perform densitometric analysis and calculate the relative protein expression level by comparing with the control (Bethesda, MD).

Statistical analysis

All the data are shown as mean ± standard deviation (mean ± SD) (or mean ± SEM). One-way ANOVA was used for the comparison of means between multiple groups. Least significance difference (LSD) test was used when the variances were uniform, while the Dunnett’s-T3 test for uneven variances; a p < 0.05 was considered as statistically significant. The statistical analyses were performed by SPSS 23.0 (IBM, Armonk, NY). All the Figures 1–7 were drawn by GraphPad Prism 6.02 (GraphPad, La Jolla, CA) and Adobe Illustrator CS6 (Adobe, San Jose, CA).

Results

The H2O2 content, level of ROS, and apoptotic rate were significantly increased, while the rate of glucose consumption decreased in the HLHG model cells compared to control cells, thus suggesting the presence of IR in the HLHG model cells. Contrary, YPHL reversed the situation, which suggested that YPHL can decrease IR in vitro (Figure 4).

Compared with the control group, serum TG, TC and pancreatic TG levels were significantly increased in T2DM-IR model rats. Moreover, the level of TG was significantly decreased in the YPHL group compared with model group. Compared with model group, the IR level on T2DM-IR model rats was significantly reduced after YPHL or SIRT1 intervention, and this effect was the most obvious in SIRT1 group (Figure 1).

Although the oil red O staining results were not very obvious, we observed varying degrees of lipid deposition in pancreatic tissues in model group and NC group contrary, this process was reduced after YPHL and SIRT1 intervention (Figure 3). In model group and NC group, large fat deposits were observed between the acinar lobules by HE staining, while acinar cell ectopic hyperplasia, and telangiectasia were observed in the pancreatic islets (Figure 2). Furthermore, TEM showed no obvious autophagy in normal organelles of the normal rats. In the HLHG model
cell, the organelles and many autophagosomes disappeared. After YPHL intervention, the early autophagosomes, enlarged mitochondrial structure and autophagy was reduced compared to model group (Figure 3). In addition, the protein expression of SIRT1 and LC3 in the pancreas tissues of model group and NC group were significantly lower than control group, while the protein expression of p62 was significantly increased. In the RIN-m5f cells of model group and si-NC group, the level of autophagy-related proteins was significantly reduced. After YPHL intervention and SIRT1 overexpression, the protein expression of LC3 and p62 increased significantly. The protein expression of SIRT1 and LC3 of model, si-SIRT1, si-NC and si-SIRT1 + YPHL groups in RIN-m5f cells were significantly lower than that of control group, while the protein expression of FoxO1 was significantly increased (Figures 5–7).

Discussion

Obesity and obesity-associated IR contribute to T2DM and metabolic syndrome (MS) (Levine and Yuan 2005). In obese patients, the secretion and expression of many inflammatory cytokines are abnormal and the inflammation signal pathway is activated. As a result, chronic inflammation may develop, interfering with the insulin signal pathway and leading to IR occurrence.

Autophagy is an important regulator of organelle function and insulin signalling. The loss of autophagy is a critical component of defective insulin action seen in obese and T2DM-IR patients (Liu et al. 2009). Autophagy is a regulatory process that maintains cell homeostasis by promoting the reuse of damaged organelles. Endoplasmic reticulum stress, chronic inflammation and oxidative stress are closely related to IR, which can lead to the dysfunction of organelles and the accumulation of damaged organelles. Autophagy vital in removing damaged organelles and is significantly inhibited in various tissues when IR develops (Kim and Lee 2014). EDL in the tissue can alter the ability of mitochondria to oxidise fatty acids, resulting in mitochondrial dysfunction and increased oxidative stress. With an aggravation of EDL in the pancreas, the function of β-cells is gradually lost and IR caused by insulin signal transduction dysfunction increases (Kobyliak et al. 2017). In the early stage of EDL, autophagy is synergistically activated by lipolysis products or metabolic derivatives of free fatty acids, as well as oxidative stress and endoplasmic reticulum stress, while the activity of autophagy will tends to increase (Kim and Lee 2014). However, with time, the fusion of autophagosomes and lysosomes are hindered due to the disturbance of the lysosomes’ acidic environment or the change in lipid droplet membrane composition (Kern et al. 2016; Lippai and Szatmári 2017). In obese mice, a severe downregulation of
autophagy and autophagy-related gene Atg7 expression has been observed in the liver, which, in turn, resulted in the defection of insulin signalling and elevated endoplasmic reticulum stress. The restoration of the Atg7 expression resulted in dampened endoplasmic reticulum stress, enhanced hepatic insulin action, which was dependent on autophagy to regulate the action of insulin in the liver (Kern et al. 2016; Lippai and Szatmari 2017). The autophagy enhancer MSL can improve the metabolic profile of obese ob/ob mice, ameliorate inflammasome activation and improves the glucose profile of ob/ob mice by accelerating the clearance of intracellular lipids (Vainshtein et al. 2014). The down-regulation of autophagy can also increases the expression of P62. Over-expression of P62 can increase the transfer of NF-κB into the nucleus, thus promoting the down-regulation of GLUT4 expression, reducing the glucose transport and extraction in islet cells, and eventually leading to T2DM and IR (Li et al. 2015).

SIRT1, a member of the Sir2u family in mammalian, is an NAD^+–dependent histone deacetylase, widely expressed in animal’s liver, muscle, blood vessels, islets, brain, fat and other tissues that have an important role in energy balance (Li and Kazgan 2011). Fork box protein O1 (FoxO1) is a member of the FoxO family whose expression is positively correlated with the level of insulin resistance. It has an essential role in regulating insulin sensitivity in pancreatic β-cells, liver cells and adipocytes. SIRT1 can deacetylate and activate autophagy proteins as Atg5, Atg7 and LC-3 in the cytoplasm in order to induce autophagy directly. Moreover, SIRT1 can deacetylate transcription factors as p53, NF-kB, FoxO1, PGC-1α in the nucleus to regulate the level of autophagy and glycolipids metabolism (Ferraro et al. 2014). SIRT1 can reduce the deacetylation of FoxO1, increase the mobilization of adiponectin, promote lipid mobilization and reduce insulin resistance in peripheral tissues. The SIRT1-FoxO1-autophagy pathway has an essential role in regulating the pathophysiological processes as diabetes, obesity and tumours (Liang et al. 2009).

Previous studies suggested that YPHL decoction could improve the blood glucose, serum lipids, BW and other related indicators of metabolism in patients with T2DM. In addition, our study suggested that YPHL could reduce the degree of fat deposition in the liver and pancreas in C57BL/KsJ-db/db diabetic mice (Chai et al. 2013).
Figure 5. Photographs of each RIN-m5f cell group under the laser confocal microscope (×400). Laser scanning confocal microscopy showed that compared with control group, the expression of SIRT1 was reduced and the expression of FoxO1 was increased significantly in model group (p < 0.05). Compared with model group, the expression of SIRT1 and FoxO1 in YPHL group had no significant difference (p > 0.05).

Figure 6. The results of SIRT1 and FoxO1 mRNA expressions. Expressions of SIRT1 and FoxO1 mRNA in pancreas tissues (A) and RIN-m5f cells (B) were detected by qRT-PCR analysis. GAPDH was used as the internal control. n = 8, ANNOT p < 0.05, ANNOT p < 0.01 vs. control group, p < 0.05 vs. model group, p < 0.05 vs. NC group, # p < 0.05 vs. si-NC group. (A) In pancreatic tissue, the relative expression of SIRT1 mRNA in model group and NC group was decreased significantly compared with control group (p < 0.05). The relative expression of SIRT1 mRNA was increased in YPHL group and SIRT1 group compared with model and NC groups, respectively (p < 0.05). On the contrary, the relative expression of FoxO1 mRNA in model group and NC group was increased significantly compared with control group (p < 0.05). The relative expression of FoxO1 mRNA was decreased in YPHL group and SIRT1 group compared with model and NC groups, respectively (p < 0.05). (B) In RIN-m5f cells, the relative expression of SIRT1 mRNA was significantly decreased in model, si-SIRT1, si-NC, and si-SIRT1 + YPHL groups compared with control group (p < 0.05). On the contrary, compared with model, si-SIRT1, si-NC, and si-SIRT1 + YPHL groups, the relative expression of SIRT1 mRNA was increased in si-NC + YPHL group (p < 0.05). On the contrary, the relative expression of FoxO1 mRNA was significantly increased in model, si-SIRT1, si-NC, and si-SIRT1 + YPHL groups compared with control group (p < 0.05). Compared with model, si-SIRT1, si-NC, and si-SIRT1 + YPHL groups, the relative expression of FoxO1 mRNA was decreased in si-NC + YPHL group (p < 0.05).
Male ZDF rats, characterized by obesity, insulin resistance and impaired glucose tolerance, have been widely used as a T2DM model (Etgen and Oldham 2000; Finegood et al. 2001). In this study, we hypothesized that YPHL could reduce IR by regulating the genes related to the SIRT1-FoxO1-autophagy pathway in ZDF rats. Briefly, ZDF rats were first treated with different concentrations of YPHL decoction (4, 2, 1 and 0.5 g/mL). We found that the drug was safe and did not induce side effects; in addition, 2 g/mL was the optimal dose for blood glucose control. Consequently, we performed a series of in vitro experiments to investigate the effect and mechanism of action of YPHL in the high-lipid high-glucose (HLHG) rat pancreatic β-cells (RIN-m5f). We used the sterile lyophilized YPHL powder instead of YPHL decoction to ensure the accuracy of the experiment. RIN-m5f cells are brown rat insulinoma epithelial cells derived from islet cell lines and are the most classic cell model for studying pancreatic β-cells in vitro. Preliminary CCK8 cell viability assay showed that the survival rate of cells treated with 10 mg/mL YPHL (24 h) was the highest when IR-RIN-m5f cells were treated by different concentrations of YPHL (p < 0.01) (Mao et al. 2019). Therefore, the 10 mg/mL YPHL was selected for further experiments.

Our data showed that serum and pancreatic TG levels in T2DM-IR rats were increased, and intervention of YPHL reduced the TG levels. The intervention of YPHL or SIRT1-overexpression also reduced the IR level in T2DM-IR rats, which is consistent with Milne et al. (2007), who discovered that the activation of SIRT1 in ZDF rats improves systemic insulin sensitivity and hepatic insulin response. In addition, the intervention of YPHL or SIRT1 overexpression could reduce lipid deposition in the pancreatic tissue of T2DM-IR rats. Under transmission electron microscopy, we observed an increase in autophagosomes and the disappearance of organelles in HLHG model cells, which is consistent with the finding reported in previous literature (Kern et al. 2016). After the intervention of YPHL, the early autophagosomes were reduced and mitochondrial structure expanded and improved. Furthermore, YPHL intervention and SIRT1 overexpression increased the expression of SIRT1 and reduced the inhibition of LC3 and p62 protein expression in pancreatic cells caused by HLHG, indicating that the mechanism

**Figure 7.** The results of protein expressions. The protein expressions of SIRT1, Foxo1, LC3, and p62 in pancreas tissues (A, B) and cells (C, D) were detected by Western blotting. The figure represents one of three experiments with similar results (A, C). In (B) and (D), *p < 0.05, **p < 0.01 vs. control group, *p < 0.05, **p < 0.01 vs. model group, *p < 0.05, **p < 0.01 vs. NC group, *p < 0.05 vs. si-NC group. (A–B) In pancreas tissue, compared with control group, the relative expression of protein SIRT1 and LC3 was significantly decreased in both model and NC groups (p < 0.05), while the relative expression of protein FoxO1 and p62 was significantly increased in model group and NC group (p < 0.05). On the contrary, compared with model group and NC group, the relative expression of protein SIRT1 and LC3 was significantly increased in both YPHL and SIRT1 groups (p < 0.05), while the relative expression of protein FoxO1 and p62 was significantly decreased in both YPHL and SIRT1 groups than that in model and NC groups (p < 0.05). (C–D) In RIN-m5f cells, compared with control group, the relative expression of protein SIRT1 and LC3 was significantly increased in model, si-SIRT1, si-NC, and si-SIRT1 + YPHL groups (p < 0.05), while the relative expression of protein FoxO1 was significantly increased (p < 0.05). On the contrary, the relative expression of protein SIRT1 and LC3 was significantly increased in si-NC + YPHL group than that in model, si-SIRT1, si-NC, and si-SIRT1 + YPHL groups (p < 0.05), while the relative expression of protein FoxO1 was significantly decreased in the si-SIRT1 + YPHL group (p < 0.05).
of YPHL to reduce insulin resistance in β-cells may be achieved by activating autophagy. To verify this effect, we used a cell model of si-RNA interference instead of overexpression intervention. We found that YPHL can reverse the decrease of SIRT1 and LC protein expression and the increase of FoxO1 protein expression in si-NC cells, thus indicating that YPHL may regulate the expression of the above genes through the SIRT1 pathway.

**Conclusions**

In the present study, we hypothesized that YPHL could reduce IR by regulating the genes related to the SIRT1-FoxO1-autophagy pathway in ZDF rats. It was observed that SIRT1 as an upstream gene can inhibit the activity of FoxO1, promote the expression of autophagy-related factor LC3, increase autophagy activity, reduce the ectopic deposition of lipids in the pancreas, regulates lipid metabolism and relieves insulin resistance. The regulatory effect of YPHL on the SIRT1-FoxO1 pathway was similar to that of SIRT1 overexpression. YPHL can increase the autophagy activity in pancreatic β-cells through the SIRT1-FoxO1 signalling pathway to reduce insulin resistance and treat T2DM. Thus, we speculate that the SIRT1-FoxO1-autophagy pathway may have an important role in the pathogenesis of EDL, insulin resistance and type 2 diabetes.

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**Disclosure statement**

The authors report no conflict of interest.

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**Data availability statement**

The data set supporting the results of this article are included within the article.

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