• 论 著 •

Improvement Effect of Insulin Resistance of *Nitraria Roborowskii* Kom in Type 2 Diabetic Mice via PI3K/AKT Signaling Pathway

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ABSTRACT: OBJECTIVE To explore hypoglycemic effect of 95% ethanol fraction of *Nitraria roborowskii* Kom(NRK-C) and its possible mechanism evaluated in the type 2 diabetes mellitus(T2DM) mice. **METHODS** The body weight, organ indices, blood glucose levels, serum biochemical indexes, as well as HE/PAS histopathological section were all analyzed to assess the hypoglycemic effect of NRK-C in T2DM mice induced by a high-fat diet(HFD) combined with six intraperitoneal injections of 35 mg·kg^{-1} of streptozotocin(STZ). The Western blotting and immunofluorescence were further applied to determine the regulatory effect of NRK-C on key signaling proteins. **RESULTS** The fasting blood glucose levels were significantly reduced after 7 weeks of administration of NRK-C. In addition, NRK-C could also significantly improve glucose tolerance, hepatic glycogen levels, and lipid levels(total cholesterol, triglyceride, low density lipoprotein and high density lipoprotein), and significantly reduced insulin resistance of diabetic mice, which played an important role in the antidiabetic effects. Further mechanism research demonstrated that phosphorylated PI3K expression was up-regulated and p-GSK3β expression was up-regulated after NRK-C intervention, indicating that NRK-C might exert a potential antidiabetic effect by modulating the PI3K/AKT signaling pathway. **CONCLUSION** All these results suggested that NRK-C might improve T2DM and had the potential to be used as an adjunctive therapy.
KEYWORDS: type 2 diabetes; *Nitraria roborowskii* Kom; glucose tolerance; insulin resistance; PI3K/AKT signaling pathway

大果白刺通过 PI3K/AKT 信号通路改善 2 型糖尿病小鼠胰岛抵抗

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摘要：目的 探究大果白刺 95% 乙醇提取物 (NRK-C) 对 2 型糖尿病 (type 2diabetes mellitus，T2DM) 小鼠的降血糖作用及其可能机制。**方法** 通过高脂饮食 (high-fat diet，HFD) 联合 6 次腹腔注射 35 mg·kg^{-1} 链脲佐菌素 (streptozotocin，STZ) 诱导 T2DM 小鼠模型，对 (T2DM) 小鼠体质量、脏器指标、血糖水平、血清生化指标以及采用 HE/PAS 染色技术对肝组织病理切片进行分析，探究 NRK-C 对 T2DM 小鼠的降血糖作用。进一步采用蛋白质印迹和免疫荧光检测测定 NRK-C 对关键信号蛋白的调节作用。**结果** NRK-C 给药 7 周后空腹血糖水平显著降低。此外，NRK-C 还能显著改善糖尿病小鼠的葡萄糖耐量、肝糖原水平和血脂水平 (总胆固醇、甘油三酯、低密度脂蛋白和高密度脂蛋白)，显著降低糖尿病小鼠的胰岛素抵抗，在治疗糖尿病方面发挥重要作用。进一步的机制研究表明，NRK-C 干预后磷酸化 PI3K 表达上调，p-GSK3β 表达上调，表明 NRK-C 可能通过调节 PI3K/AKT 信号通路发挥潜在的治疗糖尿病作用。**结论** 所有这些结果都表明 NRK-C 可能改善 T2DM，并具有作为辅助治疗的潜力。
关键词： 2 型糖尿病；大果白刺；葡萄糖耐量；胰岛抵抗；PI3K/AKT 信号通路
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Type 2 diabetes(T2DM), also known as noninsulin-dependent diabetes, was a chronic metabolic disease characterized by hyperglycaemia, accounting for more than 90 % of the incidence of all diabetics[1].

According to statistics, there were approximately 463 million people living with diabetes globally in 2019, and it was predicted that there would be as many as 750 million people living with diabetes in 2045[2]. A series of chronic complications caused by long-term persistent hyperglycemia in diabetes patients[3], could seriously endanger the patient's own health, reduce the quality of life, and even lead to death[4]. Currently, commercially available hypoglycemic drugs had toxic side effects and drug dependence on diabetic patients, so it has become the focus of diabetes research to explore natural active substance without side effect from natural plants that can improve insulin resistance, hyperglycemia, lipid metabolism disorders, as well as chronic complications of diabetes[5-6].

The phosphatidylinositol-3-hydroxykinase/ protein kinase B(PI3K/AKT) signaling pathway was thought to regulate various physiological processes associated with T2DM. Numerous studies have found that the PI3K/AKT pathway was a major insulin signaling pathway, and its activity was reduced in the presence of insulin resistance[7]. AKT was a key signaling molecule in the insulin PI3K/AKT signaling pathway[8-9]. Phosphorylation of AKT activated downstream signaling molecules such as glycogen synthase kinase 3(GSK-3β), a serine/threonine phosphokinase[10]. And its phosphorylate allowed the transport of glucose transporters from the intracellular to the cytosol membrane, thereby caused glucose uptake in liver, adipose and skeletal muscle tissue, intracellular glycogen synthesis inhibition, gluconeogenesis and glucose export, finally regulated glycogen metabolism[11].

Nitraria roborowskii Kom(NRK) was a member of genus *Nitraria*, and widely distributed in western Inner Mongolia, Qinghai, Xinjiang and northeastern Tibet[12-13]. The fruit of NRK, known as the "desert cherry", has high potential economic value as a source of edible berries. It has been found that *Nitraria tangutorum* had the lowering effects of blood lipids and blood glucose levels, and antioxidant activity[13-14]. Although NRK has a long history of medicinal use in the folk and had high medicinal value, research on its modern pharmacological activity was still in its early stage, and there has been no report on its improvement of insulin resistance(IR)[15].

In this study, the fasting blood glucose(FBG) level, glucose tolerance, insulin level, lipid level, oxidative stress and histopathological analysis were analyzed to investigate the improvement effect of NRK for glucose-lipid metabolism and insulin resistance in T2DM mice. At the same time, the hypoglycemic mechanism of NRK was further investigated through related protein expression according to the network pharmacology analysis. This study clarified the hypoglycemic effect and mechanism of NRK, providing solid data support for the high-value development and utilization of this resource.

1 Experimental materials and methods

1.1 Experimental material

PBS buffer(Beijing Solebao Technology Co., Ltd., Lot: 2309009); anhydrous disodium hydrogen phosphate/anhydrous sodium dihydrogen phosphate(Sinopharm Chemical Reagent Co., Ltd.); streptozotocin(Aladdin Biochemical Science and Technology Co., Ltd., Lot: J2129407); glucose test paper and glucose(Tianjin Kaitong Chemical Reagent Co., Ltd., Lot: 20152400921.); PI3Kp85(Lot: 4257S), p-PI3Kp85(Lot: 4228S), AKT(Lot: 4691S), GSK3β(Lot: 12456S), p-GSK3β(1∶1000, Lot: 5558S) were procured from CST; p-AKT(Lot: AF0016), anti β-actin antibody(Lot: AF7018) were procured from Affinity; the biochemical kits of triglyceride(TG, Lot: A110-1-1); malondialdehyde (MDA, Lot: A003-1); total cholesterol(TC, Lot: A111-1-1), superoxide dismutase(SOD, Lot: A001-3-1), high-density lipoprotein-C(HDL-C, Lot: A112-1-1), low-density lipoprotein-C(LDL-C, Lot: A113-1-1), catalase(CAT, Lot: A007-1-1), glutathione

peroxidase(GSH-Px, Lot: A005-1), mouse insulin (INS) enzyme reaction kit(Lot: H203-1-2) were procured from Nanjing Jiancheng Bioengineering Institute.

The total extract of NRK was obtained by squeezing the juice and concentrating, which then was eluted with 95% ethanol on macroporous resin to obtain 95% alcohol part of NRK.

1.2 Animals

Five-week-old male C57BL/6J mice(n=80) were purchased from Beijing Huafukang Bioscience Co. [Production license number: SCXK(京)2019-0008]. All mice were acclimatized and fed for one week with free access to food and water. After one week, mice were randomly selected according to their body weights and divided into two groups: the control group(n=14) was given normal sterile chow, and the diabetes modeling group(n=66) was fed a high-sugar and high-fat chow. All mice were housed in cages with 10 mice per cage in an animal house maintained at a room temperature of 22−25 ℃ and a humidity of approximately 50%, were given 12 h of light and 12 h of darkness per day and were provide sufficient food and water in an experimental environment. All the animal experiments were carried out strictly in accordance with the Guidelines for Care and Use of Laboratory Animals of the Northwest Institute of Plateau Biology, CAS and approved by the Animal Ethics Committee of Northwest Institute of Plateau Biology, CAS.

In this experiment, T2DM model was induced by the combination of high-fat diet(HFD) and streptozotocin(STZ)[16]. The mice were fed the above-mentioned high-sugar and high-fat chow for 7 weeks, and then the mice were given an injection of 1% STZ solution(0.1 mmol·L^{-1}, prepared by the citrate buffer with a PH value of 4.4) into the lower left peritoneal cavity at a dose of 35 mg·kg^{-1}, and the control group was given an injection of citrate alone. The model group was injected intraperitoneally for six consecutive times and once a day. The fasting blood glucose(FBG) levels after 72 h of modeling was measured, and mice with the value ≥ 11.1 mmol·L^{-1} for three consecutive days was considered to be successful in molding. 65 mice were modeled.

The diabetic mice were randomly divided into T2DM model group(Mod), positive drug metformin intervention group(Met, 200 mg·kg^{-1}), low-dose drug intervention group(NRK-CL, 100 mg·kg^{-1}), medium-dose drug intervention group(NRK-CM, 200 mg·kg^{-1}) and high-dose drug intervention group(NRK-CH, 400 mg·kg^{-1}). The mice in the control and model groups were gavaged with saline for 7 weeks. During the whole experiment, the mice were observed for diet, mental status, hair color, urine output, body weight and blood glucose.

1.3 Assessment of body weight, and FBG levels

The drugs were administered by gavage at the dose corresponding to the body weight of the mice every day, and the body weights of the mice were measured at the same time every week. The mice body weight change was recorded every week and documented until the end of the whole experiment, and the variability of body weights between each group was compared[17]. The FBG levels were also measured once a week after fasting for 12 h with a glucometer, and the differences in FBG levels between the mice in each group were compared.

1.4 Effects on organ indices

After the seventh week of administration, all experimental animals were weighed, and after removing the eyeball and taking blood, the mice were executed to obtain organ tissues. The liver, kidney and pancreas tissues of each group were quickly removed, rinsed in pre-cooled 0.9 % saline, and then the tissues were weighed and the organ indexes were recorded when the water was absorbed by filter paper. The formula is as follows: $\text{Organ index}=\frac{\text{organ weight(g)}}{\text{body weight(g)}}\times 100\%$.

1.5 Serum biochemical analysis

After the seventh week of administration, the blood was obtained by removing the eyeball, then centrifuged(1 788.8×g, 10 min, 4°C) to obtain serum, and stored at −80°C before analysis. The levels of TG,TC, LDL-C, HDL-C, CAT, SOD,GSH-Px and

MDA in mice serum were determined using the appropriate kits according to the manufacturer's instructions, and fasting insulin(FINS) were determined using ELISA kits. Homeostasis model insulin resistance index(HOMA-IR) was calculated according to the results of FBG and FINS levels and the HOMA-IR formula[18].

The formula is as follows: HOMA-IR= $\frac{(\text{FBG x FINS})}{22.5}$ (FBG: fasting blood glucose; FINS: fasting insulin)

1.6 Histopathological analysis of HE/PAS staining

For HE staining, the liver tissues were fixed with 4% paraformaldehyde for 24 h, then dehydrated with 30% sucrose and embedded with paraffin. Then the sections were stained with HE solution[19]. For liver PAS staining, the sections were immersed in periodate staining solution for 30 min, rinsed with double-distilled water, and then stained with Schiff's stain[20]. The stained tissues were photographed through a light microscope(200×).

1.7 Glucose tolerance test(OGTT) assay

OGTT was used to evaluate the responsiveness of mice to glucose stimulation after glucose administration[21]. After overnight fasting, all mice were gavaged with glucose(2 g·kg^{-1}). Blood glucose levels were measured at 0, 30, 60 and 120 min after glucose administration. The trend of blood glucose was plotted using prism 8.0 software and the area under the curve(AUC) was also calculated using prism 8.0.

1.8 Insulin tolerance test(ITT) assay

ITT was performed as previously described with some modifications[22]. The mice were first fasted for 5 h, and human insulin(0.25 U·kg^{-1}) was administered intraperitoneally at 2 pm. The state of diabetic mice was observed throughout the period before and after insulin injection, and blood glucose levels were measured at 30, 60, and 120 min after intraperitoneal injection administration, and the trend of blood glucose changes was plotted and the AUC was calculated by using prism 8.0 software.

1.9 Network pharmacology analysis

The gene targets of chemical components of NRK-C were obtained by using the Traditional Chinese Medicine System Pharmacology Database(TCMSP) and PubChem Database and Swiss Target Prediction[23]. The disease gene targets related to type 2 diabetes were got by searching the DisGeNet and GeneCards database. Then intersection targets of chemical components with type 2 diabetes were obtained by Venny 2.1.0. The PPI target network interaction diagram was obtained by importing the intersection targets of diseases and active ingredients into String[24]. KEGG signaling pathway analysis was conducted by importing the top 35 targets into the David database, and the result was presented in the form of enriched bubble plots[25].

1.10 Western blotting

Western blotting was used for the determination of several protein changes in the liver[26]. Then the protein concentration was determined by the BCA protein assay kit. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) gel with 5% and 8% solubilized gels, respectively[27]. Subsequently, the membrane was blocked with 5 % skimmed milk solution for 1 h at room temperature. The primary antibodies of pI3Kp85, p-PI3Kp85, AKT, p-AKT, GSK3β, p-GSK3β and anti-β-actin were respectively incubated with PVDF membrane sheets at 4 °C overnight, and the primary antibody was recovered on the next day, and the secondary rabbit antibody was incubated at 25 °C for 60 min, *β*-actin was used as an internal reference protein(1∶10 000). Protein bands were captured by the Amersham Imager 600 system. The results were calculated by Image J software.

1.11 Liver immunofluorescence

Immunofluorescence was performed according to the previous described method[28]. Liver tissue sections were incubated with primary antibody(p-GSK-3β 1∶400) overnight at 4°C and incubated with secondary antibodies for 1 h at room temperature. After adding DAPI to counterstain nuclei, an anti-fluorescence quencher was added to seal the slides[29]. Finally, fluorescence microscopy was used to observe and obtain images.

1.12 Statistical analysis

The data of this study were expressed as mean ± standard deviation. Data were statistically analyzed and plotted using GraphPad Prism 8.0 software. Statistical comparisons between the two groups were analyzed using unpaired t-tests when necessary. Multiple comparisons were compared using one-way(or two-way) analysis of variance(ANOVA) followed by Bonferroni post hoc test. Differences were statistically significant at $P<0.05$.

2 Results

2.1 Effect of NRK-C on body weight, FBG, FINS, HOMA-IR, ITT, OGTT and organ index,

The mice in the model group showed a gradual decrease in body weight with the increase of feeding days compared with that in the Con, and the mice in the Mod were found to have loose body hair, gray fur, decreased activity, and showed the typical diabetic symptoms of "three more and one less" in the experiment. As shown in Figure 1A, the body weights

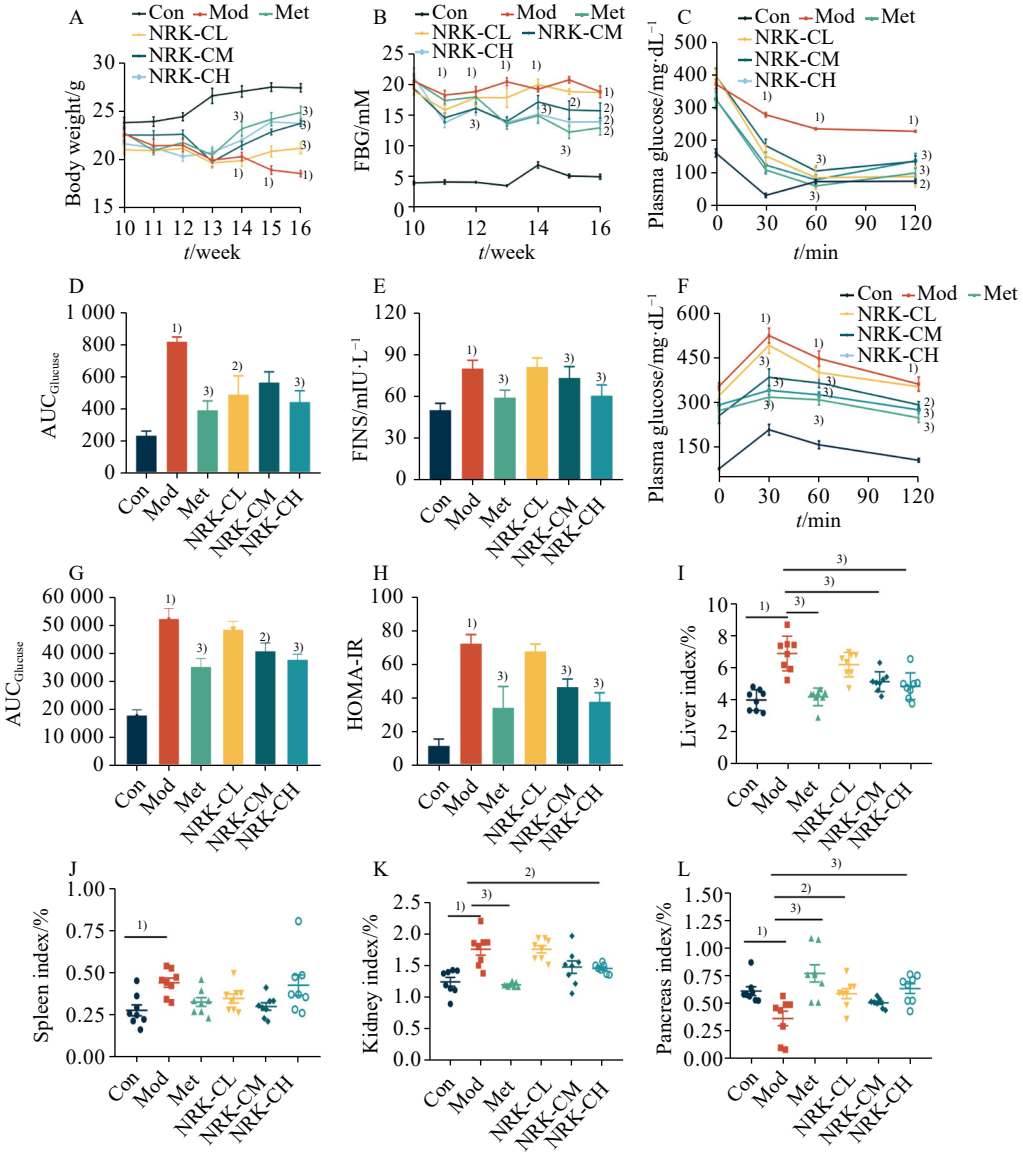

Fig. 1 Effects of NRK-C on blood glucose levels, glucose tolerance, and insulin sensitivity in high-fat diet and STZ-induced diabetic mice($\bar{x}\pm s$, n=8)

A−body weight levels; B−fasting blood glucose levels; C−ITT test; D−ITT AUC; E−serum insulin levels; F−OGTT test; G−OGTT AUC; H−HOMA-IR index; I−liver index; J−spleen index; K−kidney index; L−pancreas index. Compared with Con, $^{1)}P<0.01$; compared with Mod, $^{2)}P<0.05$, $^{3)}P<0.01$.

图 1 NRK-C 对高脂饮食和 STZ 诱导的糖尿病小鼠血糖水平、葡萄糖耐量和胰岛素敏感性的影响 ($\bar{x}\pm s$，n=8)

A−体质量水平；B−空腹血糖水平；C−ITT 试验；D−ITT AUC；E−血清胰岛素水平；F−OGTT 试验；G−OGTT AUC；H−HOMA-IR 指数；I−肝脏指数；J−脾脏指数；K−肾脏指数；L−胰腺指数。与 Con 相比，$^{1)}P<0.01$；与 Mod 相比，$^{2)}P<0.05$，$^{3)}P<0.01$。

of the Mod were significantly lower than that of the Con from weeks 14−16. At week 16, the body weights of the NRK-CL, NRK-CM, NRK-CH were increased significantly compared with that of Mod(P<0.01), but still lower than that of the Con. It indicated that the drug intervention of NRK-C had significantly improved the general symptoms of diabetic mice. As shown in Figure 1B, the FBG levels of the control mice remained stable during the experiment, while the mice in the Mod always maintained a high blood glucose level. At weeks 15 and 16, the FBG level in the positive administration group was significantly lower than that of the Mod(P<0.01). The FBG levels in the medium and high dose administration groups were also significantly lower than that in the Mod(P<0.01), but still higher than that in the control mice. The FBG values of NRK-C administration group showed an improved effect from week 13 to week 16 compared with the Mod, and the FBG improvement effect of NRK-CH was better than that of NRK-CM, indicating NRK-C could dose-dependently improve the FBG levels. As shown in Figure 1E, the FINS level of the Mod mice was highly significantly elevated(P<0.01) compared with that of the control mice. The presence of hyperinsulinemia(high blood insulin levels and still high blood glucose) indicated that the Mod mice had severe insulin resistance. The FINS levels of NRK-CM and NRK-CH were significantly lower compared with that of the Mod(P<0.01).

The steady state model is currently the most common used and very effective method for evaluating insulin resistance. As seen in Figure 1H, compared with the Con, the HOMA-IR of Mod was severe(P<0.01), and was more than four times that of the Con. After 7 weeks of NRK-CM, NRK-CH intervention, HOMA-IR were significantly improved compared with the Mod(P<0.01), indicating NRK-C had better improvement on the FINS level and insulin resistance status.

ITT assay was also performed. As shown in the Figure 1C−D, after insulin injection, although the blood glucose level of the Mod mice decreased, it significantly increased compared to the Con at 30, 60 and 120 min, and the area under the curve was also significantly elevated, indicating that the HFD+STZ induced diabetic mice showed insulin resistance. The degree of blood glucose reduction for the Mod was higher than that of the Con, showing certain insulin-dependent characteristics, indicating that the HFD+STZ induced diabetic mice had reduced insulin secretion and showed insulin-deficient characteristics. The high dose of NRK-C could effectively improve insulin tolerance abnormality, improve hyperinsulinemia or stimulate insulin secretion in diabetic mice.

In the OGTT test, as shown in the Figure 1F−G, all experimental mice showed a rapid increase in blood glucose levels at 30 min, followed by a gradual decrease in the Con to the normal level within 120 min, whereas the Mod showed a state of high blood glucose level, which even lasted until 120 min. And the AUC value of the Mod was extremely and significantly increased than that of Con(P<0.01). However, after the administration of high dose of NRK-C and Met, the blood glucose levels and AUC values were both significantly reduced, which indicated that NRK-C was able to improve glucose tolerance in diabetic mice.

As shown in Figure 1I−L, compared with the Con, the organ indexes including liver, kidney, spleen and pancreas indexes in the Mod all have undergone significant changes(P<0.01). Compared with the Mod, the liver and kidney indexes of the metformin-positive drug and NRK-CM, NRK-CH were significantly lowered, and the pancreas indexes were significantly increased. These result indicated that the intervention of metformin-positive drug and NRK-CM, NRK-CH could significantly improve the organ index of diabetes mice to some extent.

2.2 Effects of NRK-C on blood lipids and CAT, SOD, MDA, GSH-Px of diabetic mice

As shown in Figure 2A, the blood lipid levels of TG, TC and LDL-C were significantly elevated in the model mice compared with the Con(P<0.01). All these three blood lipid levels were decreased after treatment with medium/high dose of NRK-C(Figure 2A). HDL-C levels were significantly decreased in the

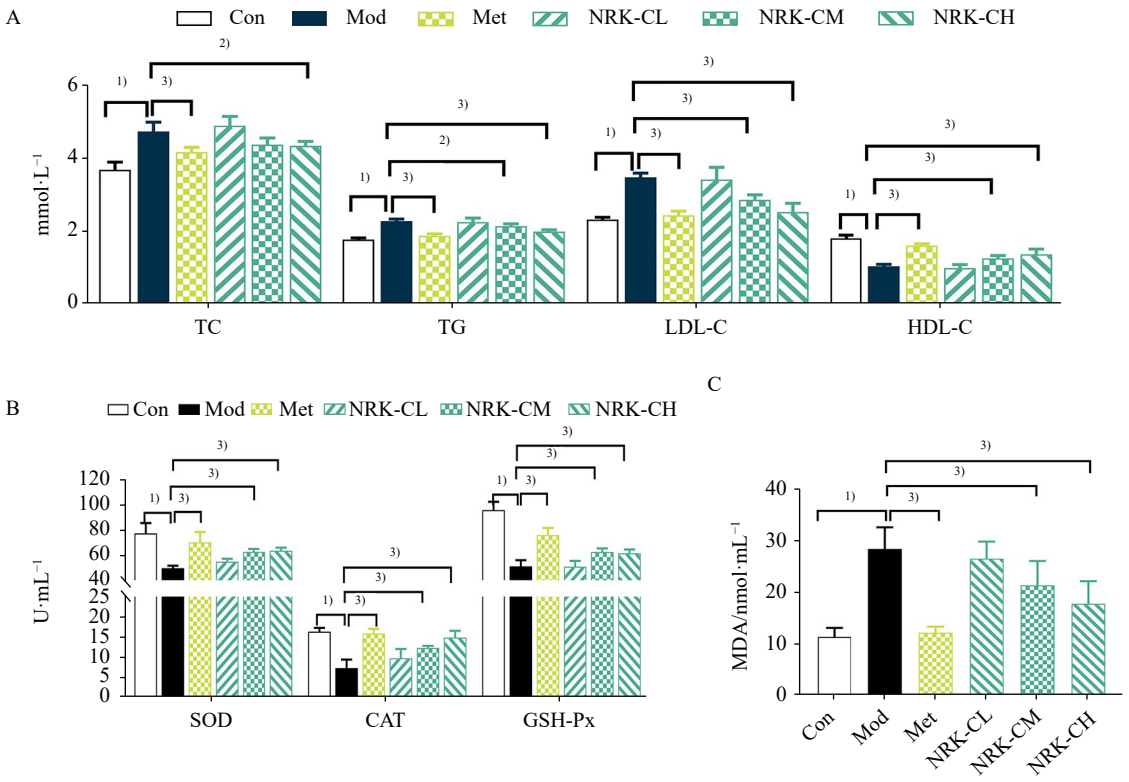

Fig. 2 Effects of NRK-C on lipid metabolism and oxidative stress in high-fat diet-STZ-induced diabetic mice($\bar{x}\pm s$, n=8)

A−levels of TC, TG, LDL-C and HDL-C in serum. B−levels of SOD, CAT and GSH-Px in serum. C−levels of MDA in the liver. Compared with Con, $^{1)}P$<0.01; compared with Mod, $^{2)}P$<0.05, $^{3)}P$<0.01.

图 2 NRK-C 对高脂饮食联合 STZ 诱导的糖尿病小鼠脂质代谢和氧化应激的影响 ($\bar{x}\pm s$, n=8)

A−血清中 TC、TG、LDL-C 和 HDL-C 水平；B−血清中 SOD、CAT 和 GSH-Px 的水平；C−肝脏中 MDA 的水平。与 Con 相比，$^{1)}P$<0.01；与 Mod 相比，$^{2)}P$<0.05，$^{3)}P$<0.01。

Mod in comparison to the Con(P<0.01), and treatment with the positive drug metformin/NRK-CH led to a significant increase in HDL-C levels(P<0.01). The above results indicated that NRK-C had a better effect on improving lipid metabolism in diabetic mice.

During oxidative stress, the body can produce excessive excess MDA to protect cells from oxidative damage, and MDA, SOD, GSH-Px and CAT can be used as important indicators to determine the degree of oxidative stress[26]. In the present experiment, the MDA, SOD, GSH-Px and CAT levels in mice serum were further determined. As shown in Figure 2B−C, compared to the Con, the MDA levels were significantly increased(P<0.01), while the SOD, CAT, and GSH-px levels were all significantly lowered in the Mod mice. Compared with the Mod, the MDA, SOD, GSH-Px and CAT levels after treatment with the positive drug metformin and NRK-CM/H were all improved, indicating that NRK-C could inhibit oxidative stress in diabetic mice.

2.3 Effect of NRK-C on liver histopathology in diabetes mice

Liver tissue HE staining was performed to investigate the effect of drugs on liver histopathology. As seen in the Figure 3, the hepatocytes of the Con mice were of moderate size, with intact structure, clearly visible nuclei and obvious blood sinusoids, and the hepatic cord and central vein were arranged in a radial and regular pattern, and there was no inflammatory cell infiltration. In the Mod, hepatocytes were dilated and enlarged, some were necrotic, blood sinusoids were dilated, hepatic cords were disorganized and inconspicuous, and inflammatory cell infiltration was obvious to the naked eye. After the intervention of positive drug metformin and NRK-C, the above lesions in the livers of diabetic mice were alleviated, indicating that NRK-C had a good protective effect on diabetes-induced liver injury by combining the serum hepatic biochemical indexes.

In addition, liver glycogen PAS staining in mice

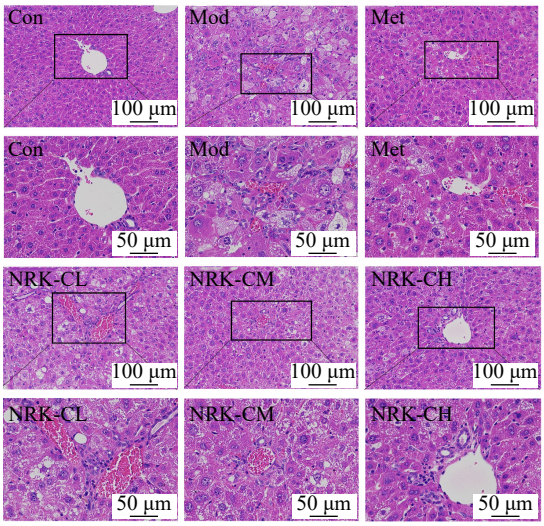

Fig. 3 Effect of NRK-C on HFD+STZ-induced histopathological changes in liver(HE, 200×, *n*=3)

图 3 NRK-C 对 HFD+STZ 诱导糖尿病小鼠的肝脏组织病理学变化的影响 (HE，200×, *n*=3)

was also carried out in this experiment. As seen in the Figure 4, the depth of purple color represents the amount of glycogen content. The color of the Mod was lighter than that of the Con, while the color of the positive drug metformin intervention group was deeper than that of the Mod, which indicated that the content of hepatic glycogen was significantly increased. The color gradually became darker after the intervention of different dosages of NRK-C by gavage, further proving that NRK-C could promote the synthesis and storage of liver glycogen.

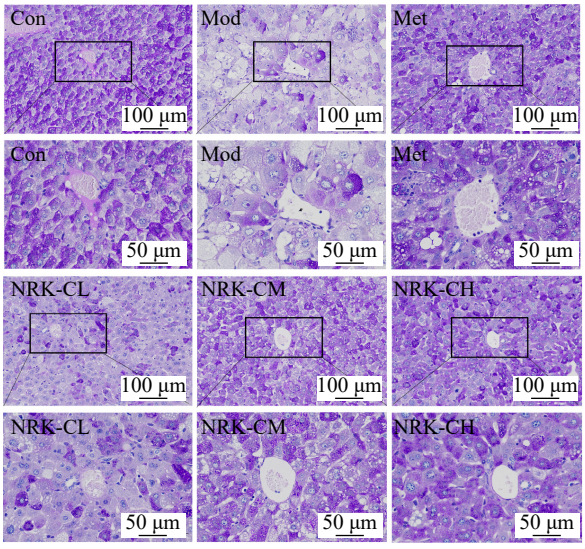

Fig. 4 Effect of NRK-C on glycogen changes in liver(PAS, 200×, *n* = 3)

图 4 NRK-C 对肝脏糖原变化的影响 (PAS，200×，*n*=3)

2.4 Network pharmacology analysis

Based on our previous UPLC-MS/MS analysis for NRK-C and relevant literature[30-31], 15 main chemical components including ferulic acid glucose, kaempferol, kaempferol 7-*O*-rutinoside, kaempferol 3-*O*-glucose, methyl kaempferol 3-*O*-rutinoside, quercetin, quercetin 7-*O*-rutinoside, isorhamnetin, isorhamnetin 3-*O*-rutinoside, isorhamnetin 7-*O*-glucose, quercetin 3-*O*-rutinoside, apigen, apigenin-7-*O*-rhamnoglucoside, apigenin-7-glucuronide and flazin were selected for network pharmacology research. As shown in Fig 5A, 164 gene targets for 15 main chemical components, 6 288 gene targets related to type 2 diabetes, and 136 intersection targets were obtained. The intersection targets mainly include PIK3R1, AKT1, NOX4, AVPR2, MMP13, etc.

To further understand the interaction of NRK-C components in the treatment of diabetes, the intersection targets were imported into String to construct target interaction network relationship diagram, and the top 35 targets based on the degree values were selected as the core targets(Figure 5B). 35 core targets were imported into David database for KEGG pathway analysis, resulting in a total of 125 analysis results. The top 26 results were displayed in the form of enrichment bubble plots. As shown in Figure 5C, PI3K/AKT signaling pathway was highly enriched, which suggested that NRK-C might interact with this pathway to play a role in the treatment of type 2 diabetes.

2.5 Effect of NRK-C on PI3K/AKT/GSK-3β insulin signaling pathway

Based on the results of network pharmacology, the expression of PI3K/AKT signaling pathway related proteins were studied(Figure 6). Western blotting results showed that the expression of P-PI3K, P-AKT and p-GSK-3β were decreased in the livers of the Mod compared with the Con(P<0.01). Compared with the Mod, the protein expression of p-PI3K, p-AKT and p-GSK-3β were enhanced after medium/high-dose administration of NRK-C. The p-PI3K/PI3K, p-AKT/AKT and p-GSK-3β/GSK-3β in the NRK-C medium/high-dose intervention group

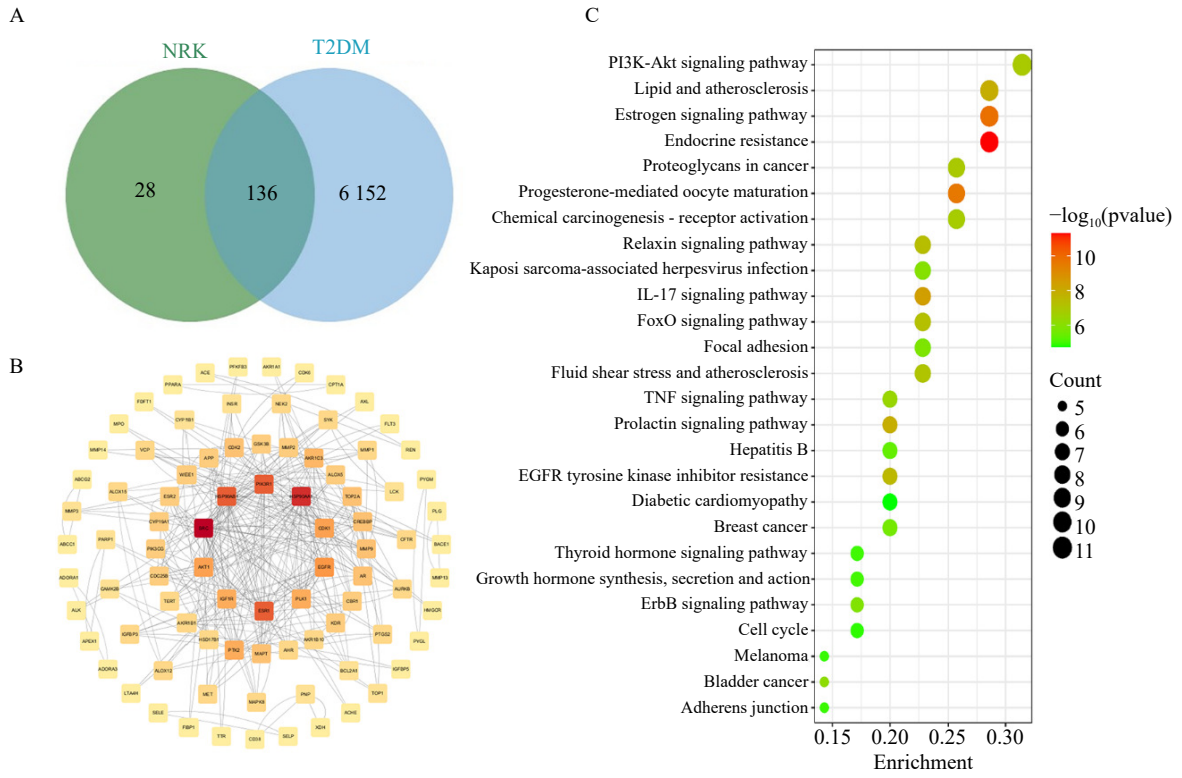

Fig. 5 Relationship between the active components of NRK-C and targets in the prevention and treatment of diabetes

A−intersection of NRK-C active components and diabetes-related target points. B−protein-protein interaction(PPI) network diagram. C−bubble plot of KEGG enrichment results.

图 5 NRK-C 活性成分与糖尿病预防和治疗靶点的关系

A−NRK-C 活性成分与糖尿病相关靶点的交集。B−蛋白质-蛋白质相互作用 (PPI) 网络图。C−KEGG 富集结果的气泡图。

were all significantly improved compared with the Mod. All these results suggested that NRK-C might activate the activity of relevant proteins in the PI3K/AKT/GSK-3β signaling pathway and thereby improve insulin resistance(Figure 6A−C).

As shown in the Fig 6D, the red fluorescence was the positive reaction signal of p-GSK3β protein, and blue fluorescence was the nucleus of liver tissue cells. The activated p-GSK3β protein was mainly localized in the cytoplasm of liver cells, and the weaker positive reaction of p-GSK3β in the cytoplasm of liver cells in the Mod and the regular morphology of the nucleus indicated that there were fewer activated p-GSK3β genes in the cytoplasm. Whereas, the stronger red fluorescence in the cytoplasm of the liver cells in the NRK-C medium/high-dose intervention group and the solidified nucleus indicated that a large number of p-GSK3β proteins in the cytoplasm of the cells were activated.

3 Discussion

T2DM is a common form of diabetes mellitus caused by insufficient insulin secretion or insulin resistance, which eventually leads to hyperglycemia. Hyperglycemia not only affects the normal functioning of the body, but also leads to microvascular and macrovascular dysfunction in diabetic patients[32]. Currently available diabetic drugs include sulfonylurea and glinides drugs that promote insulin secretion, metformins that reduce hepatic glucose output and improve peripheral insulin resistance, alpha-glucosidase inhibitors that delay digestion and absorption, and thiazolidinone diketone drugs that increase the sensitivity of target cells to insulin action[33]. Despite the increasing number of drugs related to the treatment of T2DM, many of them lead to drug tolerance and side effects affecting the health of the organism. Currently, research on traditional Chinese herbal medicine for treating diabetes has received increasing attention[34-35].

This experiment was mainly to investigate the hypoglycemic effect of NRK-C and its potential mechanism. Currently, diabetic animal models are

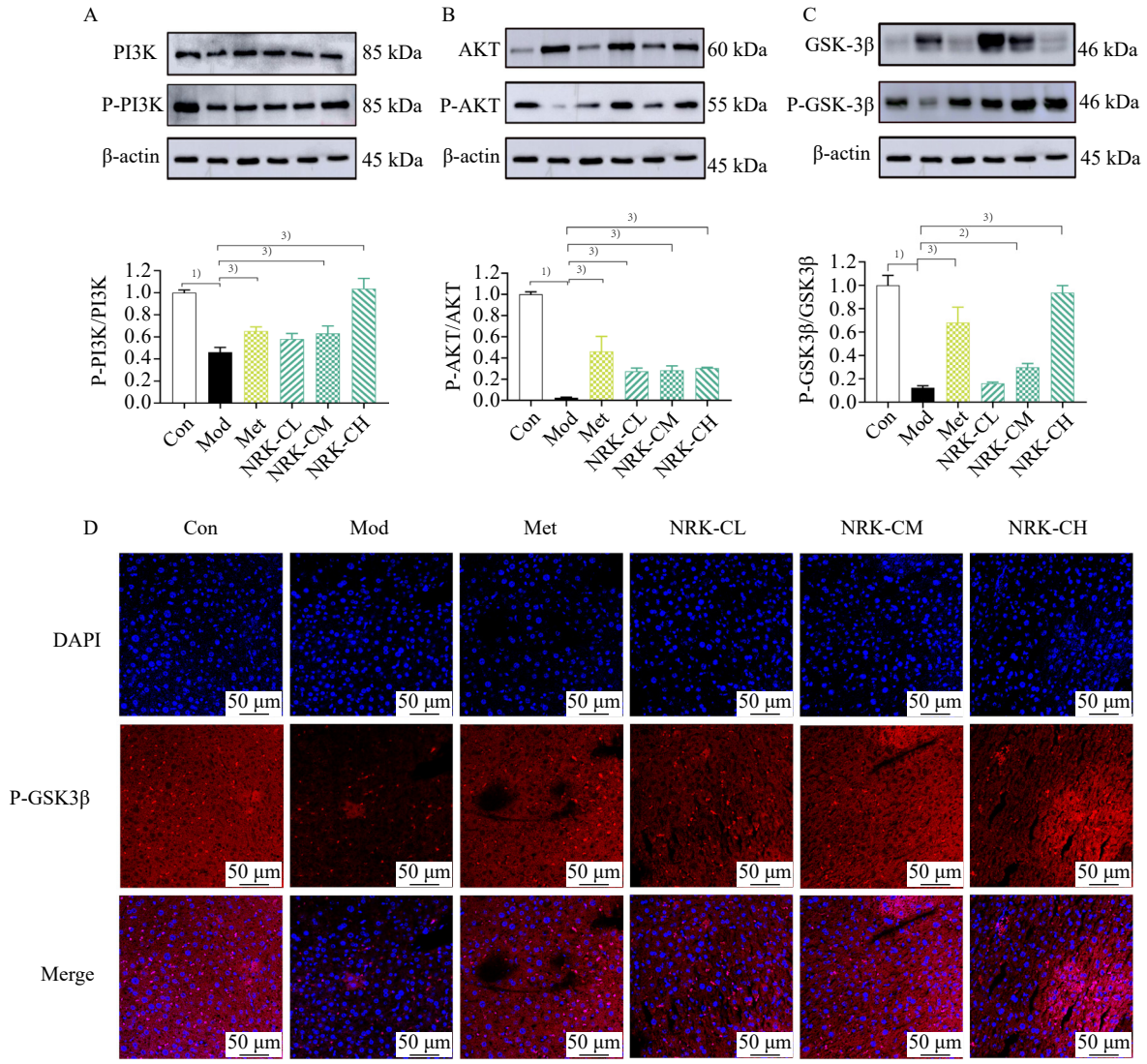

Fig. 6 Effect of NRK-C on liver p-PI3K, PI3K, p-AKT, AKT, p-GSK3β and GSK3β($\bar{x} \pm s$, n=3)

A~C−Western blotting analysis of protein expression of antibodies against p-PI3K, PI3K, p-AKT, AKT, p-GSK3β and GSK3β. D−liver immunofluorescence, liver was stained with p-GSK3β-coupled fluorescent secondary antibody(red). Nuclei were stained with 4′,6-diamidino-2-phenylindole(DAPI)(blue). Compared with Con, $^{1)}P$<0.01; compared with Mod, $^{2)}P$<0.05, $^{3)}P$<0.01.

图 6 NRK-C 对肝脏 p-PI3K、PI3K、p-AKT、AKT、p-GSK3β 和 GSK3β 的影响 ($\bar{x} \pm s$，n=3)

A−C−NRK-C 对糖尿病小鼠肝脏组织中 p-PI3K、PI3K、p-AKT、AKT、p-GSK3β 和 GSK3β 蛋白表达的影响。D−肝脏免疫荧光，肝脏用 p-GSK3β 偶联荧光二抗 (红色) 染色。细胞核用 4′，6-二脒基-2-苯基吲哚 (DAPI)(蓝色) 染色。与对照组相比，$^{1)}P$<0.01；与模型相比，$^{2)}P$<0.05, $^{3)}P$<0.01。

mainly categorized into drug-induced, high-fat diet-induced, high-fat diet combined with drug-induced, and diabetic mouse models with spontaneous gene mutations[36-38]. Among them, the method of high-fat diet feeding for several weeks combined with low-dose intraperitoneal injection of STZ has a high success rate of modeling and low lethality of animals, which is a commonly used method to establish diabetic animal models[39]. In this study, the mice were modeled by intraperitoneal injection of STZ(35 mg·kg^{-1}) for 6 consecutive days after 7 weeks of high-fat feeding. The weight of mice increased significantly after 7 weeks of high-fat feeding, and blood glucose level increased significantly after 6 consecutive intraperitoneal injections of STZ. As for related indicators of glucose and lipid metabolism, the blood lipids TG, TC and LDL-C in the model group were significantly higher than those in the control group and HDL-C was significantly lower, suggesting that 7 weeks of high-fat diet had induced lipid metabolism disorders in the mice. The FBG levels, glucose tolerance and FINS were also significantly higher than

those of the control group in the model group, indicating that the 7-week high-fat diet and six consecutive intraperitoneal injections of STZ successfully induced insulin resistance and impairing glucose metabolism in mice. Therefore, the modeling method in this study was able to disrupt the glucose-lipid metabolism of mice and successfully simulate the development of diabetes mellitus in mice.

Organ index is one of the important indicators of histopathologic changes in T2DM mice, especially liver, kidney, spleen, and pancreas[40]. Compared with normal mice, the liver, kidney and spleen indices of model mice showed a significant increase, while the pancreas index decreased, indicating that diabetic mice showed severe enlargement of the liver/kidney/spleen organs and pancreatic atrophy. These symptoms have been significantly improved after NRK-C administration.

T2DM in mice induced by high-fat diet feeding is usually accompanied by early signs of dyslipidemia, which is prominently characterized by elevated levels of TG, TC, and LDL-C, and decreased levels of HDL-C, which plays a key role in the pathogenesis of diabetes mellitus[41-42]. The present experiment once again found that prolonged supplementation of high-fat diet might lead to dyslipidemia. After another 7 weeks of NRK-C intervention, serum TG, TC and LDL-C levels were effectively reduced and HDL-C levels were increased. Thus, the lipid metabolism disorder was significantly improved.

One of the typical features of T2DM is that abnormal glucose and lipid metabolism leads to oxidative stress, and at the same time reduce the body's ability to resist oxidative stress[43-44]. In this study, the results showed that compared with the control mice, the MDA level in diabetic mice was significantly increased, and the SOD, CAT and GSH-Px levels were significantly reduced, suggesting that diabetic mice generated oxidative stress. After 7 weeks of NRK-C administration, the MDA level was significantly reduced, and SOD, CAT and GSH-Px levels were significantly promoted, suggesting that NRK-C has an inhibitory effect on oxidative stress in diabetic mice.

IR in the liver leads to disturbed glucose homeostasis, which is another important feature of T2DM[45]. PI3K/AKT signaling pathway is a hot target pathway in diabetes research. PI3K/AKT signaling molecules are involved in the regulation of normal physiological functions of the body through the activation of the downstream target genes, which is of great significance especially in glucose metabolism[37]. PI3K/AKT directly affects the phosphorylation and activation of GSK-3β, a molecule controlling glycogen synthesis, and then regulates the body's blood glucose level by promoting hepatic glycogen synthesis[46-47]. Thus PI3K/AKT/GSK-3β is a potential pathway of action in the control of diabetes in the organism. In the present study, we found that after the intervention of NRK-C, the protein expression levels of PI3K, p-PI3K, p-AKT and p-GSK-3β were all increased, indicating that NRK-C could activate the expression levels of PI3K proteins in the PI3K/AKT signaling pathway and then activate the phosphorylation levels of downstream effector proteins[27-28]. In addition, immunofluorescence experiments on liver tissues also demonstrated that NRK-C could significantly activate the protein expression of p-GSK3β. Additionally, our research revealed that administering NRK-C led to a notable enhancement in liver glycogen synthesis. This positive outcome was further supported by the results of immune staining conducted on the liver. These findings provide compelling evidence that the administration of NRK-C can effectively improve liver glucose metabolism by modulating the PI3K/AKT signaling pathway. This exciting discovery underscores the potential of targeted pharmacological interventions to optimize glycemic control and maintain metabolic balance in the liver. NRK-C has shown effective effects on the treatment of T2DM by improving insulin resistance, promoting glycogen synthesis, and enhancing the antioxidant capacity of the body.

4 Conclusion

The present study demonstrated that NRK-C

could significantly improve FBG levels, glucose tolerance, hepatic glycogen levels and lipid levels, and significantly reduced IR of diabetic mice. Further mechanism research indicated that NRK-C might exert a potential antidiabetic effect by modulating the PI3K/AKT signalling pathway. All these results suggested that NRK-C might be a potential drug for improving insulin resistance and reducing hyperglycemia in T2DM.

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