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Hypoglycemic Effect of D-Chiro-Inositol in Type 2 Diabetes Mellitus Rats through the PI3K/Akt Signaling Pathway

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Abstract: In this investigation, a model of type 2 diabetes mellitus (T2DM) was used on Sprague-Dawley (SD) rats to clarify more details of the mechanism in the therapy of T2DM. D-chiro-inositol (DCI) was administrated to the diabetic rats as two doses [30, 60 mg/ (kg·body weight·day)]. The biochemical indices revealed that DCI had a positive effect on hypoglycemic activity and promoted the glycogen synthesis. The rats in DCI high-dosage group had a blood glucose reduction rate of 21.5% after 5 weeks of treatment, and had insulin content in serum about 15.3±2.37 mIU/L which was significantly decreased than diabetes control group. Real-time polymerase chain reaction (RT-PCR) results revealed that DCI gave a positive regulation on glycogen synthase (GS) and protein glucose transporter-4 (Glut4). Western blotting suggested that DCI could up-regulated the expression of the phosphatidylinositol-3-kinase (PI3K) p85, PI3Kp110, GS as well as the phosphorylation of protein kinase B (Akt) both in the liver and the skeletal muscle. The results also revealed that DCI enhanced the Glut4 expression on skeletal muscle. Above all, DCI played a positive role in regulating insulin-mediated glucose uptake through the PI3K/Akt signaling pathway in T2DM rats.

Key words: D-chiro-inositol; diabetes mellitus; PI3K/Akt signaling pathway; liver tissue; skeletal muscle

Introduction

There has been a sharp increase of diabetes across the world in recent decades, and diabetes has become the third largest non-communicable diseases followed by tumor and cardiovascular disease, which is a serious damage to human health and even life-threatening. 90% of the diabetes cases are T2DM which is known as non-insulin-dependent diabetes mellitus.(Zhang and Moller, 2000,Buse, 2011) T2DM is characterized by insulin resistance (Hsu, Shih, Chang et al., 2015) and impaired insulin secretion due to β-cell dysfunction, (Sharma, Bharti, Goyal et al., 2011) which is a state where insulin has a reduced ability to mediate glucose homeostasis in its major target tissues, such as skeletal muscle, adipose tissue and liver. (Kahn, 2003) T2DM has many related complications, including hyperglycemia, dyslipidemia, abdominal obesity, hypertension etc. (Salas-Salvadó, Martinez-González, Bulló et al., 2011) At present, while there are many effective drugs for the treatment of diabetes, the side effects and adverse reactions of them cannot be ignored for most of these drugs would cause damage to multiple organs when they play the treatment function. Therefore, it is necessary to find a new natural active component for the treatment of diabetes with assured safety and efficiency. (Hu, Xia, Wang et al., 2014)

Many researches had shown some natural plant extracts with an effect of enhancing insulin sensitivity to the insulin resistant experimental animals. (Chang, Chou, Liao et al., 2015) DCI, a kind of isomers of inositol, was rich in many *leguminosae* plants and animal viscus. For the past few years, DCI was regarded as a kind of food additives (or food fortifier) and was allowed to be added into some functional health care products in some of the western countries. Some research reports pointed out, DCI plays a crucial role in insulin signaling processes and insulin resistance would be caused by a lack of enough DCI in vivo. (Jones and Varela-Nieto, 1999) Therefore, many DCI-enriched foods were recommended to the diabetes or prediabetes subjects by the doctor aim to assist the diabetic treatment. DCI helped to regulate the body's physiological function and metabolic balance, to reduce the prevalence of diabetes, (Larner, Brautigan and Thorner, 2010) however, its functional mechanisms of hypoglycemic effect remains to be inquired.

Liver is the most predominant visceral organ to regulate the glucose metabolism by means of plenty of signaling pathways. PI3K/Akt pathway is one of the most primary signaling pathways which thought to be a major mechanism involved in insulin resistance. (Avramoglu, Basciano and Adeli, 2006) Glut4 plays an explicit role in regulating glucose homeostasis through translocation and activation in skeletal muscle, triggered by insulin dependent PI3K/Akt pathway. (Watson, Kanzaki and Pessin, 2004) Therefore, therapeutic approaches target on these specific metabolic indexes in the management of T2DM are advocated. In this study, high-fat diet (HFD) fed-streptozotocin (STZ) - induced T2DM rats were used to clarify the effect of DCI on T2DM and its mechanisms, with focusing on the improvement of DCI on insulin sensitivity and the identification of the role of DCI in the critical pathway of insulin sensitivity.

Materials and Methods

Chemicals

D-chiro-inositol (95%) used in this study was purchased from Shanghai Newgenco Bioscience Co., Ltd. (Shanghai, China). STZ was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Insulin was obtained from Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Trizol was purchased from TransGen Biotech Co., Ltd. (Beijing, China). Antibodies PI3Kp85, PI3Kp110, Akt, phospho-Akt, GSK-3β，GS and Glut4 were obtained from Cell Signaling Technology lnc. (Danvers, MA, USA), secondary antibody was obtained from ZSGB-BIO Technology Co., Ltd. (Beijing, China). RIPA was bought from Solarbio Science & Technology Co., Ltd. (Beijing, China). ECL was bought from Advansta Inc. (Menlo Park, CA, USA). Others laboratory chemicals were of analytical grade.

Materials

A blood glucose meter and blood glucose test strips were obtained from Johnson & Johnson, Co. (New Brunswick, NJ, USA). A rat insulin ELISA kit was purchased from Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Glycogen Assay Kit was obtained from Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). PrimeScript^{TM} RT reagent kit and SYBR Premix Ex Taq^{TM} II (Tli RNaseH Plus) kit were bought from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Nitrocellulose membrane was bought from Solarbio Science & Technology Co., Ltd (Beijing, China). An SABC (Rabbit IgG - POD) kit was bought from Solarbio Science & Technology Co., Ltd. (Beijing, China).

Animals

Male SD rats (200±20 g) chosen in our experiment were purchased from the animal house of Beijing University Science Center. All rats were housed in internally flawless animal rooms with constant temperature (23±2 $^{\circ}$C) and humidity (55±10 %). The room kept 12 h light/dark cycle with unrestricted food and water. The animals were approved by the Animal Care and Use Committee, and all of the animal facilities and experimental procedures were carried out according to the Technical Standards for Testing & Assessment of Health Food (2003).

Induction of T2DM in Rats and Medicinal Dosage

The animals were divided into normal chow diet group (20 rats included) and HFD group randomly after adapted to the laboratory environment for a week. A month later, the HFD rats were injected intraperitoneally with STZ solution at a dose of 30 mg / (kg·body weight (bw)) and the normal chow diet group rats received the vehicle only. Seventy-two hours after injection, the HFD rats with blood glucose level ≥11.1 mmol/L were considered as T2DM rats and used in the future experiments.[Jiang, Wu, Wang et al., 2015,Srinivasan, Viswanad, Asrat et al., 2005,Wu, Wen, Qi et al., 2012] Then the T2DM rats were divided into three groups at random and each group contained 10 rats to receive treatment. The Recommended Daily Allowances (RDA) of DCI was 300 mg to 600 mg per day in a 60 kg human and the dose of samples given to the rat should be 6 times than humans' because of its larger surface area. After calculating, the experiment set a first group (low-dosage group, LD) received DCI at 30 mg / (kg·bw) per day, the second group (high-dosage group, HD) received DCI at 60 mg / (kg·bw) per day and the last one received distilled water only (diabetes control group, DC). Ten rats were chosen from the NC group to receive DCI at 60 mg / (kg·bw) per day as the normal high-dosage control group (NH), in order to inspect the side-effect of DCI to the normal rats.

Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT)

OGTT were performed in overnight-fasted rats from every group. NC and DC groups were administered with distilled water orally. LD, HD and NH groups were given 30 mg/kg, 60 mg/kg and 60 mg/kg DCI separately. Twenty minutes later, all the rats were orally administered glucose at a dose of 2g/kg. Blood was withdrawn from the tip of the tail at 0, 30, 60 and 120 min from all five groups to measure the blood glucose level.

ITT were performed in overnight-fasted rats from all groups 72h after the OGTT. Twenty minutes after administration of the vehicle (distilled water) and DCI, the rats were injected insulin at 0.15 U/kg intraperitoneally. Similar to the procedure for OGTT has shown that the blood samples were withdrawn at 0, 30, 60 and 120 min after the injection of insulin, and the blood glucose level was measured.

Biochemical Analysis

Rats were sacrificed at the end of the experiment and blood samples and viscera organs were stored at -80 °C in the refrigerator for future use. The fasting blood glucose (FBG) level was determined by using the blood glucose meter and test strips each week. The fasted serum insulin (FINS) was determined by ELISA kit. The concentrations of glycogen in liver and skeletal muscle were tested by Glycogen Assay Kit. All the parameters were performed according to the manufacture's instructions.

RT-PCR Analysis

The viscera organs were stored at -80 °C in the refrigerator for RT-PCR analysis. RT-PCR was performed to determine the effect of DCI on gene level. Total RNA was extracted from liver using Trizol reagent, and the concentration of the total RNA was determined by the ultraviolet spectrophotometer. The RNA was converted to cDNA by using RT-PCR kits. The specific primers which include both sense and antisense were used for the amplification of DNA (Table1).

Western Blot Analysis

The viscera organs were stored at -80 °C in the refrigerator for Western blot analysis. The expression of proteins including PI3Kp85 (85 kDa), PI3Kp110 (110 kDa), Akt (60 kDa), p-Akt (60 kDa), GSK-3β (46 kDa)，GS（81-85 kDa）and Glut4（50 kDa）were analyzed by Western blot. In brief, 100 mg tissue was homogenized in RIPA buffer for 10 min, followed by centrifugation at 4°C 10000 r/min for 5 min. Then transferred the supernatant to a new clean centrifuge tube and used the Bradford colorimetric method to determine the concentration of the total protein. The tissue protein (50-70 μg) was subjected to 10% SDS-PAGE for 1 h at 80 V and for 2 h at 100 V, in order to separate the target protein from the others. Proteins were transferred to a nitrocellulose membrane for 3 h at 300 mA by a wet transfer. The membrane was incubated in blocking solution containing 5% nonfat dried milk for 2 h at room temperature. Subsequently, the membrane was exposed to the desired the primary antibodies, phosphate buffer saline (PBS) containing PI3Kp85 antibody (1:1000), PI3Kp110 antibody (1:1000), Akt antibody (1:1000), p-Akt (Ser473) antibody

(1:2000), GSK-3β antibody (1:1000), GS antibody (1:1000) and Glut4 antibody (1:1000), incubated with the membrane at 4°C overnight; β-actin (43 kDa) was used as control protein. After incubation with the secondary antibody for 2 h at room temperature, the membrane was exposed to chemiluminescent reagent (ECL) for about 5-10 min. The expressions of the proteins on the membranes with fluorescence were exposed to X-ray photographic films in a darkroom and the band densities were quantified.

Immunohistochemistry

Liver tissues were put into 4% formaldehyde solution for 12 h and placed into 80% alcohol for immunohistochemistry use. Liver tissues embedded in paraffin were performed on 5μm paraffin sections for immunohistochemical staining. The paraffin sections were dewaxed and rehydrated before being dipped into 0.01 M citrate buffer (pH 6.0) with a boiling water reaction to retrieve antigen. The paraffin sections were incubated with 3% H_2O_2 to quench the activity of endogenous peroxidase. Then the sections were incubated in the solution containing 3% BSA to block nonspecific binding sites whereas endogenous avidin-binding activity was inhibited by continuous treatment with avidin-biotin. The following steps of the immunodetection experiment was according to the manufacture's instruction for the SABC (rabbit IgG) - POD Kit. For each antibody, a negative control was included in which the primary antibody was replaced with PBS.

Statistical Analysis

All of the data were presented as means ± SD. The *ANOVA* with post-hoc comparisons were used to exhibit the significance of differences between samples and these differences were considered significant when $p<0.05$, whereas $p<0.01$ was regarded as very significant (SPSS version 20.0, Statistical Package for the Social Sciences Software, SPSS Inc., Chicago, IL, USA).

Results

Effects of DCI on Increasing Body Weight and Reducing FBG

Table 2 reveals the body weight of all the SD rats. Body weight was measured each week and diabetic rats were very significantly lower than the normal ones after establishing the T2DM model ($p<0.01$). The DC group was very significantly lower than that of the NC group during the whole experiment ($p<0.01$). The body weight of LD and HD groups were significant higher than that of the DC group after treated with DCI for 5 weeks ($p<0.05$).

The FBG levels of all experimental rats were listed on Table 3. The diabetic rats had a very significant higher level of FBG than that of normal rats ($p<0.01$). The FBG levels of treated groups showed a 9.60% decrease in LD group and 21.50% decrease in HD group after 5-week-treatment. Besides, compared with the DC group, the FBG level of LD group showed a significant decrease ($p<0.05$) and the HD group showed a very significant decrease after 5 weeks of treatment ($p<0.01$).

As the results in Table 2 and Table 3 showed, the indexes in NH group exhibited no significant differences compared with NC group.

DCI Improved OGTT and ITT of Diabetic Rats

OGTT was performed after 4 weeks of treatment with DCI and all the experimental rats were fasted overnight. The blood glucose level increased when the rats were given to the glucose (2 g/kg) orally, and reached to the peak within 30 to 60 min. The blood glucose level of the normal rats dropped to the initial level after 120 min. However, the concentration of blood glucose stayed a high level in diabetic rats during the OGTT experiments. Treatment with DCI (60 mg / kg·bw) in diabetic rats inhibited the rise in blood glucose levels significantly after oral glucose administration (Figure 1A). The overall area under curve of glucose (AUG) over 120 min and the result of AUG expressed that HD group was very significant less than that of DC group (Figure 1B, $p<0.01$).

ITT was performed three days after OGTT and all rats were intraperitoneally injected insulin at 0.5 U/kg. The changes of the blood glucose levels at 30, 60 and 120 min were revealed in Figure 2A, and the AUG of the ITT was also calculated and

exhibited in Figure 2B. The LD and HD groups both had a very significant difference with the DC group ($p<0.01$) in blood glucose decreasing level, which suggested that DCI increased the utilization of insulin, and improved the insulin sensitivity of the diabetic rats as well.

As the results of OGTT and ITT experiments revealed that there was no significance between NH group and NC group.

DCI Enhanced Insulin Sensitivity of T2DM Rats

The insulin-sensitizing activity of DCI had been studied and the results were as the Figure 3 revealed. DC group had a very significant higher concentration of FINS than that of NC group ($p<0.01$). The supplementation of DCI given a significant decrease in concentration of FINS in LD group ($p<0.05$) and a very significant decrease in HD group ($p<0.01$), compared with DC group separately. The results also proved that DCI has a beneficial effect on enhancing insulin sensitivity. The concentration of FINS in NH group had no significance compared with NC group.

DCI Promoted the Synthesis of Hepatic Glycogen and Muscle Glycogen

It was proved that DCI had a beneficial effect on the synthesis of hepatic glycogen and muscle glycogen in this research. Rat suffered from diabetes had a lower concentration of glycogen than that of in normal rats. As the result suggested in Figure 4A, the content of hepatic glycogen was very significantly increased in HD group compared with DC group ($p<0.01$). The content of muscle glycogen (Figure 4B) showed an increasing tendency in treated groups but had no significant difference compared with DC group. Besides, both of the hepatic glycogen and muscle glycogen concentrations in NH group showed no significant difference with NC group.

DCI Enhanced the Expression of GS and Glut4 in Gene Level

The RT-PCR results in Figure 5A and 5B revealed that LD groups exhibited a significant increase on the expression of gene GS both in liver and skeletal muscle compared with DC group ($p<0.05$). Meanwhile, there were very significant differences between HD group and DC group ($p<0.01$). DCI had an ability to aggrandize the mRNA expression of Glut4 to accelerate the glucose transportation (Figure 5C). The mRNA expressions of GS and Glut in NH group showed no

significant difference compared with NC group.

DCI Enhanced the Expression of Main Proteins in PI3K/Akt Pathway

In this investigation we evaluated the expression of the main proteins in the PI3K/Akt pathway. PI3K is a kind of phosphatidylinositol kinase which comprises a catalytic subunit (p110) and a regulatory subunit (p85). The p85 subunit is coupled with tyrosine-phosphorylated receptor tyrosine kinases and the p110 subunit is required for G-protein-mediated activation of PI3K. The main proteins in PI3K/Akt signaling pathway on liver were estimated in Figure 6. DCI significantly increased the expression of p110 and p85 in diabetic rats (Figure 6A, B, $p<0.05$). Akt and p-Akt were measured in this study to determine whether PI3K downstream signaling was affected by DCI in diabetic rats. The results in Figure 6C, D suggested that DCI treatment was associated with a significant increase in phosphorylation of Akt in liver tissue compared with the DC group ($p<0.05$). To gain some insight into the molecular mechanism for the effect of DCI on glycogen synthesis, we assessed the levels of GSK-3β protein and GS protein (Figure 6E, F). The expression quantity of GSK-3β protein was decreased remarkably in DCI treated groups, and the expression of GS protein was increased.

The main proteins in PI3K/Akt signaling pathway on skeletal muscle were evaluated in Figure 7, DH group showed significant difference compared with the DC group. It is suggested that DCI significantly enhanced the expression of p110, p85, p-Akt and Glut4 in skeletal muscle. It meant that DCI accelerated the transportation and metabolism of glucose on skeletal muscle.

The expressions of all the proteins in NH group revealed no significant difference in NC group.

Immunohistochemistry Test on Paraffin Sections

In order to enhance the argument and to prove the results directly, immunohistochemistry staining was brought in to observe the phosphorylation of Akt. Figure 8 showed that all tissues were completely stained in each group and there was no obvious difference in the staining of Akt in all tissues. However, deeper immunostaining of p-Akt was observed in liver tissues of normal control rats, whereas

the staining of p-Akt was markedly diminished in the DC group. Deeper immunostaining for p-Akt in the HD group than that in DC group could be observed which meant DCI promoted the phosphorylation of Akt in diabetic rats. Furthermore, NH group showed no significant staining difference compared with DC group.

Discussion

DCI is one of the nine stereoisomers of inositol and its structure is similar to the pH2.0 insulin mediator. Lacking of DCI may cause insulin resistance or even T2DM. It is reported that DCI has an ability to increase the insulin sensitivity of the body. (Giménez-Bastida and Zieliński, 2015,Lazarenko, Geisler, Bayliss et al., 2014) Besides, it did well in treating with T2DM as well as its chronic complications.(Fonteles, Almeida and Larner, 2000) In the current study, we demonstrated that treatment with DCI for 5 weeks ameliorated blood glucose level, oral glucose tolerance and insulin tolerance in STZ-induced insulin resistant rats, which were associated with the reduction in serum insulin level. The results exhibited that DCI could significantly reduce blood glucose level and relieved insulin resistance.

DCI has a similar structure to the pH2.0 insulin mediator and the mediator is able to promote the synthesis of glycogen. Therefore, we picked PI3K/Akt signaling pathway, the main pathway for glycogen synthesis, to investigate the molecular mechanism of DCI in relieving insulin resistance and treating T2DM. PI3K/Akt pathway plays an important role in insulin's metabolic function,(Jiang, Ren, Li et al., 2014,Vareda, Saldanha, Camaforte et al., 2014) and Akt/PKB is a critical node in the downstream of the PI3K/Akt pathway. Any fluctuation of Akt activation causes the pathophysiological properties of varies of complex diseases.(Liu, Chang and Chiang, 2010,Manning and Cantley, 2007)

In this study, the expression of the main genes related to glycogen synthesis and glucose transportation were evaluated. The genes of hepatic glycogen synthase, muscle glycogen synthase and Glut4 were obviously increased in DCI treated groups. These indicated that DCI probably had an ability to promote the synthesis of glycogen, the similar function of pH 2.0 insulin mediator. Moreover, DCI could give an impetus to convey glucose from extracellular to intracellular, to make it in further metabolism.

The expressions of PI3Kp85 and PI3Kp110 subunits in DCI treated groups showed a dose-dependent increasing tendency. During the experiment, stimulated with high density insulin can cause up-regulation of PI3Kp85 protein, which would give a negative feedback on insulin sensitivity in return, and affect the activity of downstream molecules to inhibit the continuous conduction of signal.(Nolan, Damm and

Prentki, 2011) DCI also increased the amounts of phosphorylated of Akt at Ser473 in a dose-dependent manner, and p-Akt inhibited the GSK-3β protein directly. GSK-3β had a negative effect on regulating GS, which could decrease the synthesis of glycogen indirectly. The inhibiting the GSK-3β's expression through DCI meant GS was enhanced and the concentration of glycogen in liver was increased. The result of western blot in Figure 6F exhibited the protein concentration of GS in liver was in accordance with the consequence in Figure 4A. It meant that the ability of DCI in adding hepatic glycogen synthesis was proved both in macroscopic and microcosmic.

PI3K/Akt signaling pathway is the main way to regulate the absorption and metabolism of the glucose, besides, balance the concentration of the glucose in blood. (Frøsig, Rose, Treebak et al., 2007) Insulin stimulates the skeletal muscle's uptake of the glucose and synthesis of muscle glycogen. (Langfort, Viese, Ploug et al., 2003) Skeletal muscle promotes the insulin to combine with the cytomembrane receptor and activate the phosphorylation of Akt in PI3K/Akt signaling pathway. The up-regulated phosphorylation of Akt increased Glut4 transmembrane to transport glucose, in order to accelerate glycometabolism. (Gandhi, Jothi, Antony et al., 2014)

The main proteins in PI3K/Akt signaling pathway on skeletal muscle had been detected. The results suggested that DCI increased the expressions of p110, p85, p-Akt, GS and Glut4 in skeletal muscle, however, decreased GSK-3β. It is suggested that the protein concentration of GS in skeletal muscle was in keeping with the data in Figure 4B, which meant the ability of DCI in increasing muscle glycogen synthesis was proved macroscopicly and microcosmicly. Accordingly, it could be summarized that DCI promoted the transportation and the metabolism of blood glucose.

On the other hand, according to the immunohistochemistry, the expression level of Akt showed none obviously difference between normal rats and diabetic rats. However, the DCI treated groups exhibited darker ground than the DC group through the microscope. Immunohistochemistry technique was based on the high specificity between antibodies and antigens, the fluorochrome was added to the samples in order to bond with antigens-combined-antibodies and emit light. Therefore, the more antigens the samples had, the deeper the microphotograph performed.(Schofield, Lewis and

Austin, 2014) In our results (Figure 8, a-e), DH group had a deeper brown than the DC one which explained that samples in DH group had more p-Akt antigens than the DC group. It indirectly proved that DCI enhanced the phosphorylation of Akt, sequentially, promoted the expression of Glut4.

In this investigation, a NH group was set up to explore the effect of DCI on normal rats. According to the data in the results section, there was no significant difference between NH group and NC group. This indicated that DCI had no adverse effect on normal rats.

Conclusion

In conclusion, DCI relieves the insulin resistance through the PI3K/Akt pathway, thereby reducing the concentration of blood glucose. This study investigated the mechanism of DCI in relieving symptom of T2DM, and DCI could be developed into an effective drug for the treatment of T2DM when sufficient further researches on DCI would be performed.

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References

[1] Zhang, B.B. and Moller, D.E., 2000. New approaches in the treatment of type 2 diabetes, Current Opinion in Chemical Biology. 4, 461-467.

[2] Buse, J.B., 2011. Type 2 diabetes mellitus in 2010: Individualizing treatment targets in diabetes care, Nat Rev Endocrinol. 7, 67-68.

[3] Hsu, C.Y., Shih, H.Y., Chang, Y.C., Huang, Z.L., Tsai, M.J., Chia, Y.C., Chen, C., Lai, Y.K. and Weng, C.F., 2015. The beneficial effects of tetracosanol on insulin-resistance by insulin receptor kinase sensibilisation, Journal of Functional Foods. 14, 174-182.

[4] Sharma, A.K., Bharti, S., Goyal, S., Arora, S., Nepal, S., Kishore, K., Joshi, S., Kumari, S. and Arya, D.S., 2011. Upregulation of PPARγ by Aegle marmelos Ameliorates Insulin Resistance and β-cell Dysfunction in High Fat Diet Fed-Streptozotocin Induced Type 2 Diabetic Rats, Phytotherapy Research. 25, 1457-1465.

[5] Kahn, S.E., 2003. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes, Diabetologia. 46, 3-19.

[6] Salas-Salvadó, J., Martinez-González, M.Á., Bulló, M. and Ros, E., 2011. The role of diet in the prevention of type 2 diabetes, Nutrition, Metabolism and Cardiovascular Diseases. 21, Supplement 2, B32-B48.

[7] Hu, S., Xia, G., Wang, J., Wang, Y., Li, Z. and Xue, C., 2014. Fucoidan from sea cucumber protects against high-fat high-sucrose diet-induced hyperglycaemia and insulin resistance in mice, Journal of Functional Foods. 10, 128-138.

[8] Chang, C.-I., Chou, C.-H., Liao, M.-H., Chen, T.-M., Cheng, C.-H., Anggriani, R., Tsai, C.-P., Tseng, H.-I. and Cheng, H.-L., 2015. Bitter melon triterpenes work as insulin sensitizers and insulin substitutes in insulin-resistant cells, Journal of Functional Foods. 13, 214-224.

[9] Jones, D.R. and Varela-Nieto, I., 1999. Diabetes and the role of inositol-containing lipids in insulin signaling, Molecular Medicine. 5, 505-514.

[10] Larner, J., Brautigan, D.L. and Thorner, M.O., 2010. D-Chiro-Inositol Glycans in Insulin Signaling and Insulin Resistance, Molecular Medicine. 16, 543-552.

[11] Avramoglu, R.K., Basciano, H. and Adeli, K., 2006. Lipid and lipoprotein dysregulation in insulin resistant states, Clinica Chimica Acta. 368, 1-19.

[12] Watson, R.T., Kanzaki, M. and Pessin, J.E., 2004. Regulated Membrane Trafficking of the

Insulin-Responsive Glucose Transporter 4 in Adipocytes, Endocrine Reviews. 25, 177-204.

[13] Jiang, J., Wu, Y., Wang, X., Lu, L., Wang, L., Zhang, B. and Cui, M., 2015. Blood free fatty acids were not increased in high-fat diet induced obese insulin-resistant animals, Obesity Research & Clinical Practice.

[14] Srinivasan, K., Viswanad, B., Asrat, L., Kaul, C.L. and Ramarao, P., 2005. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: A model for type 2 diabetes and pharmacological screening, Pharmacological Research. 52, 313-320.

[15] Wu, D., Wen, W., Qi, C.-L., Zhao, R.-X., Lü, J.-H., Zhong, C.-Y. and Chen, Y.-Y., 2012. Ameliorative effect of berberine on renal damage in rats with diabetes induced by high-fat diet and streptozotocin, Phytomedicine. 19, 712-718.

[16] Giménez-Bastida, J.A. and Zieliński, H., 2015. Buckwheat as a Functional Food and Its Effects on Health, Journal of Agricultural and Food Chemistry. 63, 7896-7913.

[17] Lazarenko, R., Geisler, J., Bayliss, D., Larner, J. and Li, C., 2014. D-chiro-inositol glycan stimulates insulin secretion in pancreatic β cells, Molecular and Cellular Endocrinology. 387, 1-7.

[18] Fonteles, M.C., Almeida, M.Q. and Larner, J., 2000. Antihyperglycemic Effects of 3-O-Methyl-D-Chiro-Inositol and D-Chiro-Inositol Associated with Manganese in Streptozotocin Diabetic Rats, Horm Metab Res. 32, 129-132.

[19] Jiang, S., Ren, D., Li, J., Yuan, G., Li, H., Xu, G., Han, X., Du, P. and An, L., 2014. Effects of compound K on hyperglycemia and insulin resistance in rats with type 2 diabetes mellitus, Fitoterapia. 95, 58-64.

[20] Vareda, P.M.P., Saldanha, L.L., Camaforte, N.A.d.P., Violato, N.M., Dokkedal, A.L. and Bosqueiro, J.R., 2014. Myrcia bella Leaf Extract Presents Hypoglycemic Activity via PI3k/Akt Insulin Signaling Pathway, Evidence-based Complementary and Alternative Medicine : eCAM. 2014, 543606.

[21] Liu, S.-H., Chang, Y.-H. and Chiang, M.-T., 2010. Chitosan Reduces Gluconeogenesis and Increases Glucose Uptake in Skeletal Muscle in Streptozotocin-Induced Diabetic Rats, Journal of Agricultural and Food Chemistry. 58, 5795-5800.

[22] Manning, B.D. and Cantley, L.C., 2007. AKT/PKB Signaling: Navigating Downstream, Cell. 129, 1261-1274.

[23] Nolan, C.J., Damm, P. and Prentki, M., 2011. Type 2 diabetes across generations: from pathophysiology to prevention and management, The Lancet. 378, 169-181.

[24] Frøsig, C., Rose, A.J., Treebak, J.T., Kiens, B., Richter, E.A. and Wojtaszewski, J.F.P., 2007. Effects of Endurance Exercise Training on Insulin Signaling in Human Skeletal Muscle: Interactions at the Level of Phosphatidylinositol 3-Kinase, Akt, and AS160, Diabetes. 56, 2093-2102.

[25] Langfort, J., Viese, M., Ploug, T. and Dela, F., 2003. Time course of GLUT4 and AMPK protein expression in human skeletal muscle during one month of physical training, Scandinavian Journal of Medicine & Science in Sports. 13, 169-174.

[26] Gandhi, G.R., Jothi, G., Antony, P.J., Balakrishna, K., Paulraj, M.G., Ignacimuthu, S., Stalin, A. and Al-Dhabi, N.A., 2014. Gallic acid attenuates high-fat diet fed-streptozotocin-induced insulin resistance via partial agonism of PPARγ in experimental type 2 diabetic rats and enhances glucose uptake through translocation and activation of GLUT4 in PI3K/p-Akt signaling pathway, European Journal of Pharmacology. 745, 201-216.

[27] Schofield, D.J., Lewis, A.R. and Austin, M.J., 2014. Genetic methods of antibody generation and their use in immunohistochemistry, Methods. 70, 20-27.

Tables

Table1. Designed Primer Sets for RT-PCR

gene	primer	5'-3'	size (bp)
Glut4	sense	GACCTCAGGGACAGGCAAAG	270
	antisense	TGTTGCCCTGTGGTCAAGTT	
GS	sense	TTGCCAGAATGCACGCAGAA	125
	antisense	TGCCTGCATCATCTGTTGAC	
β-actin	sense	GATCGATGCCGGTGCTAAGA	367
	antisense	TCCTATGGGAGAACGGCAGA	

Primer sets designed for RT-PCR to evaluate the expressions of Glut4 and GS at gene level and we picked β-actin as the house-keeping gene. The specific primers which include both sense and antisense were designed separately and followed the designing principles.

Table2. Effect of DCI on Body Weight of Experimental Rats

group	body weight (g)			
	0 week	3 week	4 week	5 week
NC	426±14.1	479±21.6**	502±23.6**	497±27.2**
DC	420±28.6	375±26.0	381±26.9	381±30.7
LD	409±25.1	420±46.5*	431±56.2*	433±37.0*
HD	413±20.1	419±30.8*	433±22.8*	434±18.0*
NH	407±46.3	467±66.1	471±67.7	461±63.7

Data in this table showed the effect of DCI on body weight. After treatment with DCI, the rats in LD and HD groups had a significantly higher body weight than the ones in DC group. We evaluated (*)p<0.05 as significant and (**)p<0.01 as very significant compared with DC group. Values are the means ± SD, and each group contained 10 rats.

Table3. Effect of DCI on Fasting Blood Glucose of Experimental Rats

group	fasting blood glucose (mmol/l)				
	0 week	3 week	4 week	5 week	reduction rate (%)
NC	5.00±0.570**	5.00±0.560**	5.30±0.560**	4.80±1.18**	
DC	18.6±3.00	23.1±6.25	20.9±3.16	21.1±4.31	
LD	19.8±1.10	21.3±2.56	18.9±1.41	17.9±2.07*	9.60
HD	20.0±0.980	22.5±3.38	17.7±1.64**	15.7±2.72**	21.5
NH	4.60±0.480	5.60±0.650	5.10±0.630	4.40±0.330	

The effect of DCI on FBG level of the rats in LD and HD groups had a tendency to reduce during the treatment. The LD group had a significant difference compared with DC group and HD group had a very significant difference compared with DC group. We evaluated (*)$p<0.05$ as significant and (**)$p<0.01$ as very significant. Values are the means ± SD and each group contained 10 rats.

Graphics

Figure 1.

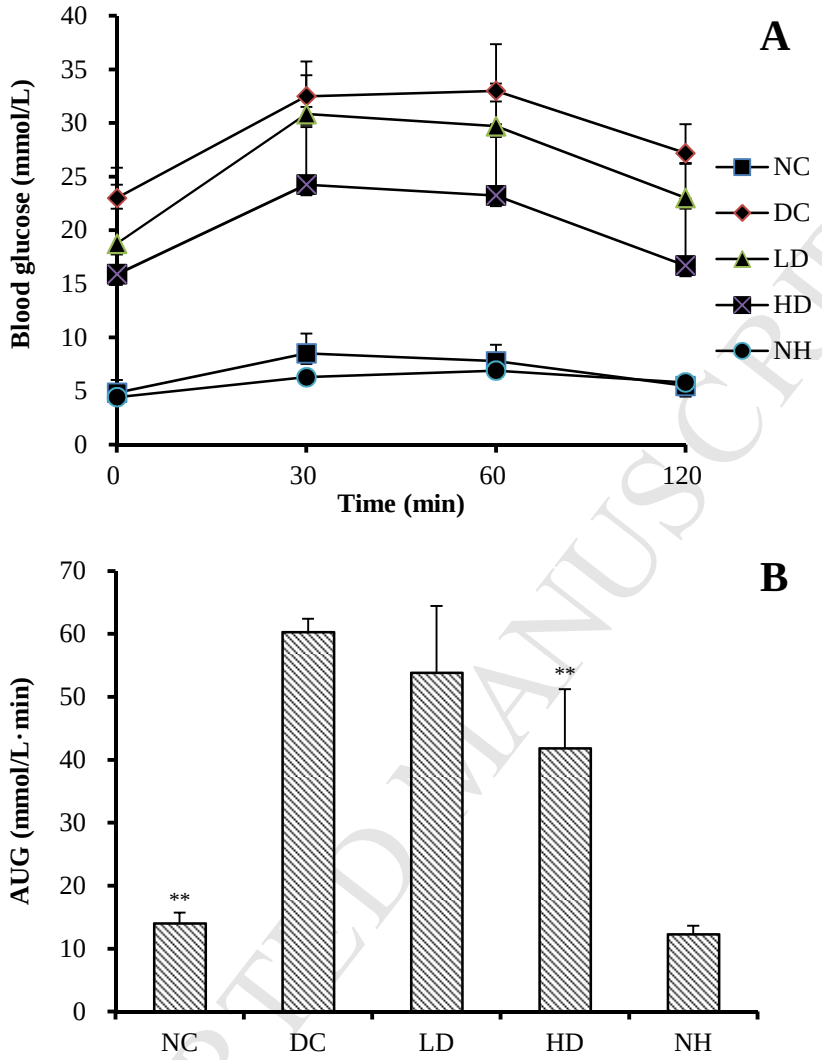


Figure 2.

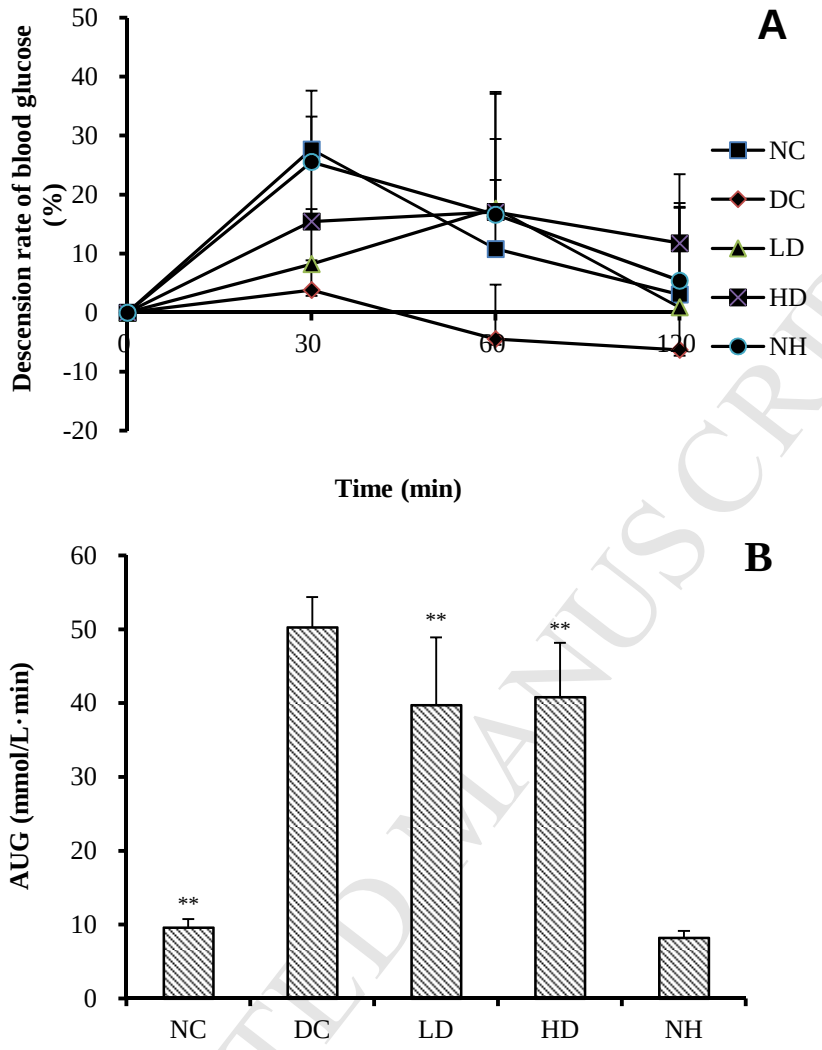


Figure 3.

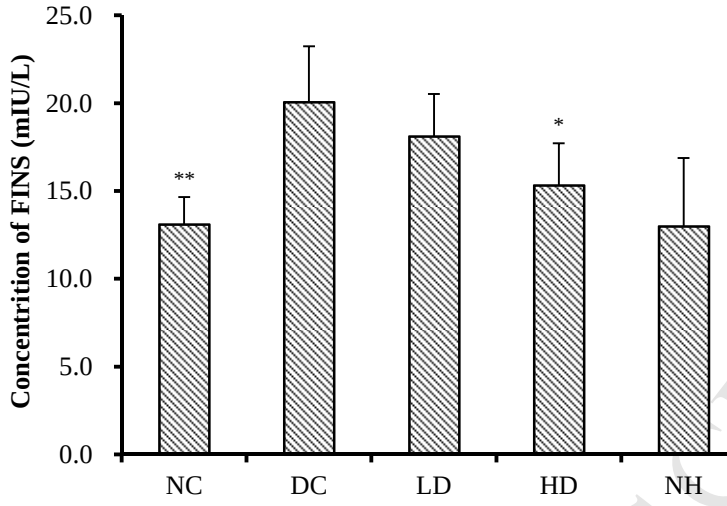


Figure 4.

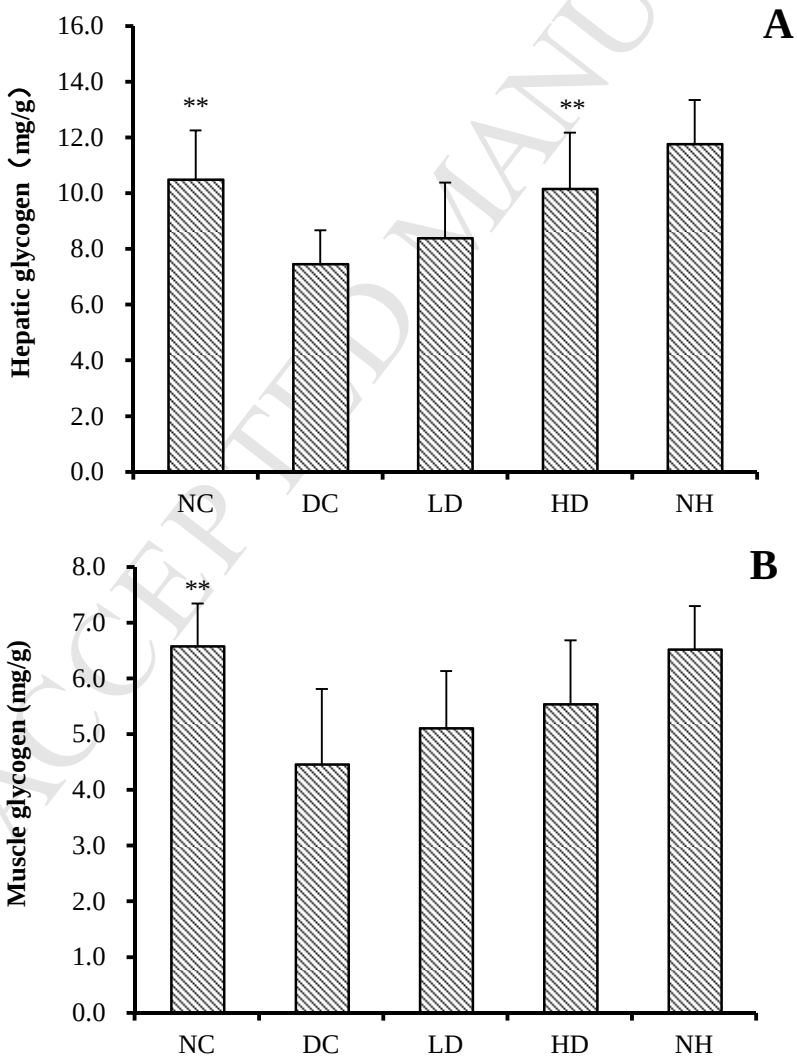


Figure 5.

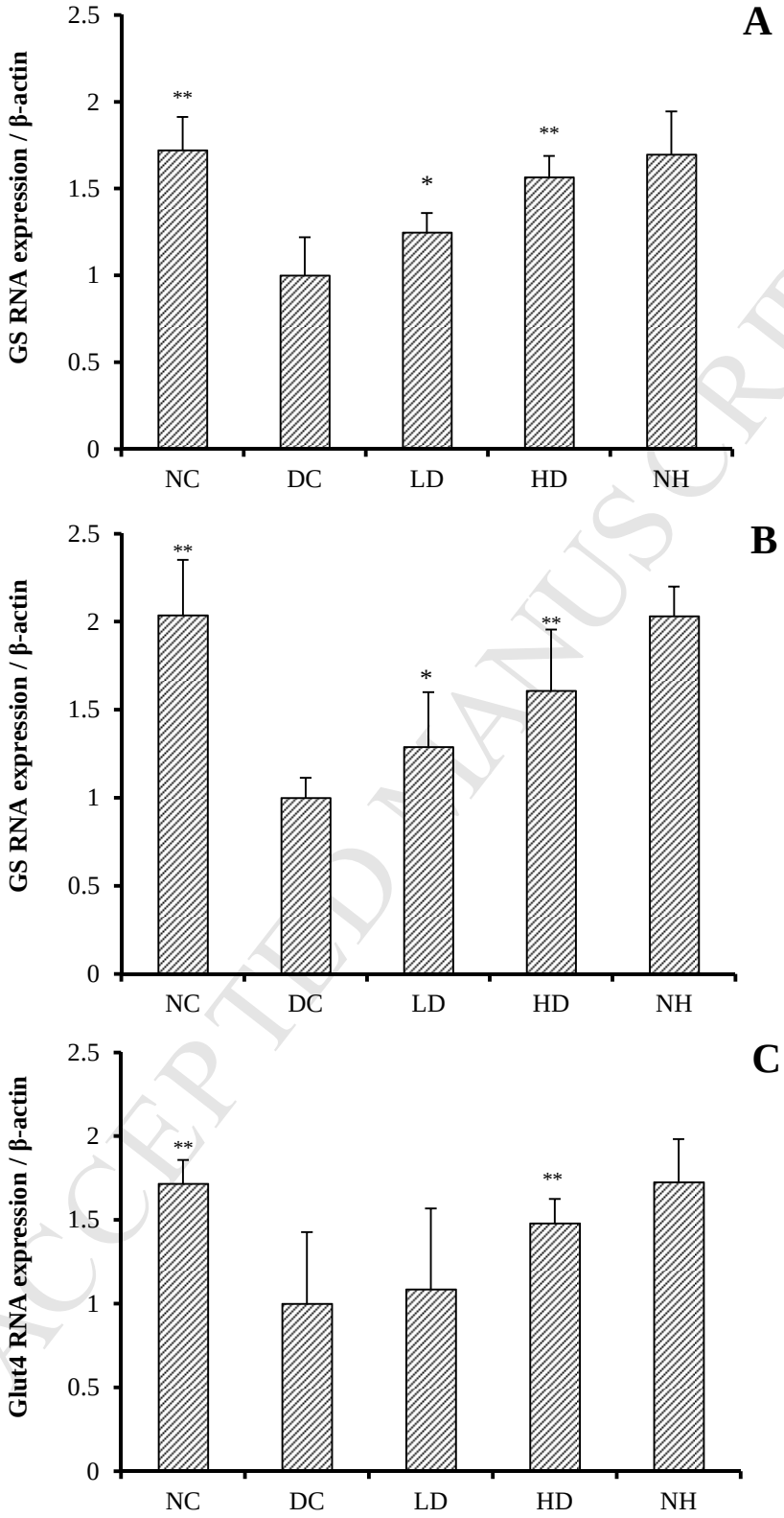


Figure 6.

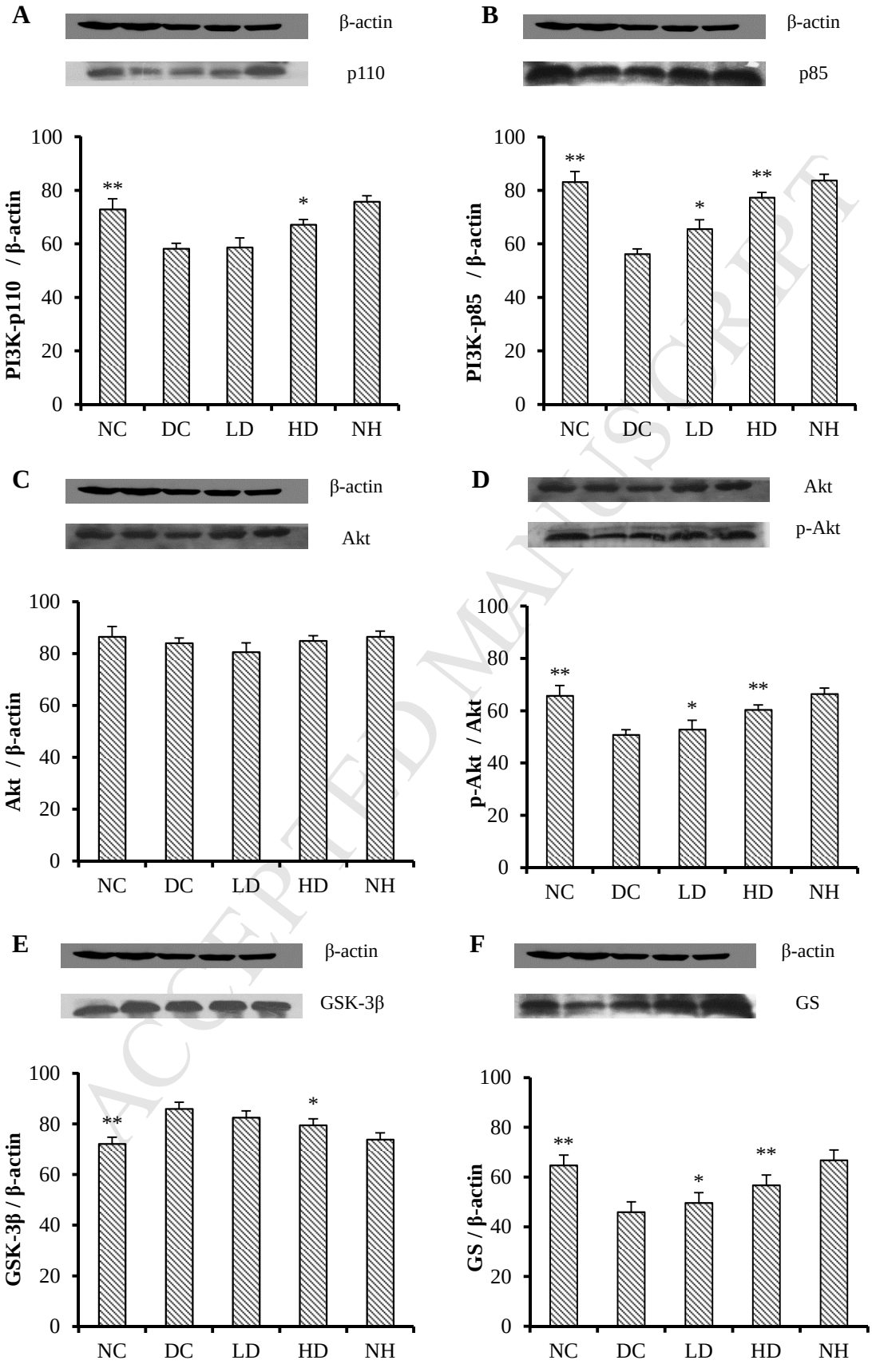
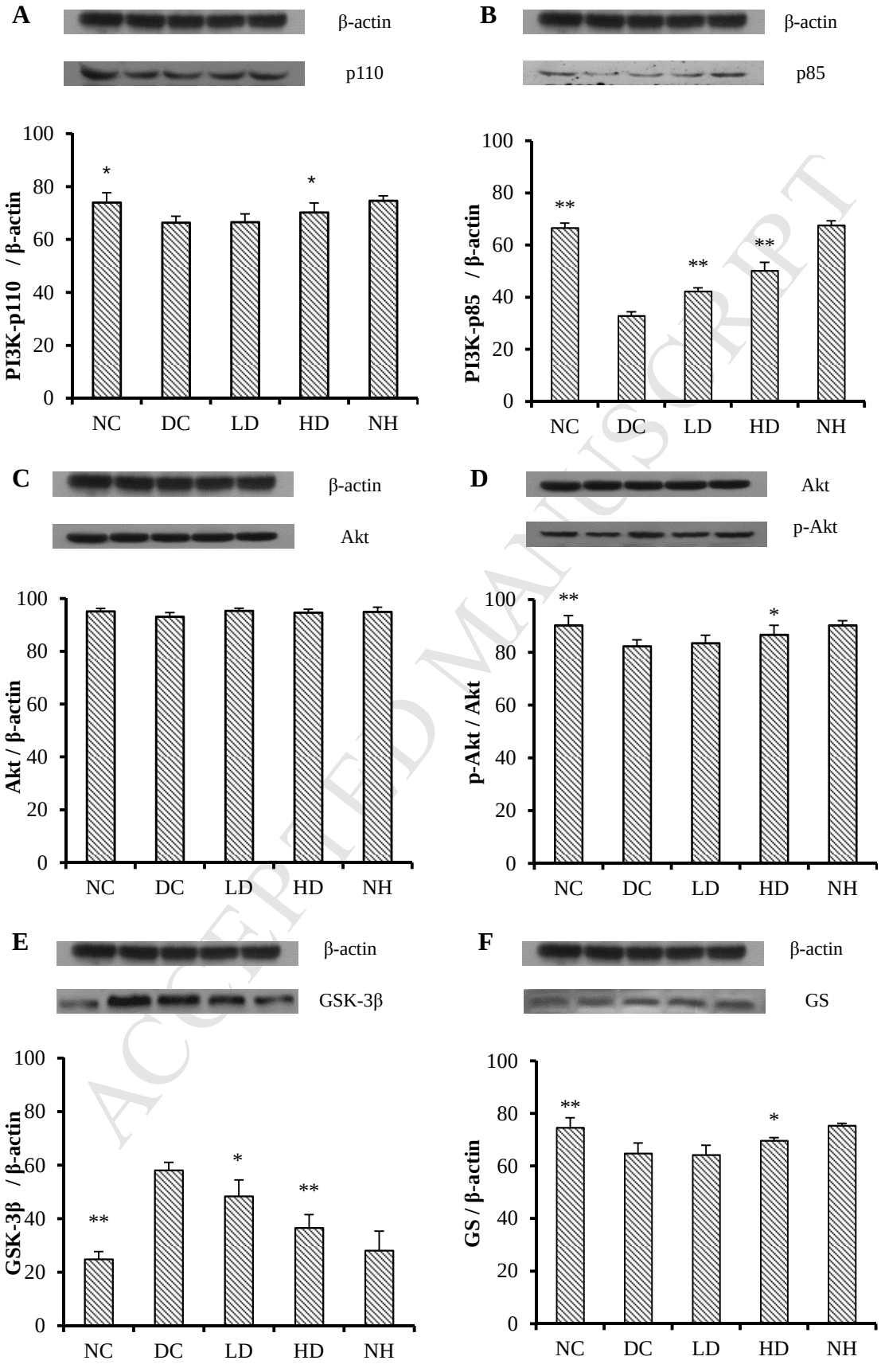

A
β-actin
p110
PI3K-p110 / β-actin
100
80
60
40
20
0
**
*
NC
DC
LD
HD
NH
B
β-actin
p85
PI3K-p85 / β-actin
**
*
**
C
β-actin
Akt
Akt / β-actin
D
Akt
p-Akt
p-Akt / Akt
E
β-actin
GSK-3β
GSK-3β / β-actin
F
β-actin
GS
GS / β-actin

Figure 7.



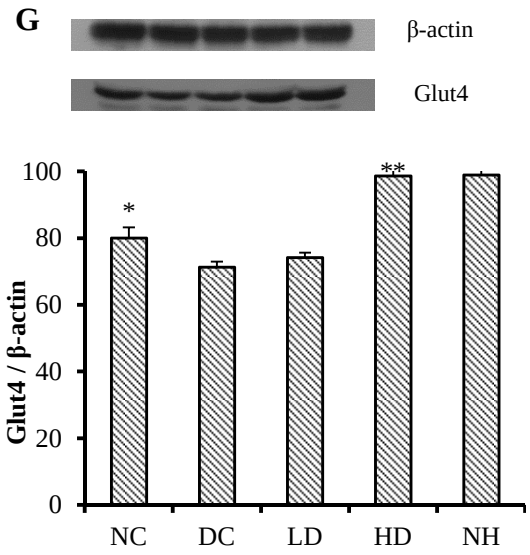


Figure 8.

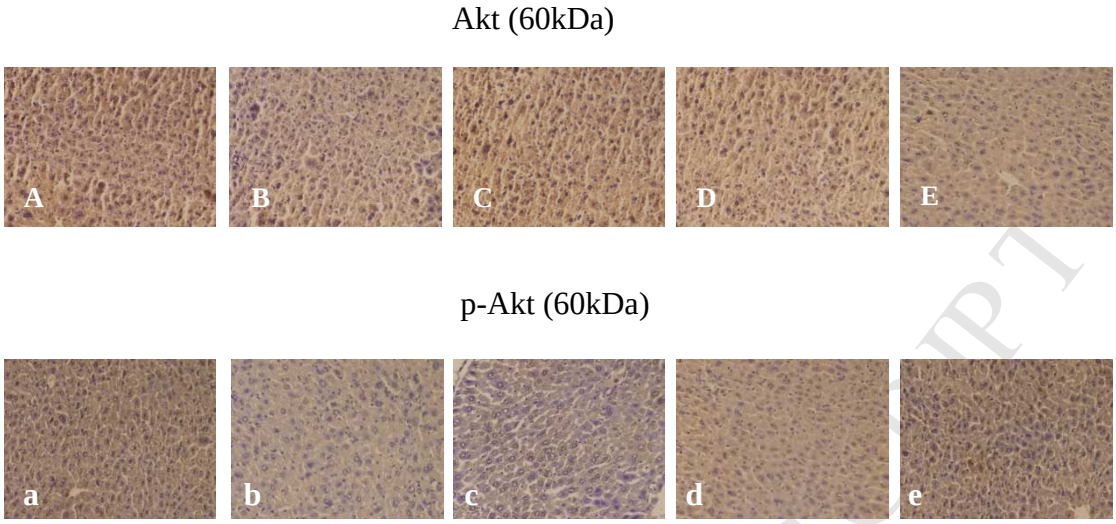


Figure Legends

Figure 1. OGTT was performed on all experimental animals after 4 weeks of treatment. DCI were administrated to the rats in LD, HD and NH groups orally and the NC and DC groups received vehicle only. Glucose at dosage of 2 g/kg were given to all the groups 20 minutes after receiving DCI. Blood glucose were tested at 0, 30, 60 and 120 minutes after giving glucose and made the curves. Each time point in Figure 1A is the means ± SD of 10 separated rats, and the area under curve of Figure 1A were calculated and revealed in Figure 1B. (**)p<0.01 was very significantly different compared with the DC group.

Figure 2. ITT was carried out 3 days after OGTT. DCI were administrated to the rats in LD, HD and NH groups orally and the NC and DC groups received vehicle only. Insulin with 0.15 U/kg was injected to the experimental rats intraperitioneally and then blood glucose were tested at 0, 30, 60 and 120 minutes after injection. Each time point in Figure 2A is the means ± SD of 10 separate rats, and the area under curve of Figure 2A was calculated and showed in Figure 2B. (**)p<0.01 was very significantly different compared with the DC group.

Figure 3. Data in this figure suggested that rats in DC group had a higher concentration of insulin in serum than that in normal groups. DCI helped to lower the contents of FINS in LD and HD groups. (*)p<0.05 was considered as significant and (**)p<0.01 as very significant compared with the DC group. Values are the means ± SD and each group contained 10 rats.

Figure 4. The concentrations of hepatic glycogen (Figure 4A) and muscle glycogen (Figure 4B) were tested and the results were exhibited in this figure. DCI had a positive effect on aggrandizing hepatic glycogen and HD group showed a significant difference from DC group. (*)p<0.05 was considered as significant, values are the means ± SD and each group contained 10 rats.

Figure 5. Total RNA was extracted from liver using Trizol reagent, and the concentration of the total RNA was determined by the ultraviolet spectrophotometer. RT-PCR was operated according to the manufacturer's instructions of the RT-PCR kits and the results in this figure demonstrated the effect of DCI on the genes of GS and

Glut4.β-actin was selected as the house-keeping gene. (*)P<0.05 compared with the DC group was considered as significant difference. Values are the means ± SD and each group contained 10 samples.

Figure 6. Western blot was used to evaluated the expression of the key protein in PI3K/Akt pathway on liver tissue. The proteins of p85 (A), p110 (B), Akt (C), p-Akt (D), GSK-3β (E), and GS (F) in liver tissue were extracted by RIPA and separated by 10% SDS-PAGE. Similar results were obtained in 3 independent experiments and the photos in this figure were representative images. Values in the figure are given as the means ± SD. (*)p<0.05 compared with the DC group was considered as significant difference. (**)p<0.01 was very significantly different compared with the DC group.

Figure 7. The proteins of p85 (A), p110 (B), Akt (C), p-Akt (D), and Glut4 (E) in skeletal muscle were tested by Western blot analysis. The proteins were extracted by RIPA and separated by 10% SDS-PAGE. Each photo in this figure was the representative image from the results of 3 independent experiments. Values in the figure are given as the means ± SD. (*)p<0.05 compared with the DC group was considered as significant difference. (**)p<0.01 was very significantly different compared with the DC group.

Figure 8. Immunohistochemical staining was performed on the 5-μm paraffin sections which embedded with liver tissues. All the steps of the staining were operated according to the manufacture's instruction of SABC (rabbit IgG) – POD Kit. A & a for NC group, B & b for DC group, C & c for LD group, D & d for HD group, and F &f for NH group. The staining outcomes were observed by microscope at the magnification of 200 times.

Highlights

1. In this study, the mechanism of DCI on hypoglycemic effect was investigated.
2. This study evaluated the effect of DCI on enhancing the synthesis of hepatic glycogen in liver through PI3K/Akt signaling pathway.
3. This study evaluated the effect of DCI on enhancing the synthesis of muscle glycogen in skeletal muscle through PI3K/Akt signaling pathway.
4. This study evaluated the effect of DCI on promoting the expression of Glut4 in skeletal muscle through PI3K/Akt signaling pathway.