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Original Research Article

COMBINATION OF GLICLAZIDE DRUG AND LUPIN SEEDS POWDER ALLEVIATE HYPERGLYCEMIA ON INDUCED-DIABETIC RATS RECEIVING HIGH-FAT HIGH FRUCTOSE/SUCROSE DIET

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Abstract: Diabetes mellitus is one of the most prevalent metabolic diseases in the world. Hyperglycemia and lipoprotein abnormalities are the characteristic clinical finding of DM. The most common legume food used in traditional medicine for the treatment of diabetes is lupin seeds powder. The aim of current research is to analyze the therapeutic effects of the combination of both hypoglycemic drug (Gliclazide) and lupin seeds powder on some biochemical parameters (serum blood glucose, serum insulin, glyclated hemoglobin and total lipid profile) and also on mRNA expression level of Glucokinase, Phosphoenolpyruvate carboxykinase (PEPCK), Insulin and Peroxisome Proliferator-Activated Receptor-Gamma (PPAR-γ) genes using relative quantitative PCR. In this study, thirty zucker male rats were divided into two groups: normal control group (six rats) and the other group expose to induce type2 DM by high-fat high fructose/sucrose diet. The diabetic groups were sub classified into 4groups (6 rats in each group), one group diabetic control and the rest was treated with Gliclazide (10mg/kg),combination of Gliclazide(10mg/kg) and lupin seeds powder (1gm/kg) and lupin seeds powder (1gm/kg) only. Combination between Gliclazide (10mg/kg) and lupin seeds powder (1gm/kg) daily for 4 weeks resulted in significant decrease in blood glucose level, glyclated hemoglobin and dyslipidemia (p<0.05), also results in significant increase (P<0.05) in high density lipoprotein-cholesterol(HDL-c) (90.66±3.8), insulin level(4.9±0.058). Also the combination of Gliclazide (10mg/kg) and lupin seeds powder (1gm/kg) tends to return biochemical parameters, lipid profile (triacylglycerol 77±3.05, total cholesterol 132±2) and the transcription of mRNA metabolic genes to normal levels (Glucokinase 1.4±0.1, insulin 1.48±0.25, PEPCK 0.49±0.25, PPAR-γ 3.1±0.2) more than the treatment with Gliclazide drug only, in type 2 diabetic rats. It is concluded that, the combination of Gliclazide hypoglycemic drug and lupin seeds powder has the most hypoglycemic effect when compared to other groups.

Key words: type2 diabetes mellitus; high-fat high fructose/sucrose diet; gliclazide; lupin seeds; total cholesterol

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Introduction

Diabetes Mellitus (DM) is a metabolic disease characterized by the occurrence of chronic hyperglycemia, which affects the metabolism of carbohydrates, lipids, and proteins (1).

In DM, the body can't manage the quantity of sugar (especially glucose) presents in the blood. Glucose is essential to the body in order to perform its vital activity. Carbohydrates convert inside the liver into glucose. The glucose is then secreted into the blood circulation. In the normal condition, a number of hormones control the blood glucose level (2). The main hormone is insulin, which is secreted by the pancreas. Insulin permits glucose to enter from the blood into cells all through the body, where it is utilized for fuel. The cause of diabetes either due to insufficient production of insulin (type 1 diabetes) or inability of proper insulin utilization (type 2 diabetes) (3). In diabetes, blood glucose levels stay high because glucose in the blood can't move effectively into cells. In this case, all cells that need the glucose for fuel suffer from starvation. Moreover, all organs and tissue exposed to destructive effect (4).

Although, there are many pharmaceutical drugs in the markets scientists tends to use medicinal plants to treat diabetes. This is due to the fact that plant medication and natural formulation are often considered to have fewer side effects than manufactured ones (5). The World Health Organization (WHO) confirmed the importance of hypoglycemic drugs originated from plants that are used in traditional medicine. The antihyperglycemic effects of natural plants are due to its ability to renovate the capacity of β-cells of pancreas, expand insulin secretion or reduce glucose absorption. Therefore, curing with medicinal plant drugs are defending β-cells and reducing variation in glucose levels (6). A large portion of the medicinal plants has been found to have antidiabetic impact substances like terpenoids, glycosides, alkaloids, flavonoids. Researchers all over the world will continue to study alternate medications, especially from the plant kingdom, for treating of DM as the disease causes many challenges not only to the doctors but also to the investigators (7).

In traditional medicine, lupin seeds are considered as antidiabetic drugs with antihyperglycaemic activity due to the presence of the protein conglutin gamma (Cγ), a glycoprotein, which has insulin-mimetic activity, allowing it to activate kinases involved in the signaling pathway of insulin, promote glucose transporter-4 (GLUT4) translocation, and contribute to muscle tissue differentiation (8).

Gliclazide is oral antihyperglycemic drugs used for treatment of type2 DM, belongs to a second era sulfonylurea, it acts by increase secretion of insulin from β-cells of pancreas, which is favored due to its rapidly absorbed from gastrointestinal tract (GIT) and lesser liability to cause hypoglycemia and weight gain. Moreover, it has generally cardiovascular protection effects (9).

In the last several years, natural herbal plants had been used for the treatment of DM, but recently scientists discovered new synthetic drugs for the treatment of DM. Advanced researches have been performed to study the effect of both natural herbal plant and antidiabetic synthetic drugs to approach and explore the best result in the treatment of DM. Gliclazide is the most commonly used positive controls in anti-diabetic animal studies. Gliclazide can stimulate insulin secretion by binding to the sulfonylurea binding site and closing the ATP-sensitive potassium channel (10). Other researchers studied the therapeutic effect of combination between lupin seeds and metformin on Type 2DM rats (11).

In the present study, type 2 DM was induced in an animal model using high fat (lard) high fructose / sucrose (HFHF)diet for a period of 12 weeks then the experimental animals were treated with lupin seeds powder and the hypoglycemic synthetic drug, Gliclazide, was used as a positive control.

Material and methods

This experiment was held at Zagazig University Veterinary labs and was approved by the university research committee NO: ZU-IACUC/2/F/24/2018

Animals

Thirty male Zucker rats, with an initial weight of 150+/-50gm, were kept at a suitable temperature of 20-25°C, 12 hours light-dark cycle and free availability of water and diet, they were randomly classified to two groups: normal control (six rats) and diabetic (24 rats).The rats from the normal control group were fed on the standard rat chow and allowed to drink water freely.

Preparation of diabetic rats

Twenty four rats were used for induction of type 2diabetesDM by feeding on HFHF. These rats were given a diet consist of lard (60%) and fructose (17%) added to the standard rat chow while sucrose 10% was added to water supplied to the rats. The experiment lasted for 12 weeks. This model was planned to mimic nearly the cafeteria diet with essentially contain high caloric value (12).

The body weight and blood glucose were taken once weekly for 12 weeks (13). Calculations of the caloric intake of HFHF diet:

Lard contains 8000 Kcal /Kg, Chow contains 3000 Kcal/Kg, and fructose contains 3000 Kcal/Kg.

Animal Grouping

a) Control group fed on normal chow (6 rats)
b) Diabetic groups classified into:
    i. Diabetic control (6 rats) fed on HFHF diet without treatment.
    ii. Diabetic (6 rats) treated with Gliclazide drug 10 mg/kg for four weeks.
    iii. Diabetic (6 rats) treated with Gliclazide drug 10 mg/kg + lupin seeds powder (1g/kg/day) for four weeks.
    iv. Diabetic (6 rats) treated with lupin seeds powder (1g/kg/day) for four weeks.

Experimental design

Preparation of lupin seed powder**:** Dry seeds of lupin of different lots were collected from the local market, mixed and purified from possible impurities and grinded to powder. Lupin powder (1g/kg/day) was given to rats for four weeks, the administration was executed using stomach tube method (feeding needle 18 gauage) (14).

Preparation of Gliclazide powder**:** Gliclazide powder was suspended in 1% (w/v) carboxymethylcellulose (CMC) suspension in normal saline and were given orally to the rats at a dose of 10 mg/kg for 4 weeks, the administration was executed using stomach tube method (feeding needle 18 gauage) (15).

Biochemical assays

After the four weeks of treatment, rats were fasted during the night then blood samples were drawn under phenobarbital 50-100 mg/kg anesthesia intraprotenial injection. The serum levels of glucose, insulin, triacylglycerol, LDL-c, HDL-c, vLDL-c, total lipid and cholesterol was determined as follow:

i. Determination of serum glucose by oxidase method using Spectrum Diagnostics glucose kit (16).
ii. Estimation of serum insulin level using Elisa rat insulin kits method (17).
iii. Ion Exchange Resin method used for quantitative determination of glycated hemoglobin (HbA1c)(18).
iv. The Direct Enzymatic colorimetric Liquid method used for determination of HDL-c, LDL-c (19.
v. Estimation of vLDL-c by Friedewald method (20).
vi. Determination of total lipid by Colorimetric Method (21).
vii. Evaluation of Triacylglycerol by GPO-PAP-enzymatic colorimetric method (22.
viii. Cholesterol liquizyme CHOD-PAP enzymatic colorimetric method used for determination of cholesterol (23).

Tissue samples for relative quantitive PCR (RQ-PCR) Analysis

Immediately after scarifying, livers were collected, weighted and wrapped in aluminum foil then, the tissues were rapidly washed with saline buffer placed immediately in liquid nitrogen container to make snap-freezing of tissue and kept at -80°C until use, for RQ-PCR analysis for the mRNA expression of insulin, Phosphoenolpyruvate carboxykinase (PEPCK),

Peroxisome Proliferator-Activated Receptor-Gamma (PPAR-γ) and glucokinase genes in liver tissue. Total RNA prepared from liver was extracted with easy-RED ™ Total RNA Extraction Kit for animal tissue (Intron Biotechnology cat.No.17063).

Conversion of RNA to complementary DNA (cDNA) was performed using TOPscript™ cDNA Synthesis Kit standard reaction conditions containing 2 µl of 10X TOPscript™ RT Buffer, 1 µl of TOPscript™ Reverse Transcriptase (200 units/µl), dNTP Mixture (2 mM each) 2 µl, Template RNA 1 µl, oligo(dT)18 Primer 1µl, RNase inhibitor (40 units/µl) 0.5 µl, add sterile RNase free water up to 20 µl, then incubated at 42°C (5 mins), 42~60℃ (60 mins), and 95 ℃ (5 mins). The cDNA was stored at -20℃ till analysis. TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Intron Biotechnology cat. No.RT500S) was used for RQ–PCR with standard reaction conditions containing 10 µl of TOPreal™ qPCR 2X PreMIX , template DNA 3 µl, 1.5 µl from forward and reverse primers (5~10 pmol/µl), and sterile RNase free water up to 20 µl. The cycling program was performed in Stratagene Mx3005P qPCR System with the following condition: initial denaturation at 95°C for 15 mins, and 50 cycles (denaturation at 95°C for 15sec, annealing at 60°C for 20sec, elongation at 72°C for 30sec). Table (1) demonstrates primers used for expression of some metabolic genes using RQ-PCR. Primers were synthesized by Bio Basic Inc., Canada. Efficiency of amplification was measured by the slope of a standard curve.

The transcription levels of target genes were normalized to those of GAPDH gene which used as reference gene. The normalized quantity of the target gene was obtained by subtracting cycle threshold for GAPDH from the CT for the target gene (ΔCT sample)(24). The same calculation was performed with controls (ΔCT control). Then ΔΔCT was calculated as the difference of these values (ΔΔCT = ΔCT sample – ΔCT control). Finally, the relative expression was expressed as fold change by $2^{-\Delta\Delta CT}$ relative to control (25).

Table 1: The nucleotide sequences of the PCR primers used to assay gene expression by relative quantitative PCR

Gene	Sequences	Gene bank ID	Annealing Temperature	Reference
PPAR-γ:	F:5′TGTGGACCTCTCTGTGATGG-3′ R:5′-CATTGGGTCAGCTCTTGTG-3'	25664	55°C	(26)
Glucokinase	F:5'-CACCCAACTGCGAAATCACC-3' R:5'CATTTGTGGGGTGTGGAGTC-3'	24385	60°C	(27)
PEPCK	F:5'-AGCCTCGACAGCCTGCCCCAG-3' R:5'CCAGTTGTTGACCAAAGGCTTTT-3'	362282	56°C	(28)
Insulin	F:5'-AGCAAGCAGGTCATTGTTCC-3' R: 5'-TTGCGGGTCCTCCACTTC-3'	24505	65°C	(29)
GAPDH	F:5'-TGCACCACCAACTGCTTAG-3' R: 5'-GATGCAGGGATGATGTTC-3'	24383	55°C	(30)

PEPCK: Phosphoenolpyruvate carboxykinase, PPAR-γ: Peroxisome Proliferator-Activated Receptor-Gamma, GAPDH: Glyceraldhyde3-Phosphate Dehydrogenase

Statistical analysis

Statistical analysis was carried out by SPSS 16 (SPSS, Chicago, Ill) program for windows. Data were expressed as mean ± SEM and was performed using one way ANOVA analysis of variance followed by post hoc test. The criterion for statistical significance was $P < 0.05$.

Results

Regarding body weight (initial and final), in diabetic control group, a significant weight loss was observed compared to normal control animals ($P<0.05$). After treatment with Gliclazide (10 mg/kg) + lupin seeds powder (1g/kg) or Gliclazide(10 mg/kg) alone, the body weight was significantly increased compared to control diabetic animals , but there was no significant change in body weight in

Table 2: The biochemical parameters in in male rats experimental groups

Group	Control	Diabetic Control	Gliclazide	Gliclazide+ Lupin	Lupin	*P* Value
BW	155.33±2.9^{a}	116.33±2.0^{d}	132.67±5.0^{c}	143.33±0.9^{b}	119.0±2.9^{d}	0.00
BG(mg/dl)	73.3±0.88^{d}	339.67±11^{a}	112.67±1.7^{c}	77.30±1.2^{d}	130.0±2.1^{b}	0.00
Serum insulin(µIU/ml)	5.0±0.057^{a}	1.26±0.088^{d}	3.20±0.20^{b}	4.9±0.058^{a}	2.20±0.37^{c}	0.00
HbA1c	4.03±0.38^{d}	13.5±0.59^{a}	6.20±0.12^{c}	3.60±0.15^{d}	7.50±0.37^{b}	0.00
TL (mg/dl)	461.3±16^{d}	1520±34.0^{a}	694.0±38.2^{c}	437.7±9.4^{d}	1056.0±38^{b}	0.00
TC(mg/dl)	125.7±3.7^{d}	314.7±16.7^{a}	173.7±9.2^{c}	132.0±2.0^{d}	232.7±11^{b}	0.00
LDL-c(mg/dl)	79.33±3.3^{d}	325.33±18^{a}	116.3±2.6^{c}	79.0±0.88^{d}	212.3±11^{b}	0.00
HDL-C(mg/dl)	74.0±5.5^{b}	22.67±2.33^{e}	59.0±4.04^{c}	90.66±3.80^{a}	41.0±3.6^{d}	0.00
vLDL-c(mg/dl)	10.5±0.98^{d}	72.6±3.9^{a}	26.67±1.1^{c}	15.40±0.61^{c}	33.4±1.5^{b}	0.00
TAG(mg/dl)	52.7±4.9^{d}	363.0±19.5^{a}	133.3±5.8^{c}	77.0±3.05^{d}	174.3±7^{b}	0.00

Means±SE in the same row and carrying different superscripts are very highly significant different at $p<0.001$.
BW: body weight, BG: blood glucose, HbA1c: glycated hemoglobin TL: total lipid, TC: total cholesterol, LDL-c: low density lipoprotein, HDL-c: high density lipoprotein, vLDL-c: very low density lipoprotein, TAG: triacylglycerol.

group treated with lupin only (Table 2). After induction of diabetes, there was significant elevation in serum glucose, HbA1c, total lipid and cholesterol, LDL-c, vLDL-c and TAG. While, the serum insulin level, HDL-c were significantly decreased in diabetic rats compared to normal control. The results also showed that, treatment with the Gliclazide drug (10 mg/kg) alone or the combination of Gliclazide drug (10 mg/kg) + lupin seeds powder (1g/kg), the blood glucose and HbA1c decreased significantly, while insulin level noticeably increased in treated diabetic groups when compared to diabetic control group. However, adding of lupin powder alone did not induce any change in levels of serum glucose and insulin and HbA1c compared to diabetic control rats (Table2). However, this improvement was more significant in the group treated with the combination of Gliclazide+ lupin seeds compared to those treated by Gliclazide alone. The current data showed that the treatment with the Gliclazide (10 mg/kg) alone and combination of Gliclazide (10 mg/kg) + lupin seeds powder (1 g/kg) significantly decreased total lipid and cholesterol, LDL-c, vLDL-c and TAG when compared to control diabetic rats. However, administration of the Gliclazide (10 mg/kg) alone and combination of Gliclazide (10 mg/kg) + lupin seeds powder (1 g/kg) increased HDL-c. Results also showed no significant change in lupin group compared to diabetic group (Table2). However, this improvement was more significant in the group treated with the combination of Gliclazide+ lupin seeds compared to those treated by Gliclazide alone.

The mRNA expression of glucokinase, Insulin and PPAR-γ genes in HFHF diet diabetic control group livers was significantly down regulated compared to control group. However, the mRNA expression level of PEPCK gene in HFHF diet diabetic group livers were up regulated compared to control group (Table 3).

In this study, the mRNA expression of glucokinase, insulin and PPAR-γ genes in HFHF diet diabetic group livers were significantly up regulated in groups treated with both Gliclazide (10mg/kg) alone and the combination of Gliclazide (10 mg/kg) + lupin seeds powder (1g/kg) compared to diabetic control group. While, the mRNA expression level of PEPCK gene in HFHF diet diabetic group livers were down regulated compared to diabetic control group (Table3). Moreover, the previous changes in the mRNA expressions were more significant in the group treated with the combination of Gliclazide+ lupin seeds compared to those treated by Gliclazide alone ($p<0.05$). Whereas, lupin treated group showed no significant change in mRNA gene expression compared to control diabetic group ($p<0.05$).

Table 3: mRNA expression level of some genes in in male rats experimental groups

Genes	Control	Diabetic control	Gliclazide	Gliclazide +lupin	Lupin	*P* value
Glucokinase	1 ± 0.1^{b}	0.091 ± 0.05^{c}	1.30 ± 0.15^{a}	1.40 ± 0.10^{a}	0.17 ± 0.40^{c}	0.00
PEPCK	1 ± 0.1^{bc}	5.83 ± 1.8^{a}	0.062 ± 0.018^{c}	0.052 ± 0.1^{c}	3.70 ± 1.24^{ab}	0.007
Insulin	1 ± 0.1^{ab}	0.43 ± 0.11^{c}	2.0 ± 0.15^{a}	1.48 ± 0.25^{b}	0.20 ± 0.10^{c}	0.00
PPAR-γ	1 ± 0.1^{b}	0.34 ± 0.5^{c}	2.80 ± 0.30^{a}	3.10 ± 0.20^{a}	0.20 ± 0.05^{c}	0.00

Means ±SE in the same row and carrying different superscripts are significant different at $p<0.05$, very highly significant different at $p<0.001$

PEPCK: Phosphoenolpyruvate carboxykinase, PPAR-γ: Peroxisome Proliferator-Activated Receptor-Gamma

Discussion

The present study demonstrated that administration of HFHF diet caused the development of type2 DM associated with metabolic disorders such as hyperglycemia, glucose intolerance, hyperinsulinaemia, hyper-triglyceridaemia and hypercholesterolemia (31). In the current study, HFHF diet induced the development and progression of the hyperglycemic state by increasing level of blood glucose and glycated hemoglobin due to inadequate secretion of insulin from the pancreas. Moreover, it induced disturbance in total lipid profile such as increased level of total lipid (TL), total cholesterol (32, bad cholesterol (LDL-c), vLDL-c and TAG, and decreased the level of good cholesterol (HDL-c).

This agrees with other researches, which concluded that dyslipidaemia and cardiovascular comorbidities and mortalities are more dominant in patients with Type 2 diabetes (33,34). Importantly, Gliclazide is sulfonylurea derivatives drugs, which is utilized for treatment of type2DM by restraining ATP-dependent potassium chann-els. It is administrated orally and has a modified release form (35).

In the present study, it was observed that administration of Gliclazide drug (10mg/kg) lowered blood glucose, glycated Hb, LDL-c, vLDL-c, TL and TC besides increasing insulin level and HDL-c. Moreover, this was associated with down regulation of mRNA expression level of PEPCK gene, upregulation of mRNA expression glucokinase, insulin, and PPAR-γ genes. An important notice, that Gliclazide caused an only slight increase in body weight.

Weight gain is one of the challenges that face type 2 DM patients due to treatment with oral hypoglycemic drugs as they improve pancreas secretion of insulin and also increase sensitivity of cells towards it (36). According to recent study, scientists proved that treatment with Gliclazide MR cause no significant change in body weight for five years of records, moreover in some trials treatment with Gliclazide MR exhibit reduction in body weight (37). The etiology of weight gain with Gliclazide treatment was not discussed before.

In consistent with the obtained results, other studies concluded that treatment with Gliclazide in type 2 DM improved glycemic parameters, insulin sensitivity and dyslipedemia (38,39). Lupin seeds contain conglutin gamma (Cγ) protein, which applies a hypoglycemic impact. Moreover, it decidedly adjusts proteins engaged in glucose homeostasis. Cγ may conceivably be utilized to oversee patients with debilitated glucose digestion (40).

In the current study, administration of lupin seeds powder 1gm/kg daily cause little change in biochemical parameters but did not reach significance. Another study concluded that lupin seeds powder can lower plasma cholesterol and triacylglycerol levels in hypercholesterolemia laboratory animals (41).

Combination of Gliclazide (10mg/kg)+ lupin seeds (1gm/kg) daily for 4 weeks, noticed that all parameters be liable to shift toward normal states such as body weight and blood glucose, also increase secretion of insulin due to upregulation of mRNA expression of insulin gene and downr egulation of mRNA expression level of PEPCK gene, also increase in good cholesterol (HDL-c), decrease in bad cholesterol (LDL-c), TAG, total cholesterol and total lipid. Moreover, enhancement in the

mRNA expression level of PPAR-γ and glucokinase gene compared to Gliclazide alone treatment group.

According to our knowledge, no other research use the combination of Gliclazide (10mg/kg) with lupin seeds powder (1gm/kg) treatment in animals after induction of type2 DM by high-fat high fructose/sucrose diet in the animal model. However, some researchers used the combination of lupin seeds and metformin (antihyperglycemic drug) in the animal model and found that adding lupin seeds to the antidiabetic drug (metformin) induced more glycemic control and potentiated metformin effects and insulin activity (42,43). In addition, Dove et al. in his study concluded that adding lupin seeds with the antihyperglycemic drug in type 2 diabetic patients reduced blood sugar level, improved glucose metabolism, and increased insulin response (44).

Conclusion

In the present study, combined treatment with Gliclazide (10mg/kg)+lupin seeds powder (1gm/kg) daily for 4 weeks resulted in improvement of total biochemical parameters, lipid profile and enhanced the transcription of mRNA gene more than the treatment with Gliclazide drug only in type 2diabetic rats. It is advisable to incorporate lupin seeds in daily diet regimen of diabetic patients such as combing grinded lupin seeds with wheat flour in bakeries. Moreover, possible future researches on adding lupin seeds in diabetes pharmacotherapy.

Conflict of interest

The authors declare that they have no conflict of interest.

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