Original Article

Evaluation of protective action of fenugreek, insulin and glimepiride and their combination in diabetic *Sprague Dawley* rats

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Abstract

The present study was conducted to assess the effect of fenugreek, insulin and glimepiride alone and their combination in diabetic rat liver. Fifty six male *Sprague dawley* rats of uniform age were randomly divided into seven groups. Group 1: Non-diabetic control; Group 2: Streptozotocin (40 mg/Kg i/p single dose)-induced diabetic control; Group 3: Insulin (4 U/kg once daily for 8 weeks) treatment in diabetic rats; Group 4: Glimepiride (4 mg/Kg orally once daily for 8 weeks) treatment in diabetic rats; Group 5: Fenugreek seed powder treatment (1 g/kg orally once daily for 8 weeks) in diabetic rats; Group 6: Insulin + Fenugreek seed powder treatment (once daily for 8 weeks) in diabetic rats; Group 7: Glimepiride + Fenugreek seed powder treatment (once daily for 8 weeks) in diabetic rats. Livers were collected at the end of experiment for histopathology and estimation of reduced glutathione (GSH), thiobarbituric acid reacting substances (TBARS), protein carbonyls, glutathione S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PD), Na^+/K^+ ATPase and Mg^{2+} ATPase, cytochrome P_{450} (CYP) and glycogen. There was an increase in the concentration of TBARS and protein carbonyls, and decrease in the concentration of GSH and glycogen, and the activity of GST, G6PD, Na^+/K^+ ATPase and Mg^{2+} ATPase in diabetic livers, while treatment groups showed significant ($P < 0.05$) increase in the above parameters. The histology of liver revealed marked changes in diabetic rats and mild changes in combination treatment groups. The treatment with fenugreek, insulin and glimepiride improved the liver parameters in diabetic rats and their combination showed a beneficial effect on liver.

Key words: Diabetes, fenugreek, glimepiride, oxidative stress

INTRODUCTION

The number of people with diabetes is increasing due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity. According to the recent edition of International Diabetes Federation Atlas in 2009, the estimated diabetes prevalence for 2010 had risen to 285 million, representing 6.6% of the world's adult population, with a prediction that by 2030 the number of people with diabetes in the world will have risen to 438 million.[1] The diabetic population in India is increasing at an alarming rate and India stands first among the world nations with 31.7 million in 2000 and is predicted to be 79.4 million by the end of 2030.[2] Diabetes mellitus (DM) is characterized by hyperglycemia, altered metabolism of lipids, carbohydrates and proteins and an increased risk of complications from vascular disease. The prolonged exposure of tissues to hyperglycemia causes most diabetic complications. Management of diabetes without side effects is a big challenge and hence leading to a demand for natural products with fewer side effects. Fenugreek is a commonly used herb and has hypoglycemic action.[3] The various constituents of fenugreek seed have

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glucose lowering action.[4] It also possesses antioxidant action.[5] Glimepiride, a second generation sulfonylurea agent, lowers blood glucose by stimulating the release of insulin from functioning pancreatic *beta* cells. The extra pancreatic glucose reducing effects include inhibition of gluconeogenesis, ketogenesis, stimulation of peripheral glucose transport, glycogen synthase activity and glycerol-3-P-acyltransferase activity. Glimepiride has antioxidant[6] and hypolipidemic[7] actions. Most of the herbs are considered safe and taken as over the counter medication. Since, herbs are pharmacologically active they might interact with modern medicine resulting in altered activity and efficacy. Hence, the present study was designed to investigate the interaction of fenugreek with insulin and glimepiride with reference to hepatoprotection in experimentally induced diabetes mellitus rats.

MATERIALS AND METHODS

Chemicals

Glimepiride was provided as gratis sample by Ranbaxy, India. Insulin (Aventis) and streptozotocin (SRL, Mumbai) were purchased. All the chemicals (for preparation of reagents and buffers) were procured from Qualigens Pvt. Ltd., Mumbai and SRL Pvt. Ltd., Mumbai.

Glimepiride was administered as suspension in freshly prepared 0.5% w/v carboxy methyl cellulose sodium salt. Streptozotocin was dissolved in 0.5 M sodium citrate, pH 4.5.

Herb preparation

Fenugreek (*Trigonellafoenumgraecum*) seeds were purchased from local market, shade dried, powdered and administered as suspension freshly prepared in 0.5% w/v carboxy methyl cellulose sodium salt.

Animals

Fifty six male *Sprague dawley* rats of uniform age (3 months) and weight were procured from National Center for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad, for the study. Feed and water was provided *ad libitum* throughout the experiment. Animals were housed in polypropylene cages in a well ventilated animal house with 12 h-12 h light - dark cycles.

Induction of diabetes

After an acclimatization period of 2 weeks, rats were randomly divided into 7 groups of 8 rats in each and blood samples were collected and serum was separated for glucose estimation. Subsequently, group 1 was kept as normal control throughout the experimental period. Remaining 6 groups were induced diabetes by intraperitoneal injection of streptozotocin @ 40 mg/kg body weight. The rats were provided with glucose water for 24 h to prevent hypoglycemia. Blood samples were collected after 72 h and serum was separated for glucose estimation. Rats with blood glucose value of >250 mg/dl (72 h after streptozotocin administration) were included in the study ($n = 8$). Treatment protocols were initiated from day 2 post-confirmation of diabetes (day 5 post-streptozotocin administration) and were continued for 8 weeks. The experimental protocol was approved by Institutional Animal Ethics Committee, India.

Experimental design

After induction of diabetes, all the groups were maintained as per the following drug and herb treatment schedule for 8 weeks. Group 1: Non-diabetic control; Group 2: Streptozotocin (40 mg/Kg i/p single dose)-induced diabetic control; Group 3: Insulin (4 U/kg once daily for 8 weeks) treatment in diabetic rats; Group 4: Glimepiride (4 mg/Kg orally once daily for 8 weeks) treatment in diabetic rats; Group 5: Fenugreek seed powder treatment (1 g/kg orally once daily for 8 weeks) in diabetic rats; Group 6: Insulin + Fenugreek seed powder treatment (once daily for 8 weeks) in diabetic rats; Group 7: Glimepiride + Fenugreek seed powder treatment (once daily for 8 weeks) in diabetic rats.

Sample collection

At the end of the experiment, 6 rats from each group were sacrificed and liver was collected in 10% buffered form a line for histopathological studies and part of liver stored at -20°C for estimation of GSH,[8] TBARS,[9] protein carbonyls,[10] glutathione S transferase (GST),[11] glucose-6-phosphate dehydrogenase (G6PD),[12] Na^+/K^+ ATPase and Mg^2+ ATPase,[13,14] Cytochrome P_{450} (CYP),[15] glycogen[16] and was estimated in the liver homogenates. The protein content was estimated by Lowry method.[17]

RESULTS

The concentration of TBARS (n moles MDA/mg protein) in liver revealed a significant ($P < 0.05$) rise in Group 2 as compared to Group 1. The groups 3 to 7 showed a significant ($P < 0.05$) decrease in TBARS concentration as compared to Group 2. The groups 6 and 7 showed significant ($P < 0.05$) decrease in TBARS concentration as compared to groups 3, 4 and 5. The concentration of protein carbonyls (n moles/mg protein) in liver revealed a significant ($P < 0.05$) rise in Group 2 as compared to Group 1. The groups 3 to 7 showed a significant ($P < 0.05$) decrease in protein carbonyls concentration as compared to Group 2. The groups 6 and 7 showed significant ($P < 0.05$) decrease in protein carbonyls concentration as compared to groups 3, 4 and 5 [Table 1].

The concentration of GSH (μ moles/mg protein) in liver revealed a significant ($P < 0.05$) decrease in Group 2 as compared to Group 1. The groups 3 to 7 showed a significant ($P < 0.05$) increase in GSH concentration as compared to Group 2. The groups 5, 6 and 7 showed significant ($P < 0.05$) increase in GSH concentration as compared to groups 3 and 4. The activity of GST (μ moles/min/mg protein) in liver revealed a significant ($P < 0.05$) decrease in Group 2 as compared to Group 1. The groups 3 to 7 showed a significant ($P < 0.05$) increase in the activity of GST as compared to Group 2. The activity of G6PD (units/mg protein) in liver revealed a significant ($P < 0.05$) decrease in Group 2 as compared to Group 1. The groups 3 to 7 showed a significant ($P < 0.05$) increase in the activity of G6PD as compared to Group 2, but significantly ($P < 0.05$) decreased when compared to Group 1[Table 1].

The activity of Na^+/K^+ ATPase (μ moles Pi liberated/mg microsomal protein/30 min) in liver revealed a significant ($P < 0.05$) reduction in Group 2 as compared to Group 1. The groups 3 to 7 showed a significant ($P < 0.05$) increase in Na^+/K^+ ATPase activity as compared to Group 2. The groups 6 and 7 showed significant ($P < 0.05$) increase among all the treatment groups. The activity of Mg^2 + ATPase (μ moles Pi liberated/mg microsomal protein/30 min) in liver revealed a significant ($P < 0.05$) reduction in Group 2 as compared to Group 1. The groups 3 to 7 showed a significant ($P < 0.05$) increase in Mg^2 + ATPase activity as compared to Group 2. The activity of CYP_{450} (n moles/mg microsomal protein) in liver revealed a significant ($P < 0.05$) reduction in Group 2 as compared to Group 1. The groups 3 to 7 showed a significant ($P < 0.05$) increase in CYP_{450} activity as compared to Group 2 [Table 2].

The concentration of glycogen (mg/g tissue) in liver revealed a significant ($P < 0.05$) reduction in Group 2 as compared to Group 1. The groups 3 to 7 showed a significant ($P < 0.05$) increase in glycogen concentration as compared to Group 2 [Table 2].

The sections of liver showed marked central vein congestion and bile duct hyperplasia in Group 2 [Figure 1], while groups 3 and 5 revealed moderate central vein congestion, mild bile duct hyperplasia and mild sinusoidal congestion [Figure 2]. Sections of Group 4 exhibited mild central vein congestion and mild degenerative changes in the hepatocytes. The sections of Group 6 showed hydropic degeneration [Figure 3], while Group 7 revealed mild sinusoidal congestion [Figure 4] as compared to Group 2, while Group 1 did not show any lesions of pathological significance.

DISCUSSION

Diabetes mellitus (DM) consists of a group of syndromes characterized by hyperglycemia, altered metabolism of lipids, carbohydrates and proteins, and an increased risk of complications from vascular disease. During hyperglycemia,

Table 1: Antioxidant profile of liver

Group	TBARS (n moles of MDA released/mg protein)	Protein carbonyls (*n* moles/mg protein)	GSH (*n* moles/mg protein)	GST (μ moles/min/mg protein)	G6PD (Units/mg protein)
Non-diabetic control	7.59 ± 0.14^{a}	2.31 ± 0.15^{a}	31.71 ± 0.32^{e}	1.43 ± 0.04^{d}	3.87 ± 0.08^{d}
Diabetic mellitus (DM) control	17.97 ± 0.24^{f}	7.34 ± 0.21^{e}	17.27 ± 0.23^{a}	0.74 ± 0.01^{a}	1.29 ± 0.05^{a}
DM+Insulin	11.74 ± 0.15^{c}	4.46 ± 0.10^{c}	26.18 ± 0.23^{b}	1.02 ± 0.01^{b}	2.30 ± 0.09^{b}
DM+Glimepiride (GM)	12.26 ± 0.22^{d}	4.93 ± 0.04^{d}	26.30 ± 0.23^{b}	1.04 ± 0.02^{bc}	2.40 ± 0.12^{b}
DM+Fenugreek (FG)	12.87 ± 0.08^{e}	4.91 ± 0.06^{c}	27.66 ± 0.13^{c}	1.02 ± 0.02^{b}	2.49 ± 0.13^{b}
DM+Insulin+FG	10.62 ± 0.18^{b}	3.35 ± 0.13^{b}	29.01 ± 0.06^{d}	1.10 ± 0.01^{c}	2.98 ± 0.05^{c}
DM+GM+FG	10.59 ± 0.20^{b}	3.20 ± 0.10^{b}	29.12 ± 0.12^{d}	1.09 ± 0.03^{c}	3.19 ± 0.11^{c}

Values are Mean±SE (*n*=6), one way ANOVA (SPSS), means with different alphabets as superscripts differ significantly (*P*<0.05)

Table 2: Liver parameters

Group	Na^+-K+ATPase activity (μ moles of Pi liberated/mg microsomal protein/30 min)	Mg^{2+} ATPase activity (μ moles of Pi liberated/mg microsomal protein/30 min)	Cytochrome P_{450} activity (*n* moles/mg microsomal protein)	Glycogen concentration (mg/g tissue)
Non-diabetic control	17.11 ± 0.14^{e}	10.91 ± 0.21^{d}	3.50 ± 0.05^{b}	58.49 ± 3.58^{d}
Diabetic mellitus (DM) control	6.37 ± 0.16^{a}	4.91 ± 0.05^{a}	1.37 ± 0.05^{a}	16.75 ± 1.84^{a}
DM+Insulin	13.65 ± 0.12^{bc}	7.70 ± 0.13^{bc}	2.58 ± 0.03^{c}	32.62 ± 0.97^{b}
DM+Glimepiride (GM)	13.36 ± 0.21^{b}	7.49 ± 0.14^{b}	2.58 ± 0.04^{c}	35.24 ± 1.54^{bc}
DM+Fenugreek (FG)	13.41 ± 0.25^{b}	7.65 ± 0.15^{bc}	2.61 ± 0.08^{c}	34.21 ± 1.84^{bc}
DM+Insulin+FG	14.08 ± 0.03^{cd}	8.03 ± 0.11^{c}	2.71 ± 0.05^{c}	38.65 ± 1.50^{bc}
DM+GM+FG	14.23 ± 0.16^{d}	7.94 ± 0.15^{c}	2.71 ± 0.06^{c}	39.37 ± 1.54^{c}

Values are Mean±SE (*n*=6), one way ANOVA (SPSS), means with different alphabets as superscripts differ significantly (*P*<0.05)

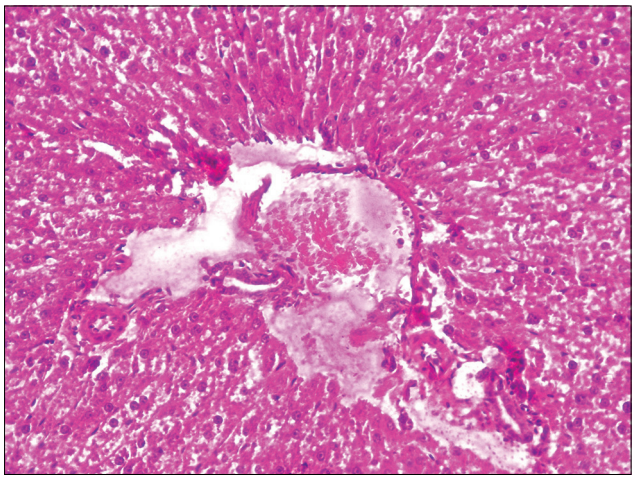

Figure 1: Photomicrograph of liver showing marked central vein congestion and bile duct hyperplasia. H and E ×200 (Group 2)

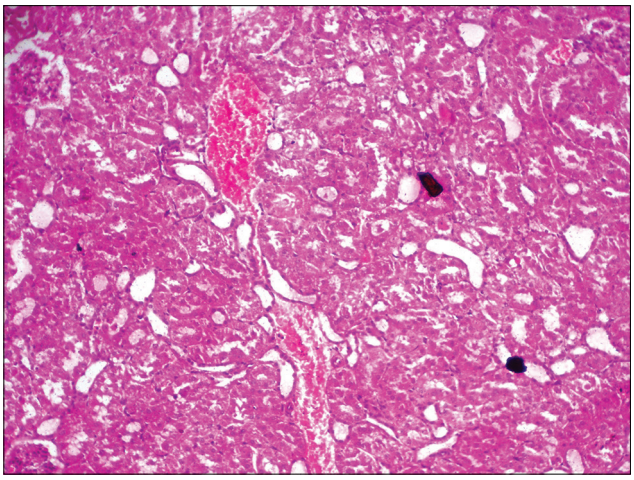

Figure 2: Photomicrograph of liver showing moderate sinusoidal congestion and dilation. H and E ×200 (Group 3)

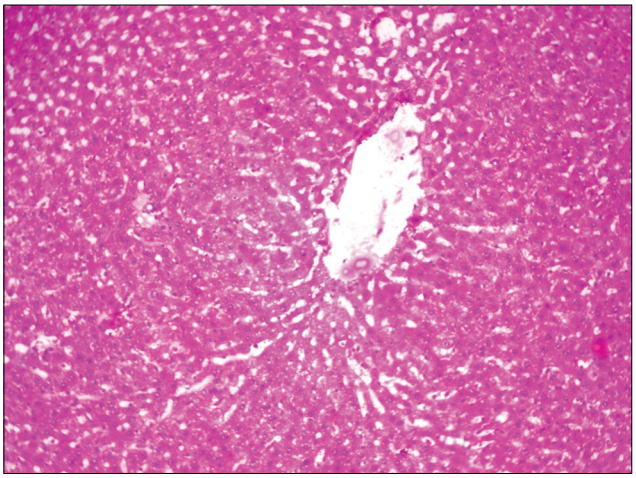

Figure 3: Photomicrograph of liver showing mild sinusoidal dilatation and congestion. H and E ×100 (Group 7)

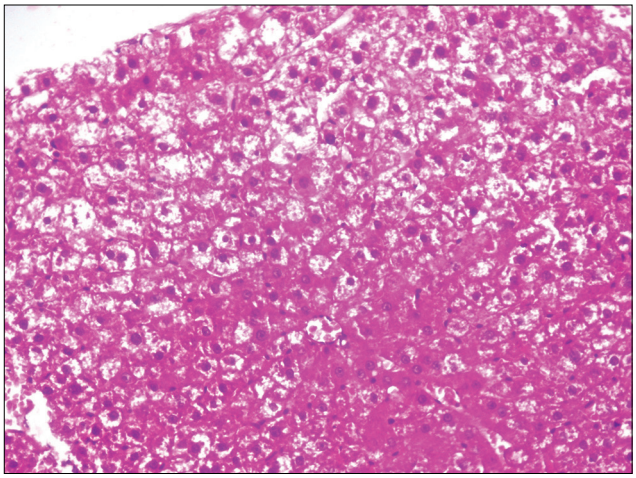

Figure 4: Photomicrograph of liver showing hydropic degeneration of hepatocytes. H and E ×200 (Group 6)

more glucose being oxidized in the TCA cycle in the cells pushes more electron donors (NADH and $FADH_2$) into the electron transport chain, thereby generating superoxide radical.[18] This superoxide inhibits glyceraldehyde-3 phosphate dehydrogenase (GAPDH) activity *in vivo*.[19] Inhibition of GAPDH leads to activation of the four pathways namely polyol pathway, hexosamine pathway, PKC activation and increased production of advanced glycation end products (AGEs). These pathways further lead to free radical generation.

The biomarkers of oxidative stress (TBARS, protein carbonyls, GST and GSH) were studied in liver to evaluate the extent of free radical-induced damage. An imbalance between pro-oxidants and antioxidants, in favor of the pro-oxidants, results in oxidative stress associated with oxidative modification of bio-molecules such as lipids, proteins and nucleic acids.[20] Hyperglycemia generates reactive oxygen species (ROS) and attenuates antioxidant mechanisms resulting in oxidative stress. During diabetes, there is increased production of free radicals through glucose auto-oxidation and protein glycation. The oxidative degradation of these oxidants could participate in the formation of lipid peroxidation products. The oxidative degradation of fructosamines may contribute to the oxidative stress found in hyperglycemia associated with diabetes mellitus. During diabetes, advanced glycation end products (AGEs) are formed, when glucose reacts with various proteins such as hemoglobin, albumin, collagen, LDL or crystalline proteins to form labile Schiff bases, which then undergo further modification to form Amadori products.[21] The rate of glycation is proportional to the blood glucose concentration. Chronic hyperglycemia induces carbonyl stress, which in turn can lead to increased lipid peroxidation.[22] The increased concentration of lipid peroxidation induces oxidative damage by increasing peroxy radicals and hydroxyl radicals.[23] Thus, lipid peroxidation is one of the characteristic features of

chronic uncontrolled diabetes. The most commonly used indicator of lipid peroxidation is TBARS.[24] The increased lipid peroxidation in the plasma and tissues of diabetic animals may be due to the observed remarkable increase in the concentration of TBARS and MDA as a main product of lipid peroxidation in the plasma and liver.[25] In the present study, the concentration of TBARS and protein carbonyls were increased in liver, while GSH and GST were reduced in diabetic rats. This was further supported by the histopathological findings of liver, which revealed marked central vein congestion and bile duct hyperplasia in diabetic control Group 2, while groups 3 and 5 revealed moderate central vein congestion, mild bile duct hyperplasia and mild sinusoidal congestion, Group 4 exhibited mild central vein congestion and mild degenerative changes in the hepatocytes. The sections of groups 6 and 7 showed mild changes as compared to Group 2. The treatment with fenugreek, glimepiride and insulin reduced the oxidative stress. There was significant reduction in concentration of TBARS and protein carbonyls, and increase in GSH and GST levels. The flavonoids present in fenugreek have antioxidant action. Reduced glutathione (GSH) is one of the most essential non-enzymatic compounds for detoxification of several exogenous and endogenous toxicants. It has a direct antioxidant function by reacting with superoxide radicals, peroxy radicals and singlet oxygen followed by the formation of oxidized glutathione (GSSG) and other disulfides.[26] The depletion of GSH seems to be the critical factor that promotes lipid peroxidation.[27] Glutathione S-transferase (GST) is GSH-dependent antioxidant enzyme that catalyzes the conjugation of GSH, via the sulfhydryl group, to electrophilic centers on a wide variety of substrates.[28] This activity is useful in the detoxification of endogenous compounds such as peroxidized lipids.

Glucose 6-phosphate dehydrogenase (G6PD) is the rate limiting enzyme of pentose phosphate pathway, which catalyses the production of ribose-5-phosphate and NADPH. NADPH is the principal intracellular reductant in the cells and G6PD is the principal source of NADPH. This NADPH is necessary for normal coupling of GSH. Decrease in NADPH leads to decreased GSH levels. Also, increased aldose reductase activity reduces NADPH as a consequence there is increase in reactive oxygen species. High glucose has been reported to inhibit G6PD activity through the activation of PKA.[29] During diabetes, there is increased polyol pathway and increased aldose reductase activity resulting in depletion of NADPH and subsequently GSH and catalase. In the present study, G6PD levels were decreased in liver, resulting in increased oxidative stress. Insulin, fenugreek and glimepiride treatment reduced the blood glucose levels, thereby reducing aldose reductase activity and increased GSH levels.

The activity of Na^+/K^+ ATPase, Mg^2+ ATPase and CYP450 in diabetic liver was reduced, which may be due to the membrane peroxidative damage induced by increased lipid peroxidation status. The levels of these enzymes were improved in treatment groups owing to their antioxidant action.

In diabetic rats, there was significant reduction in liver glycogen probably due to lack of insulin in diabetic rats, which results in inactivation of gluconeogenic enzymes in liver. The enzyme glycogen synthase system inhibition leads to decrease in liver glycogen stores. Treatment with insulin, fenugreek and glimepiride has improved the glycogen levels as compared to the diabetic rats, which could be due to reactivation of glycogen synthase system as a result of increased insulin secretion. The liver plays a significant role in glucose homeostasis through a delicate balance between hepatic glucose uptake, utilization and hepatic glucose production.[30] In liver, insulin enhances glucose uptake and utilization mainly because of increased activity of enzyme glucokinase. In STZ-diabetic rats, the glucokinase expression is lowered and insulin treatment restores the levels of glucokinase. Fenugreek treatment enhances both hepatic glucokinase and hexokinase activity in diabetic mice.[31]

In conclusion, the study revealed that addition of fenugreek seed powder to insulin and glimepiride had positive interaction in improving the liver parameters in streptozotocin-induced diabetic *Sprague dawley* rats, which was evident from greater improvement in oxidative stress parameters and liver parameters in the groups that were treated using a combination of fenugreek with either insulin or glimepiride as compared to individual agent-treated groups.

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