

ANTIDIABETIC ACTIVITY OF *TRIGONELLA FOENUM-GRÆCUM* L. SEEDS EXTRACT (IND01) IN NEONATAL STREPTOZOTOCIN-INDUCED (N-STZ) RATS

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SUMMARY

The objective of the present investigation was to study antidiabetic activity of standardized extract of *Trigonella foenum-graecum* seeds (IND01) using neonatal streptozotocin-induced rat (n-STZ) model of diabetes mellitus (DM) in rats for the first time. Streptozotocin (STZ) was injected (50 mg/kg i.p.) in pups of rats in a split dose manner (on 2nd and 3rd postnatal days). After 8 weeks, DM was confirmed by checking for 4 h fasting serum glucose (SG) levels. Effects of IND01 (100 mg/kg, oral) and standard drug, glyburide (10 mg/kg, oral) were evaluated on acute and subacute (28 days) administration in separate sets of n-STZ rats. Body weights were recorded every day. The levels of SG were recorded at 0, 2, 4, 6, and 24 h in acute study and on day 7, 14, 21 and 28 in subacute

study. Levels of glycosylated hemoglobin (HbA1c) and serum insulin were measured on day 28 in subacute study. The treatment of n-STZ in pups produced significant and progressive rise in SG levels, body weights and HbA1c during the study period of 28 days. Decrease in serum insulin levels and mean number of pancreatic islet β -cells (histology study) was also found in n-STZ control rats. IND01 (100 mg/kg, oral) and glyburide (10 mg/kg, oral) treatment showed significant reversal of n-STZ-induced changes (rise in SG, decline in body weight and rise in HbA1c). Histology sections of pancreas from the rats treated with IND01 (but not glyburide) showed increase in number and size of pancreatic islet β -cells. IND01 showed a potential to ameliorate symptoms of DM during progressive deterioration and improved glycemic functions in n-STZ induced diabetic rats.

INTRODUCTION

Diabetes mellitus (DM) is a chronic heterogeneous disease characterized by hyperglycemia resulting from both insulin resistance and insulin deficiency secondary to pancreatic beta-cell failure (1). At present, the treatment of DM mainly involves sustained hyperglycemia reduction by use of biguanides, thiazolidinediones, sulfonylureas, D-

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phenylalanine derivatives, meglitinides and α -glucosidase inhibitors in addition to insulin. However, due to unwanted side effects, the efficacies of these compounds are debatable and there is a demand for new compounds for the treatment of DM.

Recently, there have been growing interests in the application of natural components as antidiabetic agents (2). A wide range of products claiming to lower blood glucose levels or prevent and treat diabetes complications and comorbidities are marketed to the public (3). One of the most promising amongst them is *Trigonella foenum-graecum* L. (fenugreek, family Leguminosae) seeds. Fenugreek seeds have shown potential as a dietary supplement and cause a marked decrease in the symptoms of DM such as polydipsia, polyuria, urine sugar, renal hypertrophy and glomerular filtration rate (4).

Fenugreek is a spice rich in dietary fibers and has a traditional history of medicinal use in the management of DM in Egypt, southern Europe, India, Asia, and northern Africa (5). Fenugreek seeds mainly contain 4-hydroxyisoleucine (4-HI), trigonelline, galactomannan with flavonoids, carotenoids, coumarins, proteins, saponins, and lipids (6). Fenugreek seeds have previously been shown to have hypoglycemic and hypocholesterolemic effects in type 1 and type 2 DM patients (7,8) and alloxan induced diabetic animals (9,10).

Many experimental models in laboratory animals are available for understanding the pathogenesis, complications, and genetic or environmental influences that increase the risks of type 2 DM and testing of various therapeutic agents (11). Streptozotocin (STZ)-induced DM in rats is a widely used animal model for evaluation of agents against DM. STZ is a chemical substance specifically toxic to pancreatic β -cells. It is taken into the pancreatic β -cells by glucose transporter 2 (GLUT-2) (12). When injected into adult rats, STZ causes type 1 DM with severely elevated blood glucose level. However, when STZ is administered to neonatal rats, the neonates experience acute hyperglycemia within the first few days and blood glucose gradually decreases thereafter.

STZ promotes insulin resistance of neonatal rats to considerable intensity that sustains for long periods (13).

At 6-15 weeks after single dose administration to neonatal rats, STZ induces β -cell injury, limited regeneration, short-term normalization of glycemia, an impaired glucose disposal rate and significant β -cell secretory dysfunction (type 2 DM) (14). Rats injected with STZ at neonatal stage (n-STZ) exhibit decreased β -cell mass and develop features (hyperglycemia, polyphagia, polydipsia, polyuria, insulin resistance and abnormal glucose tolerance) in adulthood that closely resemble type 2 DM patients (15). Therefore, n-STZ model provides an ideal platform for β -cell regeneration study and antidiabetic drug evaluation against progressive DM (16,17).

IND01 is a standardized hydroalcoholic extract of *Trigonella foenum-graecum* seeds, which contains 4-HI, trigonelline, and galactomannan. In the past, the antihyperglycemic activity of fenugreek seed extract (18) and IND01 (19) was reported. IND01 also showed synergistic interaction with synthetic antihyperglycemic agents like pioglitazone and glyburide in alloxan induced DM in mice (20). Individual constituents of IND01 such as 4-HI (21), trigonelline (22), and galactomannans (23) demonstrated potent antihyperglycemic effects in animal models of DM through different mechanisms.

Recently, the antihyperglycemic activity of 4-HI (a major component of IND01) is proposed to be mediated through its ability to regenerate the pancreatic β -cell mass (24). However, effects of IND01 on the progression of DM (especially pancreatic β -cell mass) remain unknown. Therefore, the present work was undertaken with the objective to evaluate the potential of IND01 in n-STZ induced DM in laboratory animals. Here, we report for the first time the use of the n-STZ model for the study of the antidiabetic potential of fenugreek seeds.

MATERIALS AND METHODS

Animals

Male Wistar rats (150-200 g) were purchased from National Toxicology Center, Pune. During the experiment, rats were housed in standard housing conditions like temperature of 25 ± 1 °C, relative humidity of 45%-55% and 12 h light:12 h dark cycle. Rats had free access to food pellets (Navmaharashtra Chakan Oil Mills Ltd., Sangli, India) and tap water *ad libitum* during the experiments. All experiments were in accordance with ethical guidelines for investigations of experimental pain in conscious animal. Research protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Pune.

Chemicals

STZ (Sigma Aldrich, MO, USA), D-glucose (S.D. Fine Chemicals Ltd., India), glucose estimation kit (Accurex Biomedical Pvt. Ltd., Mumbai), enzyme-linked immunosorbent assay (ELISA) kits for measurement of glycosylated hemoglobin (HBA1c) (Crest Biosystem, Goa, India) and plasma insulin (Boehringer Mannheim, Germany) were purchased from the respective vendors. All other chemicals were of analytical grade. STZ was dissolved in 0.1 M citrate buffer (pH 4.5).

Preparation of IND01 from fenugreek seeds

Fenugreek seeds were collected in the areas of the state of Rajasthan in India during November 2007. The seeds were authenticated by Dr. A. M. Mujumdar, Agharkar Research Institute, Pune, India, and a voucher specimen was deposited at that Institute. The test composition, IND01, hydroalcoholic extract of *Trigonella foenum-graecum* seeds, is prepared by extraction with ethyl alcohol:water (70:30) by column chromatography according to the reported procedure (22) (yield: 9.0 g, 0.9% w/w). Solutions of IND01 were freshly prepared in distilled water and administered as mg *per* kg basis for further evaluation in experimental animals.

Characterization of IND01 based on 4-HI by HPLC

IND01 was characterized with the help of HPLC after derivatization for marker composite amino acid (4-HI). The mobile phase was made up of Part A (0.05 mL of trifluoroacetic acid in 100 mL with DM water) and Part B (40 mL of acetonitrile) mixed at a ratio of 60:40. The solutions were separately filtered through membrane filter paper (Nylon 6,6 membrane), degassed before mixing. Standard solution was prepared by dissolving 5 mg of reference standard (4-HI, Sigma-Aldrich, US; purity >98%) in 50 mL DM water. Sample solution was prepared by dissolving 100 mg of IND01 with 50 mL of methanol:water mixture (70:30) in 100 mL volumetric flask. The solution was stirred and pH was adjusted to 7.0 with the help of 0.1 N NaOH. The volume was made up to 100 mL with methanol:water mixture (70:30). The standard and sample solutions were derivatized in 10 mL standard volumetric flask as follows: to 2 mL of the solutions, 8 mg of 1-fluoro-2,4-dinitrobenzene (DNF) reagent was added. The volume was made up to 10 mL with buffer (pH 7.4). The flask was sonicated for 1 h, allowed to derivatize for the next 24 h, filtered through Whatman filter paper #41 and injected in HPLC system to record chromatogram. HPLC conditions were as follows: model JASCO LC 2000 with UV-2075; column: reverse phase C-18 column L1 (250 mm x 4.6 mm) as defined in USP30/NF25 with 5 μ particle size; detector: UV-VIS, injection volume: 20 μ L; method time: 30 min; flow rate: 1.5 mL/min; detector: UV at 347 nm (4-HI). Retention time and area under curve (AUC) were calculated to determine purity of sample.

Induction of experimental DM

To induce experimental DM, male neonatal rats were administered intraperitoneally (i.p.) STZ (split-dose schedule: 50 mg/kg on day 2 and day 3 of age) as *per* reported procedure (25). The site of injection was controlled to be under the inguinal fat of the neonates to prevent any leakage of the liquids. Preparation and administration of STZ solution was performed on ice and in darkness to avoid degradation of STZ. Control littermates received an injection of citrate buffer (0.1

M, pH 4.5) alone. The animals were weaned at 28 days of age. After 8 weeks, DM was confirmed by measurement of fasting (4 h) serum glucose (SG) levels. To measure SG levels, blood samples from the experimental rats were collected by retro-orbital plexus technique using capillary glass tubes. The collected blood samples were analyzed for SG levels by the glucose oxidase peroxidase (GOD/POD) method. The rats showing SG above 11 mmol/L were labeled as diabetic rats and selected for the study.

Effect of acute and subacute administration of IND01 on serum glucose level in n-STZ diabetic rats

The effects of IND01 were evaluated after acute and subacute (28 days) administration in a separate set of n-STZ rats. For each set of experiment, n-STZ induced diabetic rats of either sex were divided into 3 groups (n=6): group I, vehicle (distilled water, 10 mL/kg), group II, IND01 (100 mg/kg); and group III (glyburide, 10 mg/kg), and were administered respective treatment orally. The acute study involved estimation of SG levels at 0, 2, 4, 6 and 24 h after drug administration. The subacute study involved repeated administration of drug once daily for 28 days at prefixed times, and SG levels were estimated on day 7, 14, 21 and 28 of the study. Data were expressed as mean SG level (mmol/L).

Effect of IND01 on body weight in n-STZ diabetic rats (subacute study)

The rats were weighed on day 0 and then on day 0, 7, 14, 21 and 28 of the study period. Body weights were noted. Data were expressed as mean body weights (g).

Effect of subacute administration of IND01 on glycosylated hemoglobin (HbA1c) and serum insulin level in n-STZ diabetic rats

After completion of treatment on day 28 of the subacute study, the animals were sacrificed. Blood was collected by cardiac puncture. Levels of HbA1c and insulin level in plasma were measured using respective enzyme-linked immunosorbent assay (ELISA) kits

according to the manufacturer's instructions.

Histology of pancreas and islet morphometry

At the end of 28-day period, the rats were euthanized and the pancreata from all rats were collected for histology study. The pancreata were fixed in 10% formalin overnight and then embedded in paraffin. Sections (3 mm thickness) were cut by microtome throughout the pancreas. The sections were deparaffinized and rehydrated sequentially in xylene, xylene/ethanol, 100%, 95%, 80%, 70% and 50% ethanol, and then put in distilled water for 10 min. Pancreatic sections were stained with hematoxylin and eosin (H/E) using standard protocols. The mean number of islets in tissues was measured.

Statistical analysis

Data obtained for SG (acute and subacute administration) and body weight (subacute administration) were expressed as mean \pm SEM and analyzed separately by two-way ANOVA followed by Bonferroni test using the GraphPad Prism Version 4.03 software. Separate analysis was performed for data on HbA1c, serum insulin levels and mean number of islets *per* pancreatic tissues on day 28 by one-way ANOVA followed by Dunnet's test for significance.

RESULTS

Standardization of IND01 by HPLC

At wavelength of 347 nm, the reference standard of the marker compound, 4-HI, showed retention time of 7.47 min, whereas the sample of IND01 showed retention time of 7.41 with AUC = 37371 with assay of 31.15% (Figure 1).

Effect of IND01 on serum glucose level in n-STZ diabetic rats (acute study)

Data on the effects of acute oral administration of IND01 on serum glucose levels are presented in Table 1. Single administration of vehicle treatment to disease control (n-STZ control) rats did not show change in SG levels. Single administration of IND01 (100

Table 1. Effect of acute administration of IND01 on serum glucose levels in neonatal STZ (n-STZ) induced diabetic rats

Time of observation	Mean serum glucose level (mmol/L) ± SEM		
	n-STZ control	IND01 (100 mg/kg, oral)	Glyburide (10 mg/kg, oral)
Baseline	14.04 ± 1.24	14.60 ± 0.93	14.23 ± 1.18
2 h	14.33 ± 1.00 ^{ns}	11.95 ± 0.62**	8.20 ± 0.37***
4 h	14.09 ± 1.06 ^{ns}	11.72 ± 0.65**	6.34 ± 0.32***
6 h	14.00 ± 1.26 ^{ns}	11.67 ± 0.69**	5.46 ± 0.36***
24 h	14.91 ± 0.94 ^{ns}	12.37 ± 0.76*	9.32 ± 0.32***

Number of rats per group = 6; data were analyzed by two-way ANOVA followed by Bonferroni post tests; ns = nonsignificant; * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$ significant reduction as compared with the respective group baseline.

Table 2. Effect of subacute administration of IND01 on serum glucose levels in neonatal STZ (n-STZ) induced diabetic rats

Day of observation	Mean serum glucose level (mmol/L) ± SEM		
	n-STZ control	IND01 (100 mg/kg, oral)	Glyburide (10 mg/kg, oral)
Baseline	14.18 ± 0.81	12.98 ± 0.43	13.54 ± 0.70
7 h	19.85 ± 0.50###	9.57 ± 0.31***	11.22 ± 0.19***
14 h	20.26 ± 0.55###	9.36 ± 0.37***	10.95 ± 0.27***
21 h	19.25 ± 1.21###	8.99 ± 0.29***	9.86 ± 0.24***
28 h	20.93 ± 0.50###	8.88 ± 0.23***	9.64 ± 0.20***

Number of rats per group = 6; data were analyzed by two-way ANOVA followed by Bonferroni post tests; ###significant increase at $P < 0.001$; ***significant decrease at $P < 0.001$ as compared with the respective group baseline

Table 3. Effect of subacute administration of IND01 on body weight in neonatal STZ (n-STZ) induced diabetic rats

Day of observation	Mean body weight (g) ± SEM		
	n-STZ control	IND01 (100 mg/kg, oral)	Glyburide (10 mg/kg, oral)
Baseline	120.83 ± 5.84	123.33 ± 14.94	121.17 ± 13.83
7 h	120.17 ± 4.99 ^{ns}	126.83 ± 15.17**	122.00 ± 13.79 ^{ns}
14 h	116.67 ± 4.48###	135.33 ± 15.46***	127.17 ± 13.62***
21 h	114.50 ± 5.32###	140.67 ± 15.22***	132.33 ± 13.49***
28 h	113.67 ± 5.43###	144.00 ± 15.07***	137.00 ± 13.53***

Number of rats per group = 6; data were analyzed by two-way ANOVA followed by Bonferroni post tests; ###significant decrease at $P < 0.001$; *** $P < 0.001$; ns = nonsignificant as compared with the respective group baseline.

mg/kg, oral) treatment produced peak reduction in SG levels (antihyperglycemic effect) from the baseline level of 14.6 mmol/L to 11.67 mmol/L at 6 h (20.5% reduction, $P < 0.01$). Acute administration of glyburide (10 mg/kg, oral) resulted in 61.6% reduction (from 14.23 to 5.46 mmol/L, $P < 0.001$) in the same period. The reduction in SG level persisted even 24 h after treatment in both IND01 and glyburide treatment groups.

Effect of IND01 on serum glucose in n-STZ diabetic rats (subacute study)

Data on the effects of subacute (28 days, oral, once daily) administration of IND01 on serum glucose

levels are presented in Table 2. In the subacute study, repeated administration (once a day for 28 days) of IND01 and glyburide caused significant ($P < 0.001$) reduction in serum glucose level as compared to vehicle treated group. The n-STZ control rats showed progressive and significant ($P < 0.001$) rise in SG during the study period of 28 days (from 14.18 mmol/L at baseline to 20.93 mmol/L on day 28). IND01 (100 mg/kg, oral, once daily) treatment of n-STZ rats showed progressive reduction from baseline SG level of 12.98 mmol/L to 9.57 (26.27%), 9.36 (27.92%), 8.99 (30.73%) and 8.88 mmol/L (31.59%) on day 7, 14, 21 and 28, respectively. Similar reduction in SG levels was noted in rats from glyburide (10 mg/kg, oral) treatment group. The SG

levels were reduced from baseline level of 13.54 mmol/L to 11.22 (18.5%), 10.95 (19.12%), 9.86 (27.17%) and 9.64 mmol/ L (28.80%) on days 7, 14, 21 and 28, respectively.

Effect of IND01 on body weight of n-STZ diabetic rats (subacute study)

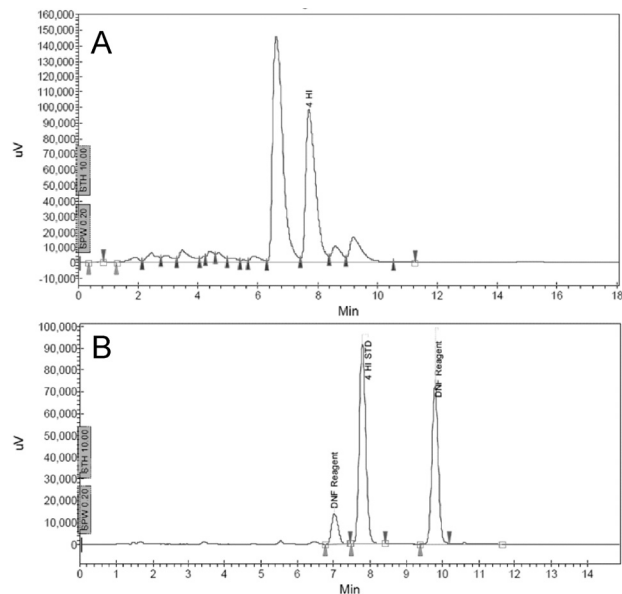
Data on the effects of subacute (28 days, oral, once daily) administration of IND01 on body weights of rats are presented in Table 3. The n-STZ control rats showed progressive and significant ($P<0.001$) reduction in body weights during the study period of 28 days (from 120.83 g at baseline to 113.67 g on day 28). Daily administration of IND01 (100 mg/kg, oral) showed significant rise ($P<0.001$) in body weight from day 7 onwards. The body weights of rats showed progressive rise in baseline SG levels of 123.33 g to 144.00 g (16.75% rise) on day 28. Similar rise in body weights (13.06%, $P<0.001$) was noted with glyburide treatment (from 121.17 g to 137.00 g) on day 28 of the study.

Effect of subacute administration of IND01 on glycosylated hemoglobin (HbA1c) level in n-STZ diabetic rats

Data on the effects of subacute (28 days, oral, once daily) administration of IND01 on glycosylated hemoglobin (HbA1c) levels in rats are presented in Table 4. The mean HbA1c levels of normal (nondiabetic) rats was 6.28%. Rats from n-STZ control group showed significant rise in mean HbA1c (9.20%), which indicated poor glycemic control. Significant reduction in mean HbA1c levels was

recorded with 28-day IND01 treatment (6.48, $P<0.01$) and glyburide (5.32, $P<0.001$) as compared to rats of n-STZ control group (9.20%).

Figure 1. HPLC fingerprint at 347 nm for the marker compound, 4-hydroxyisoleucine (4-HI) identified in (A) IND01 (retention time = 7.41) and (B) reference standard (retention time = 7.47).



Effect of subacute administration of IND01 on serum insulin levels in n-STZ diabetic rats

Data on the effects of subacute (28 days, oral, once daily) administration of IND01 on the mean serum insulin levels of rats are presented in Table 4. At baseline, the mean insulin level in normal (nondiabetic) rats was 5.11 μ IU/mL. Diabetic rats (n-STZ control) showed significantly ($P<0.001$) reduced

Table 4. Effect of subacute administration of IND01 on glycated hemoglobin (HbA1c) and insulin levels in serum of neonatal STZ (n-STZ) induced diabetic rats on day 28 of the study

Treatment	Serum parameter value on day 28, mean \pm SEM		
	Glycated hemoglobin (HbA _{1c}) (%)	Insulin (μ IU/mL)	Mean number of pancreatic β -cells in histology
Normal	6.28 \pm 0.13	5.11 \pm 0.43	10.5 \pm 0.22
n-STZ control	9.20 \pm 0.10 ^{###}	1.06 \pm 0.06 ^{###}	4.67 \pm 0.56 ^{###}
IND01 (100)	6.48 \pm 0.45 ^{**}	3.59 \pm 0.75 ^{**}	8.33 \pm 0.21 ^{***}
Glyburide (10)	5.32 \pm 1.27 ^{***}	3.91 \pm 0.81 ^{**}	4.33 \pm 0.21 ^{ns}

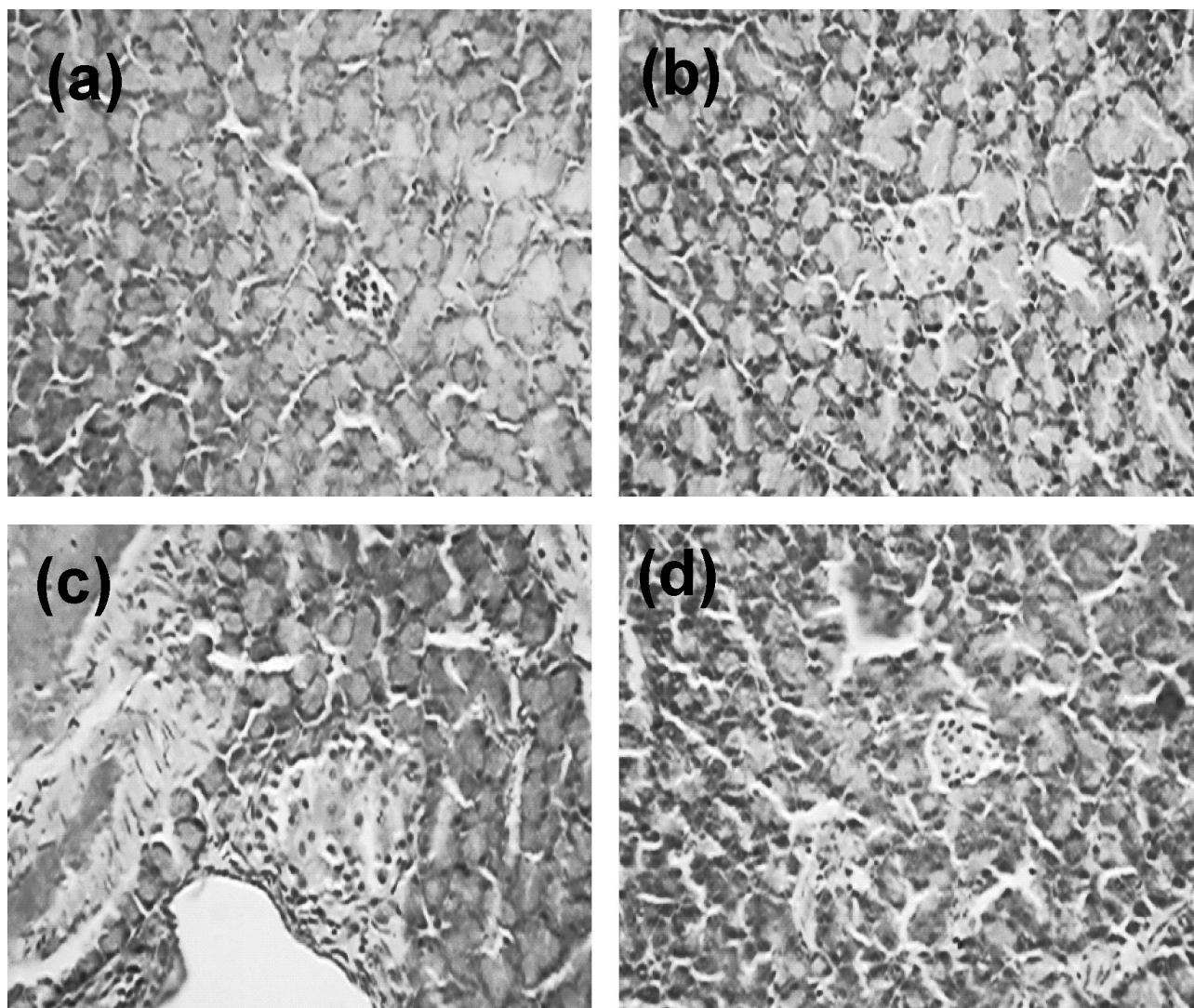
Number of rats per group = 6; data on each parameter were separately analyzed by one-way ANOVA followed by Dunnett's test; ^{##} $P<0.05$, ^{###} $P<0.001$ as compared with normal group; ^{**} $P<0.01$, ^{***} $P<0.001$; ns = nonsignificant as compared with vehicle treated group.

levels of serum insulin (1.06 $\mu\text{IU/mL}$, 79.25% reduction) at the end of the 28-day study period. Daily oral administration of IND01 and glyburide in n-STZ rats showed serum insulin levels of 3.59 $\mu\text{IU/mL}$ (29.74% reduction) and 3.91 $\mu\text{IU/mL}$ (23.48% reduction) as compared with normal (nondiabetic levels). After 28-day treatment with IND01 and glyburide, serum insulin levels were significantly ($P < 0.01$) higher than that of n-STZ control rats (1.06 $\mu\text{IU/mL}$).

Histological studies of n-STZ diabetic rat pancreas

Histological examinations of the rat pancreas sections under study are presented in Fig. 2. The section of the pancreas of normal rats showed a mean number of 10.5 islet β -cells. The rats from n-STZ control group showed a significantly ($P < 0.001$) reduced mean β -cell number of 4.67 cells. Moreover, the β -cells were smaller in size and markedly

Figure 2. Photomicrograph from pancreatic sections of (a) normal rats showing dense β -islet cells of Langerhans with well preserved cytoplasm and nucleus; (b) neonatal streptozotocin (n-STZ) intoxicated rats showing loss of cell integrity and islet mass, damaged islets, and acini degradation; (c) IND01 (100 mg/kg, oral) treated rats showing normal tissue architecture with mild damage; and (d) glyburide (10 mg/kg, oral) treated rats showing damaged islet mass, loss of cell integrity and islet mass, and damaged islets. (H&E, X40)



degenerated with necrosis of pancreatic islets. Pancreatic β -cells from IND01 treated group (mean = 8.33 cells) were larger in size (less damage) and significantly greater in number (8.33 cells, $P < 0.001$) as compared with those of n-STZ control group. Pancreatic β -cells of rats in glyburide treated group showed smaller sized islets (mean number = 4.33 cells), which indicated inability to protect the islets from progressive damage due to n-STZ during the 28-day study period.

DISCUSSION

Alloxan and STZ are widely used to induce experimental diabetes in animals. The mechanism of their action in β -cells of the pancreas has been intensively investigated and now is quite well understood. IND01 has been reported to have antidiabetic action against alloxan (20) and STZ (26) induced DM in rats. However, it remains to elucidate whether IND01 has potential towards progressive deterioration of glycemic functions and pancreatic β -cell functions in diabetic animals.

The cytotoxic action of both these diabetogenic agents is mediated by the reactive oxygen species, however, the source of their generation is different in case of alloxan and STZ. Alloxan and the product of its reduction, dialuric acid, establish a redox cycle with the formation of superoxide radicals formed by Fenton reaction with massive increase in cytosolic calcium concentration, causing rapid destruction of β -cells. On the other hand, STZ causes β -cell destruction by necrosis. STZ enters β -cell *via* a glucose transporter (GLUT2) and causes alkylation of DNA, enhanced ATP dephosphorylation to form superoxide radicals. Furthermore, STZ liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage. Fenugreek seed extract is known to induce a rapid, dose-dependent stimulatory effect on cellular glucose uptake by activating cellular responses that lead to GLUT4 translocation to the cell surface (27). Therefore, we selected n-STZ model of DM over n-alloxan induced model for the present study. Moreover, n-STZ model has been well studied, validated and reviewed in the past (17).

The insidious process of DM such as hyperglycemia development, glucose intolerance, and mild hypoinsulinemia in n-STZ induced rats is asymptomatic at the beginning and becomes manifested in adult age and so resembles the natural course of DM in humans. Herein, we showed for the first time that IND01 significantly attenuated hyperglycemia and improved glucose homeostasis (decreased HbA1c, and increased serum insulin levels and pancreatic β -cell functions) in n-STZ model.

The split-dose schedule of n-STZ administration used in the present study offers the following advantages (25): 1) reduction in mortality, 2) less number of animals required (90% of animals develop desired level of hyperglycemia, and 3) it is economical (lower amount of STZ is needed). This regimen probably leads to β -cell damage in a graded fashion, sparing some beta cell mass, and allows time for regeneration in-between the two injections. Enhancement of glucose uptake into the skeletal muscle, changes in transporters, alterations in the sensitivity of peripheral tissues and oxidative stress are other possible mechanisms responsible for hyperglycemia shown by n-STZ rats (28).

The administration of STZ to neonatal rats (n-STZ control) showed sustained unaltered basal hyperglycemia, glucose intolerance, raised HbA1c, and strong reduction of insulin levels (Tables 1-4). These results are in line with earlier reports of n-STZ model in Wistar rats (17,29). IND01 (100 mg/kg) and glyburide in n-STZ induced rats showed peak reduction in SG at 6 h of acute administration with the onset at 2 h (Table 1). The subacute study indicated a period of 7 days to be required for the reduction of SG in the blood to show sustained antihyperglycemic effects in n-STZ rats during the study period of 28 days (Table 2). Glyburide is a potent, second generation oral sulfonylurea antidiabetic agent used to control SG levels in patients with DM. The antihyperglycemic effects and increased insulin levels observed after glyburide (Tables 2 and 4) are in line with its known action of stimulation of pancreatic islets cells and also validates the n-STZ as a model of DM.

In the present study, rats in n-STZ control group

showed significant and progressive reduction in body weight, which is a symptom of DM (Table 3). Progressive reduction of body weight occurs due to protein waste and negative nitrogen balance. The reduction in body weight induced by n-STZ was reversed by IND01 and glyburide during the study (Table 3). The beneficial effects appear to be due to antihyperglycemic and prevention of muscle wasting effects of IND01 and glyburide.

In the present study, subacute treatment with IND01 (28 days) resulted in increased levels of serum insulin (3.59 μ IU/mL) as compared to n-STZ control (1.06 μ IU/mL) (Table 4). In the past, fenugreek seeds showed stimulation of insulin signaling pathways *in vitro* (adipocytes and liver cells) (27) and in erythrocytes (DM patients) (30). IND01 perhaps stimulates insulin release from the pancreas to improve peripheral glucose utilization, which reduced SG levels observed in the present study.

The insulinotropic action of IND01 can be attributed to 4-HI, a major amino acid component of fenugreek seeds (31). 4-HI has been reported to possess an interesting glucose-dependent insulin-releasing activity *in vitro* during static incubations of isolated islets, pancreas in rats as well as human islets through pancreatic and extra pancreatic mechanisms (32). Furthermore, acute *in vivo* administration of 4-HI improved glucose-stimulated insulin secretion in normal rat and dog and showed improvement in insulin secretion and/or glucose tolerance, and reduced hyperglycemia in a moderate type 2 diabetic rat model (33). Our results from subacute administration in a progressive model of type 2 DM (n-STZ model) are in agreement with the reported acute effects. The administration of 4-HI resulted in biphasic and glucose dependent pattern of insulin secretion that occurs in the absence of any change in pancreatic α - and δ -cell activity and without interaction with other agonists of insulin secretion (such as leucine, arginine, tolbutamide, glyceraldehydes) (34). 4-HI is able to interact and induce additive insulinotropic effects only in the presence of supranormal glucose concentrations (34) and so is not expected to have the side effect of hypoglycemia shown by synthetic oral antihyperglycemic agents.

In DM, there is an increased glycation of a number of proteins including hemoglobin. Hemoglobin is highly susceptible to nonenzymatic glycation. In diabetic condition, the excess of glucose present in the blood reacts with hemoglobin to form glycated hemoglobin (HbA1c), which has altered affinity for oxygen and this may be a factor in tissue anoxia. Measurement of HbA1c is an effective means to screen for glycemic control in DM (35).

In the present study, HbA1c levels were measured on day 28 (Table 4). IND01 showed improved glycemic control (significant decrease in HbA1c) on day 28 of treatment as compared with n-STZ control rats. Fenugreek seeds have been reported to improve glycemic control and decrease glycated hemoglobin (HbA1c) status in DM patients (7). Our results confirm effective glycemic control in n-STZ model by IND01 and are in line with earlier reports. These results further explain the correlation between effective glycemic control and insulin stimulation. Glucose uptake by target tissues of insulin is the rate limiting step in hyperglycemic conditions and it is facilitated mostly by translocation of glucose transporters from an intracellular site to plasma membrane (36).

In recent years, a line of diabetes research has focused on the development of new strategies addressed to recover β -cell function and mass arising from our increasing knowledge on adult pancreas plasticity (37). The n-STZ rats (10- to 16-week-old) showed complete loss of β -cell sensitivity to glucose (38). Adult n-STZ rats are characterized by low insulin release *in vivo* in response to glucose (39). In the present study, the mean number of pancreatic β -cells in n-STZ rats significantly decreased as compared with normal rats, whereas IND01 (but not glyburide) treated rats significantly reversed n-STZ-induced depletion of pancreatic β -cell mass, indicating β -cell protection or regeneration potential of IND01 (Fig. 2). The β -cell mass restoration property of IND01 can be attributed to one of the major amino acids, 4-HI. In the past, 4-HI was reported to have pancreatic β -cell regenerative potential *in vitro* in pancreatic ductal stem cell culture model (24). The presence of more pancreatic β -cells in IND01 treated rats than in n-STZ

control rats provided *in vivo* evidence for pancreatic β -cell regenerative potential of IND01.

In conclusion, standardized hydroalcoholic extract of *Trigonella foenum-graecum* seeds showed reversal of symptoms and effective glycaemic control in progressive model of type 2 DM (n-STZ) in rats by improving glucose homeostasis probably through insulinotropic properties.

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