Regenerative potential of pancreata in alloxan induced diabetic mice by 4-Hydroxyisoleucine, comparision with pioglitazone

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Abstract
4-Hydroxyisoleucine (4HI) isolated from seeds of fenugreek produced antihyperglycaemic effects in diabetic mice. However, its role in pancreatic regeneration is not well understood. The objective of the present study was to evaluate the antihyperglycaemic, pancreatic regenerative potential of 4HI and compare it with pioglitazone. Alloxan (80 mg/kg, i.v.) induced diabetic mice were fed orally with 4HI (40 mg/kg) once a day for consecutive 28 days with or without pioglitazone (Pgz, 10 mg/kg). Serum glucose levels and body weight were measured every 7 days whereas on 35th day; pancreatic, serum insulin levels and glycosylated haemoglobin levels were estimated. On 35th day mice were sacrificed, pancreas removed and histological studies were done. Potential for islet neogenesis and adipogenesis of 4HI and Pgz were also observed against in pancreatic ductal stem cell culture in vitro. 4HI treated group showed gradual decrease in serum glucose levels and attended normoglycemia at the end of treatment. Histopathological picture of the pancreas in 4HI treated mice exhibited presence of tiny islets indicating islet neogenesis from pancreatic duct/ progenital cells. This observation was further strengthened by in vitro islet neogenesis and adipogenesis obtained after treating the pancreatic ductal stem cells with 4HI. The higher levels of pancreatic and serum insulin observed in 4HI treated animals further supports the regenerative potential of 4HI. Glycosylated haemoglobin levels were significantly reduced by 4HI. Thus, the present data demonstrates for the first time the pancreatic regenerative potential of 4HI and also provides evidence for its known antihyperglycaemic activity compared to untreated diabetic mice which showed uncontrolled hyperglycaemia.

Keywords: 4-hydroxyisoleucine. Antihyperglycaemia. Glycosylated haemoglobin. Pancreas regeneration.

INTRODUCTION
Diabetes mellitus is a group of syndromes characterized by hyperglycaemia, altered metabolism of lipids, carbohydrates and protein; and an increased risk of complication from various diseases (Davis et al., 1996). Plants have been used for the treatment of diabetes in developing countries where the cost of the conventional medicines represents a burden to the population (Zheng et al., 2007). In search of new compounds from natural sources, the insulin like and insulin releasing effect of the isolated compounds such as 4- hydroxyisoleucine have been evaluated (Broca et al., 2004). 4-hydroxyisoleucine (called as 4-HI) is an original amino acids, especially found in Trigonella species (Broca et al., 2000). 4-HI is an amino acid isolated from the seeds of Fenugreek (Trigonella foenum graecum L.), a plant traditionally used to treat diabetes (Broca et al., 2004). The aims and objectives of the present study was to investigate the regenerative potential of pancreatic by 4-HI with pioglitazone (called as Pgz) in vivo and in vitro.

MATERIALS AND METHODS
In Vivo Studies
Collection and authentication of plant
The seeds of Trigonella foenum graecum (L.) were purchased from local market in Indore, Madhya Pradesh, India and authenticated at Agharkar Research Institute, Pune, India. The voucher specimen was deposited at that Institute.

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Drugs and chemicals
Alloxan monohydrate (Spectrochem, India), glucose estimation kit (Accurex Biomedical Pvt. Ltd., India), D-glucose (S.D. Fine-Chem. Ltd, India), pioglitazone (Alembic Ltd. India), n-hexan and ethanol (GR grade) (Merck, India) were purchased from the respective vendors. Pancreatic and serum insulin was measured by ELISA method using the Medgenix – Ins - EASIA kit was purchased from Biosource, Europe S.A.

(2S,3R,4S)-2-amino-4-hydroxy-3-methylpentanoic acid
2S, 3R, 4S-4-hydroxyisoleucine

Figure 1: Structure of 4-hydroxyisoleucine

Experimental animals and research protocol approval
Swiss albino mice (25-30 g) of either sex were purchased from National Toxicology Centre, Pune, India. They were maintained at a temperature of 25±1°C and relative humidity of 45 to 55% under 12-h light:12-h dark cycle. The animals had free access to standard food pellets (Chakan Oil Mills, Pune, India) and water was available ad libitum.

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Isolation of 4-hydroxyisoleucine from fenugreek seeds
100 g of fenugreek seeds were defatted using 300 ml n-hexane and extracted by aqueous alcohol (70:30, ethanol: water) 500 ml at room temperature for 8h of circulations. The extract was filtered and concentrated under vacuum to yield 9 g of crude. The crude was dissolved in 500 ml deionized water and passed through strong acid cation exchange resin (100 ml) (T-42 M.P from Thermax, India). The resin bed was washed free of colours. The adsorbed compounds were eluted and showed presence of amino acid major 4HI and Trigonelline (Tri) with minor saponins. The solution was concentrated at 45 °C in Buchii Rota evaporator to give 900 mg of mixture. This mixture was dissolved in 50 ml of hot isopropyl alcohol followed by hydrochloric acid gas saturation in the solution at 35 °C. On cooling to -5 °C, trigonelline hydrochloride was separated and filtered. The clear filtrate containing 4HI as hydrochloride was passed through strong base anion resin (Tulsion A-13, Thermax, India) to bind acid. The free amino acid solution was concentrated under vacuum at 40 °C to a paste (590mg). The paste was dissolved in water and the clear filtrate was passed through amberlite XAD-16 (non polar adsorbant from Rohm and HAAS) to remove all adhering saponins and the clear column remanant was freeze dried to get 4HI (425 mg).

Acute oral toxicity study
Healthy adult Swiss albino mice of either sex were subjected to acute toxicity studies as per guidelines (AOT No. 425) suggested by Organization for Economic Co-operation and Development (OECD, 2001).

Induction of experimental diabetes
Diabetes was induced in Swiss albino mice by a single intravenous injection of aqueous alloxan monohydrate (80 mg/kg, i.v.) solution. After 48h, the animals showing serum glucose level above 300 mg/dl (diabetic) were selected for the study.

Experimental design
The non-fasted diabetic mice were divided into five groups (n = 6) viz; Group I - Control (non diabetic), Group II - Only alloxan (80mg/kg, i.v), Group III- Pgz ) (10mg/kg), Group IV– 4HI (40 mg/kg), Group V - 4HI (40mg/kg)+Pgz (10mg/kg). All drugs were administered orally. Acute study involved
determination of serum glucose level at 0, 2nd, 4th, 6th and 24th h after 4HI, Pgz alone and their concomitant administration. Subacute study involved repeated administration of 4HI, Pgz and their concomitant administration for 28 days (once a day) at predetermined time and serum glucose levels were determined in samples withdrawn after 2nd h of 4HI, Pgz and their concomitant administration on 7th, 14th, 21st and 28th day. At the end of 28 days the 4HI, Pgz and their concomitant administration were stopped and a rest period of 7 days was given to the animals. Serum glucose level and the regenerative ability of pancreas was determined on 35th day. The data was represented as mean serum glucose level ± standard error of mean (S.E.M.).

**Determination of serum glucose level, pancreatic and serum insulin and glycosylated haemoglobin (HbA1c) level**

Serum glucose level was determined by glucose oxidase peroxidase method. At the end of 7 days rest period to the animals, the pancreas was isolated from the mice and homogenized in ice cold acid: ethanol (1:4) and then centrifuged for 30 min at 12,000 r.p.m. at 4°C (Sitawad et al., 2000). Pancreatic and serum insulin was assayed by the Medgenix – Ins - EASIA kit. Glycosylated haemoglobin was measured by ion exchange chromatography (Chandalia et al., 2002).

**Effect of body weight**

During the study period of 35 days, the mice were weighed daily and their body weights were recorded. From this data, mean change in body weight and S.E.M. were calculated.

**Histology of pancreas**

After seven days rest period, pancreas were isolated and used for histological examinations using haematoxylin & eosin staining.

**In Vitro study**

**Preparation of pancreatic ductal stem cell culture**

Pancreatic ductal cultures were prepared by previously reported Katdare et al., (2004). Pancreas was aseptically removed and subjected to collagenase digestion (1 mg/ml, Sigma) for 20 min at 37 degrees C. From the digestion mixture the pancreatic ductal pieces were hand picked and seeded onto collagen coated petri dishes and incubated at 37 °C in serum free nutrient medium but without keratinocyte growth factor (KGF). The monolayer of epithelial cells obtained was treated with different concentrations (100 - 800μl of 5mM concentration) of 4HI for 24 to 48 h. The drug was removed and the monolayer was further observed for one week for presence of islet like cell clusters.

**Statistical analysis**

Data was expressed as mean ± S.E.M. Statistical analysis was carried out by One-way ANOVA with post hoc Tukey’s test performed using GraphPad InStat version 3.00 for Windows VistaTM BASIC, GraphPad Software, San Diego California USA. P value was considered significant when <0.05.

**RESULTS**

**In Vivo Studies**

**Acute oral toxicity studies**

Acute toxicity studies revealed that the 4-HI was safe up to a dose level of 5000 mg/kg of body weight. No lethality or any toxic reactions were observed up to the end of the study period.

**Effect of 4HI, Pgz alone and their concomitant administration on serum glucose level in diabetic mice**

Single dose administration of 4HI (40 mg/kg), Pgz (10 mg/kg) alone significantly (p<0.01) reduced serum glucose level at 2nd and 6th h after administration respectively. The onset and peak antihyperglycaemic effect of 4HI was observed at 6th h but the antihyperglycaemic effect waned at 24th h. The onset antihyperglycaemic effect of Pgz was observed at 2nd h; peak effect at 6th h and the effect waned at 24th h. The onset and peak antihyperglycaemic effect of concomitant administration of 4HI (40 mg/kg) with Pgz (10 mg/kg) was observed at 6th h but the antihyperglycaemic effect waned at 24th h. The maximum reduction in serum glucose level from basal value (before) at 6th h after 4HI, Pgz and their concomitant administration were 318.81, 211.56 and 248.97 mg/dl respectively (Table 1 [Supplementary data]).

In the subacute study, repeated administration (once a day for 28 days) of 4HI, Pgz and their concomitant administration caused significant (P<0.01) reduction in the serum glucose level as compared to vehicle treated group. The reduction of serum glucose level was sustained on day 35th i.e. after 7 days rest period. The reduction in serum glucose level of 4HI, Pgz alone and their concomitant administration were 365.96, 335.09 and 302.83 mg/dl respectively on the 35th day (Table 1).

**Determination of Serum Glucose Level, pancreatic and serum insulin, and Glycosylated haemoglobin (HbA1c)**

In the present investigation pancreatic as well as serum insulin levels in alloxan induced diabetic mice significantly (P< 0.001) decreased compared to non-diabetic mice. 4HI treated group showed significant
(P< 0.001) increase in the pancreatic as well as serum insulin levels compared to vehicle treated group. Pgz treatment significantly (P< 0.01) increased pancreatic insulin level but did not significant decrease serum insulin level (Table 2 [Supplementary data]). The HbA1c value of non-diabetic mice was 6.28 g%. In alloxan induced diabetic mice the of glycosylated haemoglobin increased to 10.56g%. In 4HI treated animals the value of glycosylated haemoglobin was 6.71 g% (Table 2).

Effect on Body weight

Body weight of vehicle treated diabetic mice decreased during study period. 4HI, Pgz and their concomitant administration significantly (P< 0.01) prevented the decrease in body weight of diabetic mice. Indeed, an increase in body weight was seen which indicated a beneficial effect of the 4HI, Pgz and their concomitant administration (Fig. 2).

Histology of pancreas

The pancreatic sections of normal animals (A) showed normal population of islets in the vicinity of the duct. The diabetic animals (B) showed occasional islets which were negligible in the vicinity of the duct. The pancreatic sections of Pgz treated animals (C) showed few small islets in the proximity of the duct. 4HI treated pancreatic sections (D) showed many islets in the proximity of the duct whereas 4HI + Pgz treated group (E) showed few ducts and many small islets. Islets obtained from regenerating pancreas were small in size but many in number. Occurrence of large number of small islets indicated pancreatic regeneration (Fig. 3).

In Vitro study

Islet neogenesis from pancreatic ductal stem cell culture

The monolayer of epithelial cells was obtained from non-diabetic pancreatic duct cell cultures (Fig. 4A). Addition of 4HI resulted into formation of small islet like clusters in a concentration dependent manner maximum being obtained at the dose of 800 ul (Fig. 4B). The identity of islet like cluster was further confirmed by dithiazone (DTZ) staining which is specific for islets (Fig. 4C). In addition to the islet neogenesis 4HI treatment also led to the formation of adipocytes which were confirmed by Oil Red O staining (Fig. 4D).

DISCUSSION

In the present study, 4HI an amino acid extracted and purified from Trigonella foenum graecum (fenugreek) seeds is reported to have antihyperglycemic activity. This amino acid is not found in mammalian tissues and present in some plants. In the experiment performed on isolated ex vivo glucose perfused rat pancreas, 4HI was reported to increase glucose-induced insulin release (ranging from 100 μmol/l to 1 mmol/l) through a direct effect on the isolated islets of Langerhans in both rats and humans (Sauvaire et al., 1998). This pattern of insulin secretion was biphasic, glucose dependant, occurred in the absence of any change in pancreatic alpha and delta cells activity and without interaction with other agonists of insulin secretion (such as leucine,
arginine, tolbutamide, glyceraldehydes) (Sauvaire et al., 1998). The present study differs from that of Broca et al., (2004) with respect to animal species and the parameters viz; serum and pancreatic insulin levels, histology of pancreas and glycosylated haemoglobin levels and its concomitant administration with oral insulin sensitizer (Pgz). In the present study, mice pretreated with either 4HI or Pgz for 28 days showed significant reduction in serum glucose levels when given alone (Table 1). Further, subacute treatment of 4HI showed significant increase in pancreatic insulin levels (Table 2). It appears that insulin secreted by newly formed β cells in the pancreas might have contributed to the antihyperglycaemic effects shown by 4HI. These results are further substantiated by the histology of pancreas which showed many more islets in the proximity of the duct after sub acute treatment of 4HI, Pgz and their concomitant administration. The tiny islets represent the newly formed islets (Banerjee et al., 2005). Since the newly generated islets are not responsive to glucose, 4HI did not show significant reduction in serum glucose levels at 7th or 14th day (Table 1).

In the present investigation, it was observed that a latency period of about two weeks is required before the antihyperglycaemic effects of 4HI or its concomitant administration with Pgz. This period may be required for the formation of new pancreatic β cells and to become viable and secrete insulin. A rest period of seven days after treating the animals for 28 days was therefore included in the study to assess the control of serum glucose levels. This notion is supported by our results of sustained antihyperglycaemic effects by 4HI, Pgz or concomitant administration on after 7 days of withdrawal of drugs on 28th day (Fig. 4).

Pioglitazone is a member of thiazolidinediones derivatives (TZDs). TZDs appears to improve sensitivity to insulin in liver, muscle and adipose tissue but do not directly stimulate insulin secretion (Gumieniczek, 2003). They decrease glucose levels while simultaneously reducing circulating insulin and free fatty acids (Chakrabartik et al., 2004). The ability of 4-HI, pioglitazone alone and their concomitant administration to protect against body weight loss seems to be due to its ability to reduce hyperglycaemia (Gumieniczek, 2003).

Glycosylated haemoglobin (HbA1c) test indicates the average blood glucose concentration over the past three months and is being suggested as screening tool for progression of diabetes and increased risk of heart disease and overall mortality (Khaw et al., 2001). In our study, the glycosylated haemoglobin levels were measured on 28th day. Significant reduction in glycosylated haemoglobin levels by 4HI suggests regeneration of pancreatic β cells as primary

Figure 4: Photomicrograph of islet cell neogenesis
(A) Ductal epithelial monolayer (B) Formation of small islet like cluster (C) islets with DTZ staining (D) formation of Adipogenesis stained with Oil red O.
mechanism of action of 4HI. Our results of histopathology of pancreas (Fig. 4) and presence of islet cells in vitro study (Fig. 4) further supports pancreatic regenerating potential of 4HI. Moreover, the 4HI treatment also resulted in adipogenesis (Fig. 4D) and therefore probably account for the reversal of weight reduction of diabetic mice. The role of 4HI in adipogenesis may be beneficial in reducing the insulin resistance in type 2 diabetes as the newly formed adipocytes are sensitive to glucose uptake.

**CONCLUSION**

Our findings demonstrate that 4HI (an active principle from *Trigonella foenum graecum* seeds) has antihyperglycaemic activity per se and with Pgz. It further prevents weight loss (probably by adipogenesis) in diabetic mice. Pancreatic regenerative potential and adipogenesis is suggested to be mechanism of antihyperglycaemic effect of 4HI as evidenced by histopathological and in vitro islet neogenesis of pancreatic stem cells culture studies. With these unique features, 4HI can be attractive candidate for treating diabetes.

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**References**


