Immunohistochemical Evidence of Pancreatic β-cell Regeneration in streptozotocin-induced type 2 Diabetic Rats treated with Gymnema sylvestre Extract

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Abstract

The effective dose Gymnema sylvestre (GS) leaves methanolic extract (400 mg/kg) was administered orally to neonatally streptozotocin-induced non-obese type 2 diabetic rats for 28 days and fasting glucose, serum insulin and β-cell function were measured after the treatment periods. Pancreatic β- and α-cells were identified by immunohistochemistry and β-cells number was evaluated by morphometric analysis. GS extract showed anti-diabetic activity through improving serum insulin and β-cell function. The β-cells number was increased significantly in the GS-treated diabetic rats. The immunohistochemical data revealed presence of newly formed insulin positive β-cells in the GS-treated diabetic islets. This is the direct immunohistochemical evidence of β-cells regeneration and/or neoformation in pancreatic islets by GS extract. Cluster of β-cells without any α-cells were appeared in GS-treated diabetic rats, not in STZ-induced diabetic control rats, suggesting β-cells regeneration in GS-treated pancreas rather than stem-cell induced growth.

Keywords: Gymnema sylvestre; β-cell function; β-cell regeneration; Immunohistochemistry

Abbreviations: GS: Gymnema Sylvestre; STZ: Streptozotocin; DAPI: 4',6-Diamidino-2-Phenylindole; Db: Diabetic; GB: Glibenclamide

Introduction

Gymnema sylvestre (GS) has been used for the treatment of diabetes in Indo-Pak region for centuries. GS extract has been reported to have anti-diabetic effects in different animal models of diabetes by increasing serum insulin levels and attenuating blood glucose responses during oral glucose tolerance tests [1-13]. The mechanism of anti-diabetic effect of GS extract has been explored in some extent and β-cells regeneration was postulated one of the possible mechanisms. However, these studies lack the direct evidence of pancreatic β-cells regeneration. The present study was designed to explore pancreatic β-cells regeneration by GS extract in streptozotocin-induced non obese type 2 diabetic rats by immunohistochemical method for triple staining of insulin, glucagon and nuclei along with morphometric analysis.

Methods

Preparation of extract

GS leaves (2 kg) were purchased from a LaluKhet market of Karachi, Pakistan. Leaves were authenticated by a taxonomist, University of Karachi and voucher specimen (No. 86478) was deposited in Karachi University Herbarium, Pakistan. The powder of the leaves was soaked into aqueous 80% methanol for 72 h at room temperature 2 times. Pooled extracts were filtered, combined and evaporated to dryness under vacuum by using a rotary evaporator. Finally, the crude extract was freeze dried to give the experimental extract (140.5 gm). The extract was dissolved in water to make suspension and given to experimental rats.

HPLC analysis of GS extract

Standardization of methanolic extract of GS was performed by RP-HPLC (Agilent ChemStation 261100, USA) system comprising of binary pump with degasser, auto sampler with column oven, and DAD detector. Data acquisition and integration was controlled by ChemStation software. A Merck C-18 (Hiber 250 × 4.6 Purosphere STAR RP-18e (5 μm)) column was used. The mobile phase was composed of A (water: acetic acid, 98: 2) and B (methanol) in the gradient mode so that the concentrations of mobile phase B became 0-100% in 15 minutes. Flow rate was 1.0 ml/min throughout the analysis. The samples and standard were injected through an auto sampler. The injection volumes of standard GA and methanolic extract of GS was 20 μl. Column oven temperature was maintained at 25°C. The detection wavelength of UV was 257 nm. A standard gymnemic acid (GA) solution was prepared by weighing 10 mg of standard in 5 ml of 50% (v/v) aqueous methanol. The solution of methanolic extract of GS was prepared by dissolving 1 g in a 100 ml volumetric flask and made up volume up to 100 ml with mobile phase. All samples were filtered with 0.45 μm Milllex-HN syringe driven filter unit (Millipore, Bedford, USA) and preserved at 4°C prior to analysis.

Development of non-obese type 2 diabetic rats

A freshly prepared streptozotocin (STZ) solution of 90 mg/kg in citrate buffer (pH 4.5) was injected intraperitoneally to 2-day-old Wistar pups to obtain non-obese type 2 diabetic rats. After three months of STZ induction, an oral glucose tolerance test was performed with 3 g/kg glucose. The rats having fasting blood glucose levels of 7.7–12.1 mmol/l at 0 min and showed the maximum rise at 45 min (12.7–19.5

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mmol/l) were included in this study. The use of experimental animals in this study was conducted under the guidance of the basic standards in the care and use of laboratory animals, which has been prepared and published by the National Institutes of Health. The study protocol has been approved by the Research Ethics committee at ICCBS, University of Karachi, Pakistan.

**Acute effect of GS on blood glucose**

An acute blood glucose lowering effects of GS extract was performed in type 2 diabetic rats to select the dosage for chronic study. Diabetic rats were fasted overnight and divided into 5 groups (7 rats/group). Gr 1 (Db), diabetic control rats received only water; Gr 2 (Db+GS-200), Gr 3 (Db+GS-400), and Gr 4 (Db+GS-600), diabetic rats received 200, 400 and 600 mg/kg GS extract, respectively. Gr 5 (Db+GB), diabetic rats received 5 mg/kg glibenclamide. The extract and glibenclamide were given by gavage needle to the diabetic rats. Control rats were given an equivalent volume of water. Blood glucose was measured at 0, 1, 2 and 3 h after oral 29 administration of GS extract.

**Chronic extract treatment, collection of blood samples and pancreas for biochemical and immunohistochemical analysis**

The GS extract (400 mg/kg/day) and glibenclamide (5 mg/kg) were given orally once daily by gavage needle to the diabetic rats. After 28 days of treatment, rats were sacrificed after giving anesthesia (sodium thiopental, 60 mg/kg) and their venous blood were collected. Blood samples were centrifuged and serum was separated within 30 min, aliquoted and kept at -80°C for biochemical assay. After collection of blood, pancreata were excised and tissue were fixed in buffered formalin, sectioned and processed for immunohistochemical analysis. Serum insulin was measured using rat ELISA kit (Crystal Chem Inc., IL, and USA). Pancreatic β-cell function (HOMA %B) was calculated from fasting glucose and fasting insulin by HOMA-CIGMA software [14]. Insulin and glucagon immunostaining and morphometry were performed as described previously [15,16].

**Statistical analysis**

Statistical analyses were performed by using the SPSS 12.0 statistical package for Windows (SPSS, Inc., Chicago, IL, USA). All values were expressed mean ± S.E.M. To compare data between and within group unpaired and paired t-tests (2-tailed) were performed, respectively. Differences were considered significant at p<0.05.

**Results and Discussion**

**Standardization of methanolic extract of GS**

GS was standardized with reverse phase liquid chromatography by using GA as a marker compound. Figure 1 shows chromatogram of GS extract. GS extract showed the presence of several peaks eluted at various retention times, 2.7, 3.3, and 4.6 min. The peak of GA appeared as a major peak at retention time of 2.7 min in the GS chromatogram. It was found that the concentration of GA was 10% (w/w). In this way the consistency of GS extract could be done by adjusting the concentration of the marker compound. The concentration of GA found in our HPLC analysis also in line with studies of Pandey and Yadav [17].

**Acute effect of GS on the blood glucose level in experimental diabetic rats**

The effects of oral administration of three different doses of the GS extract on blood glucose levels of STZ-induced type 2 diabetic rats are presented in Figure 2. Very little change in blood glucose levels were observed in water treated diabetic rats. In sharp contrast, oral administration of GS extract lowered blood glucose in a dose- and time-dependent manner. The blood glucose lowering effect of 200 mg/kg dose reached significance (p<0.01) at 3-h of GS treatment (18.9 ± 3.3% reduction). Interestingly, both 400 and 600 mg/kg doses significantly decreased the blood glucose levels at 2 h and 3 h. A maximum decrease of 31.5% (p<0.01) was observed with 400 mg/kg of GS extract at 3 h. Hence, the dosage was selected as 400 mg/kg/day for chronic study.

**Chronic effect of GS extract on blood glucose, serum insulin and β-cell function**

There was a little change in fasting blood glucose in case of untreated diabetic rats during the experimental periods (Table 1). The blood glucose of the GS-treated diabetic rats significantly decreased compared with untreated diabetic rats (7.24 ± 0.33 vs. 11.06 ± 1.22 mmol/l, p<0.001). Standard drug glibenclamide also lowered the blood glucose level (5.75 ± 0.53 mmol/l) significantly (p<0.001) compared with untreated diabetic rats. Very little alteration of serum insulin was observed in the untreated diabetic rats during the experimental periods.
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to direct regeneration and/or neoformation or indirectly by stimulating
neoformation by GS extract. The generation of β-cells by GS may be due
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few newly formed β-cells in the pancreas with no surrounding α-cells
reduced in ratio to β-cells in GS-treated group. The GS treatment showed
sparkling staining of insulin positive β-cells (Figure 3B). The α-cells are
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Immunohistochemical and morphometric studies on the
pancreas
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the NGN 3.1, Mafa, PDX-1/4 or other proliferating factor, that stimulate
β-cell regeneration. The intense staining of nuclei reflecting nuclear
activity of non-islet pancreas generate another hypothesis that newly
formed β-cells mass without surrounding α-cells is generated by non-
islet cells of pancreas. The morphometric data showed that the β-cells
number in the islets of the GS-treated rats was significantly higher than
that of the untreated diabetic rats (Figure 3C). This further suggests that
GS may have a role in the regeneration or revitalization of the β-cells or
in the recovery of the partially damaged β-cells. Our data suggest that
GS extract treatment produced regeneration in STZ-induced diabetic
pancreas rather than embryonic like stem cell differentiation to β-cells.
If the β-cells growth is due to very small embryonic like stem cells then it
should appear in STZ-induced diabetic control rats as well. Cluster of
only β-cells were appeared in GS-treated pancreas, not in STZ-induced
diabetic control pancreas, suggesting that β-cells regeneration in GS-
treated pancreas and this regeneration is not due to stem-cell induced
growth. Additionally, if the stem cell induced growth occurred in GS-
treated pancreas, then regeneration should result in both β-cells and
α-cells. However, in our study we found that cluster of β-cells without
any α-cells in GS-treated pancreas (Figure 3B). These findings further
confirmed the regeneration of β-cells in GS-treated pancreas and
exclude the possibility of very small embryonic like stem cells induced
growth of β-cells.

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interest that could be perceived as prejudicing the impartiality of the
research reported.

Table 1: Effect of GS extract on fasting blood glucose, fasting serum insulin and β-cell function in diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fasting glucose (mmol/l)</th>
<th>Fasting serum insulin (pmol/l)</th>
<th>β-cell function (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic</td>
<td>9.65 ± 1.04</td>
<td>39.05 ± 2.25</td>
<td>24.8 ± 2.7</td>
</tr>
<tr>
<td>GS-treated</td>
<td>11.06 ± 1.22</td>
<td>34.87 ± 2.48</td>
<td>19.7 ± 3.9</td>
</tr>
<tr>
<td>GB-treated</td>
<td>12.45 ± 1.02</td>
<td>28.6 ± 2.7</td>
<td>108.5 ± 8.7</td>
</tr>
</tbody>
</table>

All values were expressed as mean ± SEM. Results represent the average from 6–8 rats with
an average of 60–100 fields per rat. *P<0.01 versus diabetic group.

Figure 3: Immunohistochemical (A, B) and morphometric (C) analyses on the pancreas in different experimental groups. Sections in rat pancreas stained immunohistochemistry for insulin-positive β-cells (red), glucagon-positive α-cells (green), and nuclei (blue) of pancreatic islets in (A) untreated diabetic; (B) GS-treat diabetic rats. Magnification: 20X. Morphometric analysis of β-cell number (C) in pancreatic islets of diabetic and GS-treated rats. All values are expressed as mean ± SEM. Results represent the average from 8–8 rats with
an average of 60–100 fields per rat. *P<0.01 versus diabetic group.
References