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Bitter-Gourd Attenuates Pre-Diabetes Induced Lens Abnormalities in Neonatal Streptizotocin Induced Rat Model

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ABSTRACT

The main purpose of our study is to evaluate the impact of bitter gourd on long term pre-diabetic induced lens abnormalities caused by neonatal streptozotocin (nSTZ). Male Sprague Dawley (SD) rat pups of two-day old were taken and injected with STZ (90 mg/kg body weight) dissolved in 0.1M citrate buffer, pH 4.5. Control pups received only vehicle. All rats were maintained on AIN 93G/M diet in individual cages and a subset of pre-diabetic animals received 5% bitter gourd in the diet. Majority of n-STZ injected rats developed impaired glucose tolerance (IGT) by 2 months and maintained PD state even 11 months. The eye of all groups of animals was examined after dilation of pupils using slit lamp microscope during the experimental period and all three group rat eye lens are clear and not developed any lenticular opacfications. A set of lenses was cultured in modified TC199 medium in the presence of 55mM glucose for a period of four days. Interestingly, untreated pre-diabetic rat lens developed early opacification when compared to control animal lens. PD rats shown increased oxidative stress and antioxidant enzymes evidenced by accumulated MDA and SOD in nSTZ-PD rat lens when compared to control rat lens. Bitter guard treated PD rats marginally reduce blood glucose, IGT along with opacification, polyol intermediates (AR, Sorbitol), oxidative stress marker (MDA) and antioxidant enzyme (SOD). In conclusion, nSTZ-PD rat model could aid to investigate IGT-associated lens abnormalities and bitter gourd considerably reduce PD lens abnormalities might be a hypoglycemic or antioxidant property.

Key words: *Pre-diabetes, Cataract, Bitter gourd, STZ.*

INTRODUCTION

Pre-diabetes, typically defined as blood glucose levels above normal but below diabetes thresholds, is a risk state that defines a high chance of developing diabetes. The prevalence of pre-diabetes is increasing worldwide and it is projected that more than 471 million people will have pre-diabetes in 2035[14]. Pre-diabetes is associated with the simultaneous presence of insulin resistance and β-cell dysfunction, abnormalities that start before glucose changes are detectable.

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Increases in glycaemia have resulted in a rise in pre-diabetes prevalence, although in some populations IGT has not raised despite increasing diabetes incidence, probably because increases in obesity influence FPG more than 2-h glucose and because of improved detection of diabetes[1]. As with pre-diabetic models provide a window opartunity to identifying individuals at risk of developing diabetes near future based on parameters available to the general practitioner. A categorization of persons as either 'normal' or 'prediabetic' (IFG, IGT) neglects the fact that diabetes risk significantly increased. Thus, in diabetes risk prediction, glycaemic measures (fasting and 2-h glucose) may perform better if treated as continuous traits rather than categorical variables[24,39]. Furthermore, there is some evidence to suggest that incorporating postload glucose into a model that already includes FPG improves prediction.

Catarcat is without doubt one of the main cause of blindness globally, associated with numerous risk factors such as diabetes, IGTand IFG or pre-diabetes[32]. Since many epidemiological studies had shown the association of cataract with IGT/pre-diabetes in aged population[11,16,7,26]. Cataract, characterized by the opacification of the eye lens that interferes with transmission of light onto the retina, is one of the earliest complications of diabetes. Cataract is the leading reason of visible impairment internationally and accounts for 47.8% of the blind humans on this planet[25]. Studies indicate that the incidence of cataract is much higher in diabetic than in nondiabetic individuals[6,29]. Though the etiology of cataract will not be absolutely understood.Oxidative damage to the constituents of the eye lens is considered to be a most important mechanism in the initiation and progression of quite a lot of varieties of cataracts, together with diabetic cataract[28]. Many reports indicate the role of oxidative stress within the development of diabetic complications including cataract due to glucose autoxidation, nonenzymatic glycation of proteins leading to advanced glycation end products (AGE), and enhanced glucose flux through polyol pathway[5,13,19]. More recent studies indicate that the polyol pathway is likely a main contributor to oxidative stress, at the least, within the lenses and nerves of diabetic mice and it was once pronounced that aldose reductase inhibitor reduces oxidative stress enhancement in sugar cataract. The polyol pathway may be involving hyperglycemia brought on oxidative stress, and there may be a metabolic connection between the polyol pathway and oxidative stress.

Herbal food supplements/ alternative therapies or natural/plant based approach are being used by the humans to deal with more than a few metabolic issues from the previous centuries. In recent years, these treatment options have more popular in addition to the routine pharmacological interventions in diabetic subjects[20,9,21,4] to manage hyperglycaemia[9,21], especially subjects with blood glucose levels at borderline where treatment has not yet started. Momordica charantia is commonly known as bitter gourd, which is widely consumed as a vegetable all over the world including India. Consumption of bitter gourd has been associated with variety of health-promoting benefits, which include hypoglycaemic property both in type-1 and type-2 diabetic patients[23]. Most reports, except a few have demonstrated BG beneficial effects[17,10,23,27]. Earlier study had reported that consumption of bitter gourd juice increases glucose uptake by tissues in vitro[48]. Moreover, it also increases glycogen storage in the liver, stimulates secretion of insulin by isolated beta cells of pancreas[37]. and also reduces systemic arterial blood pressure[27]. The main purpose of this study is to assess the beneficial effects of freeze dried bitter gourd in vivo against pre-diabetes induced lens abnormalities using nSTZ model which we developed earlier[22,33].
Even though, in this nSTZ-induced PD rat model has been more compatible to gain knowledge of long term pre-diabetes induced lens abnormalities and also focus effect of BG on pre-diabetes induced lens complications. These kind of models have now not been validated through any anti-diabetic or relevant

drugs which reduced the risk at pre-diabetes stage.

MATERIAL AND METHODS

Forty five male Sprague-Dawley rat pups weighing 8-9 g (obtained from the National Center for Laboratory Animal Sciences, National Institute of Nutrition Hyderabad, India) were used in this study.

Dietary ingredients

Cellulose, AIN 95G/M vitamin and mineral mixtures were obtained from MP Biomedicals (USA). All other dietary ingredients (Casein, Corn starch, Sucrose, Groundnut oil, fiber, L-Cystine, Choline, Tert-butylhydroquinone) required for AIN 93 dietary preparation, were obtained from the National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition, Hyderabad.

Preparation of Bitter Gourd (BG) Powder

Fresh BG was obtained from the local market and lyophilised. The lyophilised BG was pulverized to powder form and added to an AIN-93 diet (Reeves *et al* 1993), which was fed to the experimental animals.

Induction of PD and Experimental Groups

Two-day old male Sprague Dawley rat (SD) rat pups (n = 35) obtained from the National Center for Laboratory Animal Science (NCLAS), National Institute of Nutrition, Hyderabad, India, were injected with a single intraperitoneal injection of STZ dissolved in 0.1M citrate buffer (pH 4.5) at a dose of 90 mg/kg body weight. Control pups (n = 9) received only vehicle (citrate buffer). The pups were weaned after 21 days and maintained on AIN-93G/M diet in individual cages. After two months, nSTZ-injected rats having postprandial blood glucose levels between 140–199 mg/dL and/or fasting blood glucose levels between 110–125 mg/dL were considered as pre-diabetic (PD) and were further divided into two groups as shown in Fig. 2.1: Pre-diabetic untreated group (PD) and PD rats treated with Bitter gourd (PD+BG group). Control (n=9) and PD untreated (PD, n = 12) rats maintained on an AIN-93M diet and PD+BG group rats maintained on an AIN-93M diet containing 5% BG (PD+BG, n = 12). These animals were maintained in their respective diets and terminated after 10 months because some of the STZ injected rats developed diabetes.

Animal Care and Maintenance

All rats were maintained in temperature and humidity-controlled rooms, with 12:12-h light-dark cycles. All rats had free access to water. Regular food intake and monthly body weights of the experimental animals were monitored during experiment. All experimental procedures adhered to the guidelines of the Institutional Animal Ethics Committee (P29/IAEC/NIN/2012/7/PS/Rats-SD/Male-76) and followed the guidelines of ARVO for the use of animals in ophthalmic and vision research.

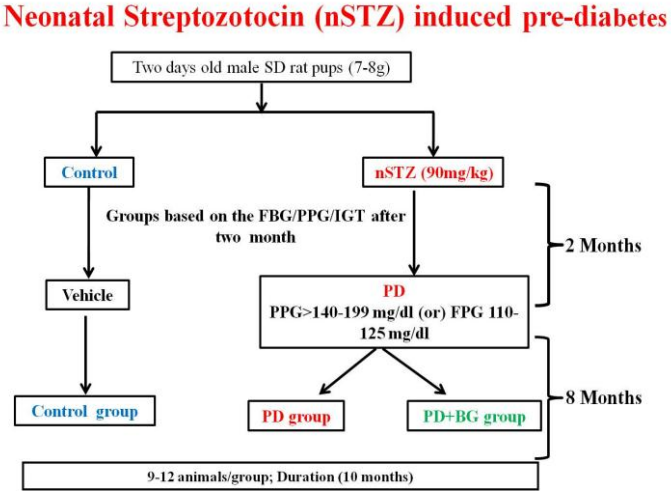

Figure 1: Experiment-1 design. SD, Sprague Dawley; nSTZ, neonatal-streptozotocin; FBG, fasting blood glucose; PPG, postprandial glucose; IGT, impaired glucose tolerance; FPG, fasting plasma glucose; PD, pre-diabetes; BG, bitter gourd.

Oral Glucose Tolerance Test

Oral glucose tolerance test (OGTT) was conducted at two and ten months after the STZ injection in overnight fasted rats by administering glucose orally as a bolus at a dose of 2.0 g kg^{-1} body weight. Blood samples were collected at 0, 30, 60 and 120-minute intervals to estimate plasma insulin and glucose concentrations to assess IFG, IGT and insulin resistance. Heparin tubes were used for collecting the blood samples and these samples were centrifuged at 4000rpm/15min at 4^0C and plasma was collected. Glucose, insulin, lipids and nephropathy parameters were estimated in these plasma samples as described below. During blood collection, care was taken not to damage the eyeball by passing the capillary from side to side.

Estimation of Plasma Glucose

Plasma glucose was estimated using the glucose oxidase (GOD)-peroxidase (POD) method with a kit obtained from Biosystems (Barcelona, Spain) according to the manufacturer's instructions.

Principle: The GOD-POD method involves oxidation of the GOD enzyme to D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in the presence of the POD enzyme oxidizes phenol, which reacts with 4-amino-antipyrene to produce a red-coloured quinonimine dye. The intensity of the colour produced is proportional to the concentration of glucose in the sample.

Method: Working enzyme reagent (1.0 mL) was added to a 0.01 mL of standard/sample, mixed gently and then absorbance was measured at 500 nm against a blank within 30 min.

Sample glucose concentrations were calculated by comparing optical density (OD) of the sample to those of the standard ODs and are represented as mg/dL.

Estimation of Plasma Insulin

Insulin was estimated in plasma samples using a radioimmunoassay (RIA) kit purchased from the Board of Radiation and Isotope Technology (BRIT), Bhabha Atomic Research Centre (BARC) Mumbai, India.

Principle: The radioimmunoassay method is based upon the competition between unlabelled insulin in a standard or sample and radio iodinated (^{125}I) insulin for limited binding sites on a specific antibody. After incubation, bound antibodies and free insulin are separated with a second antibody using a polyethylene glycol (PEG)-aided separation method. The concentration of insulin in the samples is quantified by measuring the radioactivity associated with the bound fraction of sample and standards.

Procedure: Assay buffer (0.3 mL) was added to the individual plasma samples (100 µL). For standards, 100 µL of different insulin concentrations (0, 12.5, 25, 50, 100 and 200 µU/mL) was added to insulin-free serum (100 µL, provided in the kit); assay buffer was then added for a final volume of 0.4 mL. To both samples and standards, 0.1 mL insulin antiserum was added, mixed gently and incubated overnight at 4°C. The next day, 0.1 mL ^{125}I insulin was added to all tubes and incubated for 3 h at room temperature. After incubation, 0.1 mL of second antibody was added, followed by 1.0 mL PEG, and gently mixed. All tubes were kept at room temperature for 20 min followed by centrifugation at 1500 × g for 20 min. The supernatant was carefully decanted without disturbing the pellet. Radioactivity was counted in the precipitate using a gamma counter. Percent binding of radiolabelled insulin antibody was calculated.

Calculations: Percent binding was calculated by setting the binding values measured for the blank samples as 100%. Standard log-logit curves were plotted for percent binding of standards versus concentration of standards. Sample concentrations were calculated from the standard curve and are presented as µU/mL insulin.

Slit Lamp Examination

We have studied effect of long-term PD on development of lens opacification in these animals by slitlamp examination. Eyes were examined every week using a slit lamp biomicroscope (Kowa SL-15 Portable, Japan) on dilated pupils with ophthalmic solution (atropine sulphate).

Lens Collection

After the rats were sacrificed, the eyeballs were collected and then dissected using the posterior approach; lenses were frozen at -80°C until further analysis.

Lens Organ Culture

Eyes were enucleated from rats immediately after sacrifice by CO_2 asphyxiation. Lenses were dissected from the eyes by posterior approach. A set of isolated lenses from each group was incubated in 2 mL modified TC-199 medium with antibiotics (filtered through 0.2 µm Millipore disc filters) and then incubated at 37°C under 95% air and 5% CO_2 with 55 mM glucose for a period of 4 days[31,40]. Lens images were captured during the culture period and the pictures represented were at the end of fourth day of the culture.

Lens Weight and Protein Content

Lenses were weighed individually from each group and a 10% homogenate was made from 4 to 5 pooled lenses in 50 mM phosphate buffer (pH 7.4). Before centrifugation, a set of aliquots were prepared for estimation of total protein, malondialdehyde (MDA) and sorbitol. The remaining total homogenate was centrifuged at 10,000 × g for 30 min at 4^0 C. The supernatant is referred as the soluble fraction. Protein content in total and soluble fraction was estimated using the Lowry method as described below and the percentage of soluble protein was calculated.

Protein Estimation by Lowry's Method

Principle: This method is based on the Biuret reaction in which protein peptide bonds react with copper under alkaline conditions producing Cu^{++}. The Cu^{++} reacts with Folin reagent (known as the Folin-Ciocalteu reaction), which is then reduced to hetero-polymolybdenum blue by the copper-catalysed oxidation of aromatic amino acids. The reaction results in a strong blue colour, which depends partly on the tyrosine and tryptophan content. The method is sensitive to approximately 0.01–1.0 mg/mL protein.

Procedure: The volume of various aliquots of bovine serum albumin (BSA, as a standard protein, 0.5 mg/mL, 20–100 µg) and unknown samples (lens total and soluble fractions) in duplicate was adjusted with distilled water to a final volume of 400 µl. Similarly, blanks were prepared with 400 µL distilled water. Then, 2 mL reagent A (2% Na_2CO_3, 0.1 N NaOH) was added to all tubes followed by incubation at room temperature for 10 min. Folin's phenol reagent (200 µl, 1N) was then added to the tubes, followed by incubation for another 40 min at room temperature. Finally, distilled water (900 µl) was added and absorbance was measured at 540 nm in a spectrophotometer (Spectra Max).

Calculation: A standard graph was plotted from standard absorbances versus concentration after correcting with the blank values. The concentration of proteins in the samples was determined from the standard graph and is presented as µg/mL.

Lens Protein Pattern by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Principle: Electrophoresis was carried out based on the discontinuous buffer system in the presence of SDS using a mini-protein slab gel (Bio-Rad). The principle involves separation of proteins by their size, where mobility of a protein in the gel is inversely proportional to the logarithm of its molecular weight. SDS binds to proteins in a stoichiometry of one SDS/two amino acids, imparts a negative charge and disrupts all non-covalent interactions. The presence of β-mercaptoethanol in the sample buffer reduces all disulphide bonds present in the protein.

Procedure: A 12% acrylamide gel [1.9 mL water, 1.7 mL acrylamide-bis-acrylamide (30: 0.8%), 1.3 mL resolving buffer (1.5 M Tris-Cl pH 8.8), 50 µL 10% SDS, 50 µL 10% ammonium per sulphate and 5 µl TEMED] was cast in a vertical gel apparatus. A 5% stacking gel was used [1.4 mL water, 0.33 mL acrylamide-bis-acrylamide, 0.25 mL stacking gel buffer (1.0 M Tris-Cl pH 6.8), 20 µl 12% SDS, 20 µl 10% ammonium persulphate and 2 µl TEMED]. Approximately 20 µg of lens soluble proteins were mixed with 4X sample buffer and boiled for 5 min. The protein samples were then loaded in separate wells and electrophoresed at a constant current of 30

mA using tris-glycine-SDS running buffer (25 mM Tris, 160 mM glycine, pH 8.3 containing 0.25% SDS) until the dye front reached the bottom of the gel. A standard molecular weight marker was also loaded into each gel. The gels were stained with coomassie brilliant blue R 250 (0.25% in methanol: acetic acid: water in a ratio of 5:1:4) and destained in methanol, acetic acid and water in a ratio of 5:1:4. Images were obtained by densitometry (BioRad DS800).

Measurement of AR Activity in Lens

AR activity was assayed according to a previously described method[31]. The assay mixture (1 mL) contained 50 µmol potassium phosphate buffer (pH 6.2); 0.4 mmol lithium sulphate; 5 µmol 2-mercaptoethanol; 10 µmol DL-glyceraldehyde; 0.1 µmol NADPH, and the enzyme preparation (lens soluble protein). Appropriate blanks were used for corrections. The assay mixture was incubated at 37^0C and the reaction was initiated by the addition of NADPH at 37^0C. Changes in absorbance at 340 nm, attributable to NADPH oxidation, were followed in a lambda 35 UV/Vis spectrophotometer (Perkin Elmer). Aldose reductase (AR) activity was expressed as μmoles of NADPH oxidized/h/100 mg protein.

Sorbitol Estimation in the Lens Homogenate

Sorbitol was extracted by rehomogenizing the rat lens total protein fraction by mixing with equal volume of 0.8 M perchloric acid. The homogenate was centrifuged at 5,000 × g at 4°C for 10 min and the supernatant pH was adjusted to 3.5 with 0.5 M potassium carbonate. The sorbitol content of the supernatant was measured using a spectrofluorometer (Jasco-FP-6500, Tokyo, Japan). The reaction mixture (1 mL), consisting of 50 µmol glycine buffer (pH 9.4), 2 µmol magnesium chloride, 0.2 µmol NAD and protein-free supernatant was incubated for 5 min at 37°C; the reaction was initiated by the addition of 0.5 U sorbitol dehydrogenase. Relative fluorescence, attributable to NADH formation, was measured in a fluorimeter with an excitation wavelength of 360 nm and an emission wavelength of 452 nm. Sorbitol was expressed as μmoles/g lens.

MDA Estimation

MDA was estimated according to the method of Bhuyan *et al* (1981). A 10% lens total homogenate (250 µL) was mixed with 20% trichloroacetic acid (TCA, 500 µL) and re-homogenized. The sample was then heated in a water bath at 70 ^{0}C for 10 min and cooled to room temperature, followed by centrifugation at 30,000 × g for 10 min at 25 ^{0}C. The protein free supernatants (250 µL) of each sample was mixed with 10% TCA (250 µL) and 0.5% (w/v) thiobarbituric acid (TBA) reagent, covered with a glass marble and heated in boiling water bath for 10 min. The tubes were then cooled to room temperature and absorbance of the pink colour was measured at 553 nm in a Spectra Max 190 microplate reader (Molecular Devices, USA). Standards (ranging from 0 to 0.5 nmol) were prepared with 1,1,3,3, tetraethoxy propane in a similar experimental conditions and unknowns were calculated from this standard curve.

Measurement of Superoxide Dismutase (SOD) Activity in the Lens

SOD activity was measured in the lens total soluble protein (TSP) fraction according to the method of Marklund and Marklund (1974). The assay mixture was prepared in a final volume of 1 mL containing 83 mM tris HCl buffer (pH 8.2); 1 mM diethylenetriaminepentaacetic acid (DTPA); 50 mM tris-ethylene diaminetetraacetic acid (EDTA) buffer (pH 7.4); 0.01 N HCl; 1 mM pyrogallol; and the enzyme source (lens soluble protein). The reaction was initiated by adding pyrogallol and the increase in absorbance was recorded for 3 min at 420 nm. SOD activity is measured in units, where 1 unit of activity is considered as amount of enzyme that inhibits 50% pyrogallol auto-oxidation.

Measurement of Tryptophan Fluorescence in the Lens TSP

Tryptophan present in proteins has the property of autofluorescence. During tertiary structural alterations, this amino acid get exposed to outside environment or get buried

inside and gives fluorescence, which can be measured from 300 to 400 nm upon excitation of 280 nm. Fluorescence measurements were performed using a spectrofluorimeter (Jasco, FP-7500). For all the measurements, 0.15 mg/mL lens protein in 20 mM sodium phosphate buffer (pH 7.4) was used.

Measurement of Non Enzymatic Glycation in the Lens TSP

The extent of non-enzymatic glycation was studied by measuring AGE-specific fluorescence using a spectrofluorometer (Jasco, FP-6500). For all of the measurements, 0.15 mg of lens protein/mL in 20 mM sodium phosphate buffer (pH 7.4) was used. Age-related non-tryptophan fluorescence of glycated protein was monitored by exciting the protein samples at 370 nm and emission was recorded between 400 and 500 nm. Results were represented in the form of fluorescence at 440nm (emission maxima for AGE) along with fluorescence spectra.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 software. All quantitative data are expressed as mean ± standard error (SE). Differences were analysed by independent t-test between two groups and analysis of variance (ANOVA) LSD post hoc test for more than two groups. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Effect of BG on Food Intake and Body Weights

Food intake and body weights of control, PD and PD+BG rats are shown in Fig 2. There was an increase in the average food intake of PD rats when compared to control rats (Fig. 2A). Despite of the increased food intake, the body weights of PD rats were significantly reduced during the entire experimental period (except at 10 months) (Fig 2B) when compared to control rats, whereas BG had no effect on food intake and body weights (Fig.2).

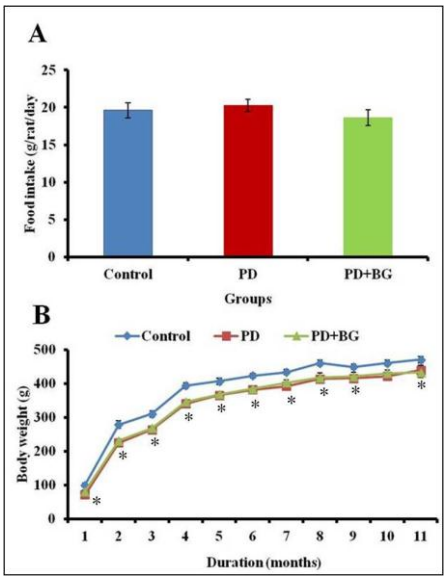

Figure 2: The average food intake (A) and body weights (B) of control, PD and PD+BG group rats. Values are mean ± SE, n=9-12 animals per group. *$p<0.05$ vs. Control. $p<0.05$ was considered as statistically significant by ANOVA.

Effect of BG on Glucose and Insulin Response during OGTT

Impairment of glucose tolerance in animals was assessed by OGTT conducted at two and ten months after STZ injection. The OGTT graph showed significantly higher plasma glucose levels at all time points (Fig. 3A) except at 0 min in PD rats compared to control rats. However, insulin levels were lower in the untreated PD rats than in control rats at all time points except at 120 min (Fig. 3C and D). Increased plasma glucose and reduced insulin

levels in STZ injected rats at 2nd and 10th month indicating the development of impaired glucose tolerance (IGT) or PD by two months which was maintained up to 10 months. Moreover, 10 months after nSTZ injection, BG-fed PD rats showed slightly lower plasma glucose levels at 30, 60 and 120 min of OGTT at ten months and also lower levels of insulin compared to the PD rats indicating utilization of insulin to reduce the glucose levels (Fig. 3B and D).

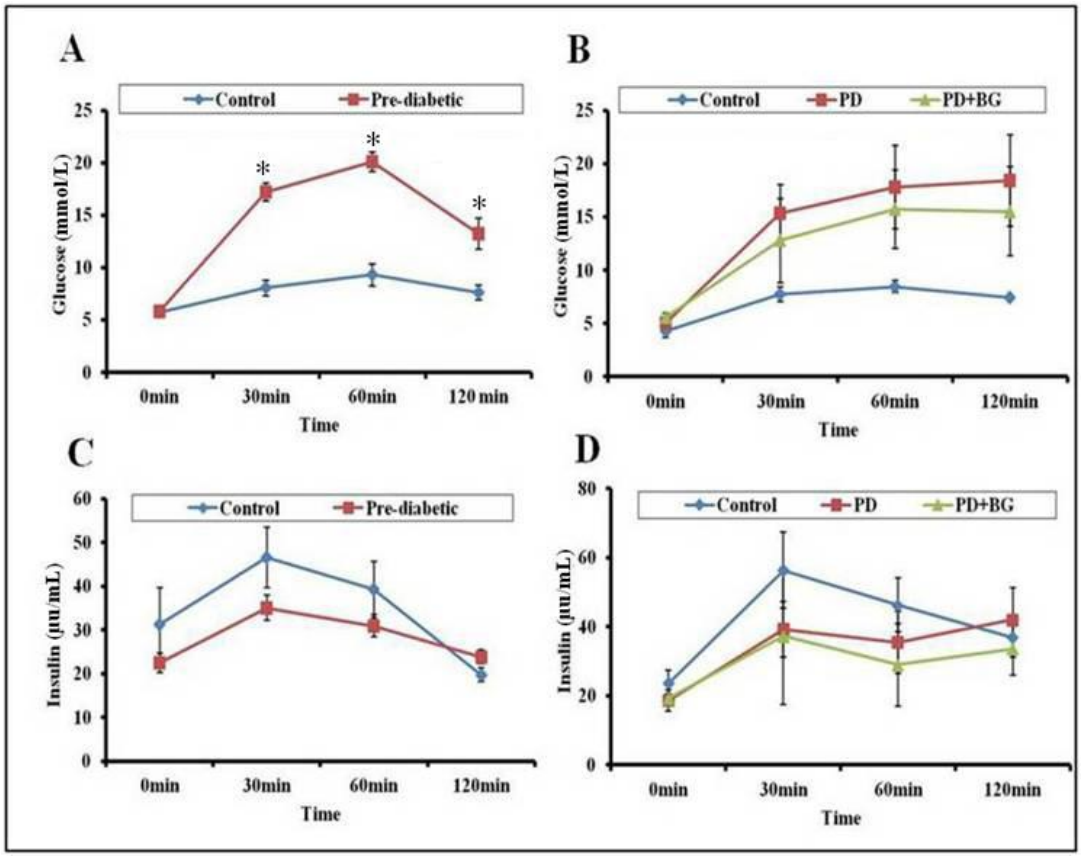

Figure 3: Glucose (A and B) and insulin (C and D) responses at various time points (0, 30, 60 and 120 min) during OGTT on overnight-fasted rats. OGTT was conducted in control, PD and PD+CN group rats after 2nd and 10th month of nSTZ injection. Values are mean ± SE, n=9–12 animals per group. *p<0.05 vs. Control. p<0.05 was considered as statistically significant by independent t-test. OGTT, Oral glucose tolerance test.

In addition, a significant increase in the area under the curve (AUC) for glucose, ratio of AUC of gucose/ AUC of insulin (GAUC/IAUC) and a significant decrease in AUC for insulin were observed in PD animals at the 2nd and 10th month when compared to those parameters in control group rats (Table 1) further support the IGT characteristic of nSTZ-PD animals. BG-fed PD rats exhibited marginally lower AUC for glucose, AUCG/AUCI ratio and AUCI at the 10th month than PD rats (Table 1).

Insulin insensitivity/ insulin resistance is a hallmark of T2D and degree of insulin resistance was calculated by HOMA-IR. The HOMA-IR index marginally decreased in untreated PD rats when compared to that of controls at the 2nd and 10th month indicating that nSTZ rats did not developed insulin resistance. Interestingly the HOMA-IR index was restored in BG-fed PD rats when compared to untreated PD rats (Table 1). These observations were in line with previous study where they observed lowering of glucose and insulin levels during OGTT at 15, 30min and also improvement in the AUC for glucose and Insulin[36]. This could be due to hypoglycemic property of BG[2,12,18].

Table 1: Plasma OGTT parameters of control, PD and PD+BG group rats at 2nd and 10th month of experimental period

Parameters	Groups				
	2 months		10 months		
	Control	PD	Control	PD	PD+BG
GAUC (mmol/h)	12.56±1.20	24.56±1.28*	11.42±0.82	22.61±4.37*	19.85±4.69
IAUC (µmol/h)	66.03±7.49	48.56±3.78*	87.33±13.91	71.94±10.60*	62.08± 8.88
GAUC/IAUC	0.20±0.02	0.61±0.09*	0.14±0.04	0.35±0.11*	0.31±0.04
HOMA-IR	0.93±0.10	0.74±0.07	0.85±0.14	0.70±0.24	0.80±0.27

Values are mean ± SE, n=9–12 animals per group. *p<0.05 vs. Control. p<0.05 was considered as statistically significant by independent student t-test and ANOVA. GAUC, glucose area under the curve; IAUC, insulin area under the curve; GAUC/IAUC, ratio of glucose area under the curve/ insulin area under the curve; HOMA-IR, homeostasis model assessment for insulin resistance

Slit-Lamp Examination

Eyes were examined weekly for the development of lens opacity from the age of one month to till the end of the experiment. Though nSTZ-PD rats developed IGT at 2 months after STZ, they did not develop lens opacification even at the age of 10 months (Fig 4). These observations were well correlated with our previous short duration (4 to 7 months) nSTZ models[22,32], where we did not observed any lens opacifications in any of these groups during the experimental period (Fig 4).

Figure 4: Representative eye images from control, PD and PD+BG group rats after 10 months of experimental period.

Lens Organ Culture

Since untreated PD animals did not develop lens abnormalities by the end of the experimental period, we have studied effect of PD on risk of cataract using Ex-Vivo model.In this model, we cultured a set of lenses (from each group) in modified TC199 medium in the presence of 55 mM glucose for a period of 4 days. The untreated PD rat lenses developed opacification earlier than lenses from the control rats, indicating that IGT associated PD is a hidden risk factor for cataract. The development of lens opacification was slower in the BG-fed PD rat lens when compared to untreated PD rat lens (Fig. 5).

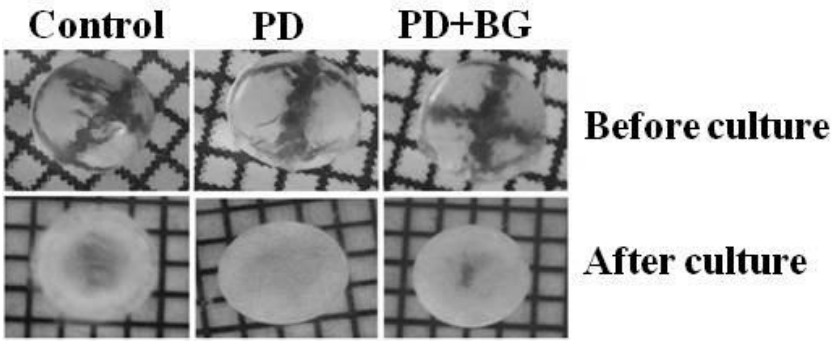

Figure 5: *In vitro* organ culture of control, PD and PD+BG group rat lenses before and after culture in modified TC199 medium with 55 mM glucose for 4 days.

Lens Weight and Protein Content

Aggregation of soluble lens proteins and increased insolubility of proteins are likely major biochemical changes leading to cataractogenesis. Therefore, we estimated total and soluble proteins and calculated the percentage of soluble protein content in the lenses. Though, the total protein content of PD rat lens was significantly higher than the control rat lens, there is no difference in soluble and percent soluble protein content between control and untreated PD rat lens. In addition there were no differences in soluble protein content between PD+ BG rats and untreated PD rats (Table 2).

Table 2: Protein content in eye lenses from control, PD and PD+BG group rats.

Parameters	Groups		
	Control	PD	PD + BG
Total protein (mg/g lens)	509±11.02	547±12.21*	543±13.43
Soluble protein (mg/g lens)	326±13.78	344±11.28	343±17.68
Percent of soluble protein	64	63	63

Values are mean ± SE, n=9–12 animals per group. *p<0.05 vs. Control. p<0.05 was considered as statistically significant by ANOVA

Soluble Lens Protein Profile by SDS-PAGE

Apart from the protein content in the lens, we have also studied protein profile using SDS-PAGE. As expected there was no high molecular weight cross-linked and aggregated soluble proteins among all three groups of rat lenses, because these animals did not developed any lenticular opacifications (Fig. 6).

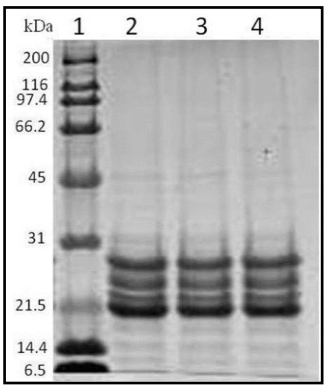

Figure 6: Subunit profile and protein cross-linking of the soluble fraction of control, PD and PD+BG group lenses. Lane 1: molecular weight markers (kDa); lane 2: control group; lane 3: PD group; lane 4: PD+BG group.

Polyol Pathway Intermediates in the Lens

Activation of the polyol pathway has been linked with various diabetic complications including diabetic cataract. AR is a key enzyme in the polyol pathway, converts excess glucose to sorbitol, the accumulation of sorbitol is related to many secondary complications of diabetes. In the present study, AR activity was marginally higher and sorbitol levels were significantly elevated in PD rat lenses than that of control rat lens indicating activation of this pathway at PD state and these results are consistence with our previous studies[22,35]. This increased sorbitol levels are due to enhanced activity of AR in PD rat lens could be one of the reasons for early opacification than the control rat lens in organ culture study. Interestingly feeding of BG to PD rats resulted in a lowered accumulation of sorbitol in these lens and this could be due to

its AR inhibitory potentials, which in turn delayed early opacification when we cultured these lens at high glucose medium in organ culture study (Fig. 7). Based on these results, it appears that BG is effective against osmotic stress caused by PD.

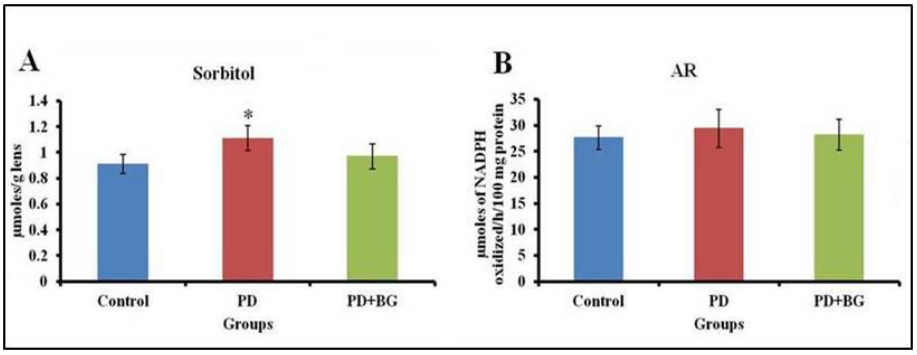

Figure 7: Sorbitol levels (A) and Aldose reductase (AR) activity (B) in control, PD and PD+BG group rat lens. Sorbitol was expressed as μmoles/g lens and aldose reductase (AR) activity was expressed as μmoles of Nicotinamide adenine dinucleotide phosphate (NADPH) oxidized/h/100 mg protein. Values are mean ± SE, n=3 estimations. *p< 0.05 vs. Control. p<0.05 was considered as statistically significant by ANOVA.

Oxidative Stress and Antioxidant Parameters in the Lens

Oxidative stress is another important factor which is known to play a key role in the development of various complications during diabetic state. In the present study, there was a marginal increase in MDA levels, and a marginal increase in the activity of an antioxidant enzyme, i.e., superoxide dismutase (SOD), in PD rat lenses when compared to that of control rat lenses indicating association/involvement of oxidative stress in PD rat lens and these results are consistence with our previous studies[22,32]. Interestingly, feeding of BG to PD rats had prevented oxidative stress by inhibiting MDA levels in lens (Fig. 8) probably through its anti oxidant property[3],

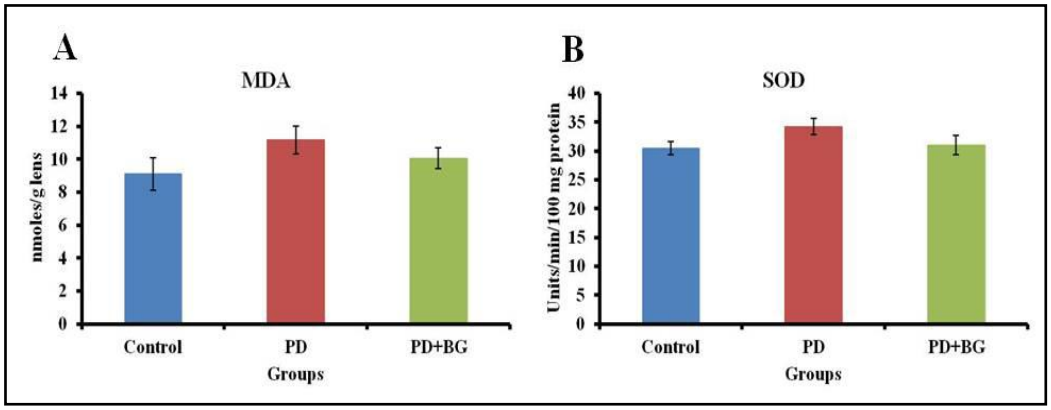

Figure 8: Malondialdehyde (MDA) levels (A) and superoxide dismutase (SOD) activity (B) in control, PD and PD+BG group rat lens. Lipid peroxidation [malondialdehyde (MDA)] levels and superoxide dismutase (SOD) activity in rat lenses. Values are mean ± SE, n=3 estimations.

Protein Structural Alterations by Tryptophan fluorescence in the Lens TSP

Increased tryptophan fluorescence is associated with an alteration in tertiary structure of proteins as a consequence of exposed aromatic amino acids that otherwise would be buried in the core of protein. In this study we assessed the structural alterations in lens proteins by measuring tryptophan fluorescence with an excitation of 280 nm and recording the emission from 300 nm to 400 nm. There was no difference in tryptophan fluorescence among all the three groups (Figure 9).

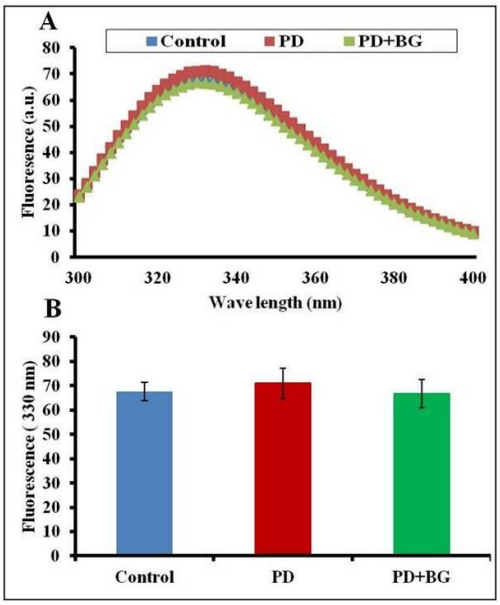

Figure 9: Tryptophan fluorescence of lens soluble protein in control, PD and PD+BG groups. Lens protein (0.15 mg/mL) in 0.05 M sodium phosphate buffer (pH 7.4) was excited at 280 nm and its emission was monitored from 300 to 400 nm (A). The average fluorescence intensity at 330 nm (B). Values are mean ± SE, *n*=3 estimations.

Non-enzymatic Glycation in the Lens Soluble Protein

Non-enzymatic glycation is also an important molecular mechanism responsible for the development of complications in diabetes. Here, we used non-tryptophan fluorescence as an index of non-enzymatic glycation. Advanced glycation end products give specific fluorescence at 440nm when they are excited at the wavelength of 370nm. However, we did not observe significant increases in AGE fluorescence in the lens proteins among the three groups studied (Figure 10).

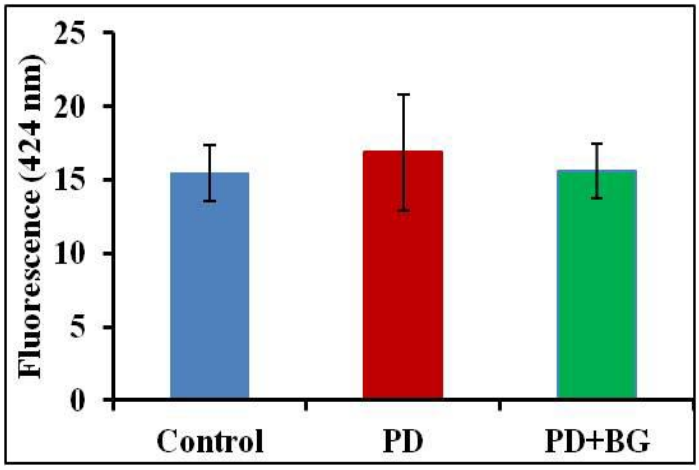

Figure 10: Advanced glycation end product (AGE) fluorescence of lens soluble proteins in control, PD and PD+BG groups. Lens protein (0.15 mg/mL) in 0.05 M sodium phosphate buffer (pH 7.4) was excited at 370 nm and emission was measured at 424 nm. Values are mean ± SE, *n*=3 estimations.

CONCLUSION

In conclusion, we evaluated that bitter guard intervention protects PD induced lens abnormalities via hypoglycemic and anti-oxidant property. Due to BG is dietary agent and safety, which could offer a new therapeutic strategy for early clinical application in PD. Among potentiality of BG one of the most important thing is early intervention strategy.

Conflicts of interest

The authors declare no conflict of interest.

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