International Journal of Agriculture Sciences
ISSN: 0975-3710&E-ISSN: 0975-9107, Volume 8, Issue 50, 2016, pp.-2166-2170.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000217

Research Article

AMELIORATING EFFECT OF BITTER GOURD (*Momordica charantica*) ON PRE-DIABETES INDUCED RETINAL ABNORMALITIES IN NEONATAL STREPTOZOTOCIN (NSTZ) INDUCED RAT MODEL

KESAVARAO SIVA K.[1], SANTHOSH V. SAI[1], RAJKUMAR P. LAKSHMI[1], UDAYKUMAR CH.[2], RAGHAVARAO T.[3], REDDY G. BHANUPRAKASH[2] AND SURYANARAYANA P.*[1]

[1]Lipid chemistry Division, National Institute of Nutrition, Hyderabad, Telangana, India
[2]Department Biochemistry Division, National Institute of Nutrition, Hyderabad, Telangana, India
[3]Department of Biochemistry, Andhra University, Vishakhapatnam, Andhra Pradesh, India
*Corresponding Author: Email- suryampnin@gmail.com

Received: May 23, 2016; Revised: July 27, 2016; Accepted: July 28, 2016; Published: October 24, 2016

Abstract- The main aim of this study is to investigate the impact of bitter gourd on long term pre-diabetic induced retinal abnormalities caused by neonatal streptozotocin (nSTZ). Male Sprague Dawley (SD) rat pups (n=35) 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 (n=10) received only vehicle. Oral glucose tolerance test (OGTT) was conducted at 2nd and 10th month. All rats were maintained on AIN-93G/M diet in individual cages and a sub set of pre-diabetic animals received 5% bitter gourd in the diet. Functional abnormalities of retina were studied by electroretinogram (ERG) and other biochemical alterations by histology, immunoblotting and gene expression. Majority of nSTZ rats exhibited impaired glucose tolerance (2h glucose>140mg/dl) or pre-diabetes by 2 months and maintained up to 11th month. ERG data showed a decrease in amplitude of scotopic b-wave and also scotopic oscillatory potentials in pre-diabetic rats indicating development of retinal functional abnormalities. Increased expression of vascular endothelial growth factor (VEGF), Glial fibrillary acidic protein (GFAP) and decreased expression of rhodopsin (Rho) in pre-diabetic animals indicate development of angiogenesis and retinal degeneration respectively. Further there was an increase in 4-hydroxynonenal (4-HNE) in retina indicating association of oxidative stress with pre-diabetes. Feeding of bitter gourd to these pre-diabetic rats partially prevented these changes. Pre-diabetes induced retinal abnormalities were prevented by bitter gourd through its hypoglycemic and antioxidant potential. This study will give an additional insight into molecular action of bitter gourd in preventing the pre-diabetic abnormalities thereby leading to the development of therapeutic strategies in controlling diabetes.

Keywords- Pre-debates, Diabetic retinopathy, bitter gourd, STZ.

Citation: Kesavarao Siva K., et al., (2016) Ameliorating Effect of Bitter Gourd (*Momordica charantica*) on Pre-Diabetes Induced Retinal Abnormalities in Neonatal Streptozotocin (nSTZ) Induced Rat Model. International Journal of Agriculture Sciences, ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 8, Issue 50, pp.-2166-2170.

Academic Editor / Reviewer: M. Hoque, Anubhav Khurana, Krishna N.V.V. Hari, Dr Jasmeet Singh

Introduction

Pre-diabetes is a high-risk state, which can lead to diabetes and is associated with impaired glucose tolerance (IGT) and impaired fasting glucose. According to the previous studies, nearly 5–10% of IGT/IFG or pre-diabetic subjects will progress to diabetes early and the same proportions are becoming normal. In addition, the prevalence of IGT is increasing all over the world and the projected estimation of these subjects will be more than 472 million by 2030 [1-4]. Pre-diabetes is associated with insulin resistance and β-cell dysfunction and these stages occurs well before detectable elevated glucose levels. Several evidence shows that pre-diabetes is associated with early forms of various diseases like non-proliferative or background form of retinopathy, kidney disease, small fiber neuropathy, nephropathy, and macro vascular disease. For these individuals, various early interventions including pharmacological and lifestyle modifications, herbal remedies etc. will reduce relative-risk to develop diabetes. Hyperglycaemia is a major event, which is most commonly associated with pre-diabetes and diabetes mellitus [5–7]. Pre-diabetes establishes early with non fasting hyperglycaemia which does not meet the criteria for diabetes diagnosis but all these subjects go through such pre-diabetic stage (IGT/IFG) before entering into full fledged diabetes mellitus [5, 6]. Various studies indicated that both pre-diabetic and diabetic individuals are at risk of cardiovascular diseases and other complications [7–10] and there is an increase in its incidence, which is a great burden on global healthcare [11, 12]. There are many pharmacological interventions to control hyperglycaemia [13], which were associated with several adverse effects including hypoglycaemia, increase in weight, gastrointestinal and liver related problems [14–18]. In many cases, these pharmacological therapies fail in controlling tight blood glucose levels and this is mainly due to inappropriate self-care behaviour, poor compliance, and other psychological problems [19]. Herbal food supplements/ alternative therapies or natural/plant based approach are being used by the humans to treat various metabolic disorders from the past centuries. In recent years these therapies have become more popular in addition to the routine pharmacological interventions in diabetic subjects [20–23] to manage hyperglycaemia [21, 22], 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 individuals [26, 27]. Most reports, except a few have demonstrated BG beneficial effects [24–28]. Earlier study had reported that

consumption of bitter gourd juice increases glucose uptake by tissues *in vitro* [29]. In addition, it also increases glycogen storage in the liver, stimulates secretion of insulin by isolated beta cells of pancreas [30] and also reduces systemic arterial blood pressure [27, 28]. The main purpose of this study is to assess the beneficial effects of freeze dried bitter gourd *in vivo* against pre-diabetes induced retinal abnormalities using nSTZ model which we developed earlier[31].

MaterialsandMethods

Animals: 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.

Preparation of bitter gourd powder: Fresh bitter gourd was obtained from the local market and was lyophilized. The dried bitter gourd was pulverized to powder form and added to AIN-93 diet and was fed to the experimental animals.

Induction of pre-diabetes and experimental groups: Female pregnant rats which were obtained from the NCLAS (National centre for laboratory animal science) were maintained on stock diet and followed till delivery. After 2 days of delivery, male pups were selected for this experiment. These two-day old rat pups (n=35) were injected with a single intraperitoneal injection of streptozotocin (STZ) at a dose of 90 mg/kg body weight which was dissolved in 100mM citrate buffer of pH 4.5. Control pups (n=10) received only citrate buffer. Only nSTZ injected rats having postprandial blood glucose levels more than 140 mg/dL and fasting blood glucose levels between 100-125 mg/dL at 2 months after STZ injection were considered as pre-diabetic and included in the study. Pre-diabetic rats were further divided into two groups; pre-diabetic untreated (PD, n=9) rats maintained on AIN 93 diet and pre-diabetic rats fed with 5% bitter gourd mixed with AIN 93 diet (PD+BG, n=9). Control rats were maintained on AIN 93 diet. All rats were maintained in the temperature and humidity controlled rooms with 12 hours light-dark cycle. All rats had free access to water. Body weights of rats were monitored once in a month throughout the study.

Fasting and postprandial blood glucose: Fasting and postprandial blood glucose levels in these experimental animals were monitored every month by glucometer (One Touch Horizon).

Oral glucose tolerance test (OGTT): OGTT was performed after 2nd and 10th month of STZ injection on overnight fasted rats by administering glucose orally as a bolus, at a dose of 2.0 g/kg of body weight. Blood samples were collected at 0, 30, 60 and 120 minutes time intervals for estimating plasma glucose and insulin concentrations to assess impaired glucose tolerance (IGT) and insulin resistance.

Clinical parameters: Plasma glucose was estimated in OGTT samples by the GOD-POD method with a commercially available kit (Biosystems). Plasma Insulin levels were estimated by RIA kit (BRIT-DAE, Mumbai, India). HbA1C was measured using kit (Biosystems).

Homeostasis model assessment (HOMA): Development of Insulin resistance in these animals was assessed by homeostasis model assessment (HOMA)-IR as reported earlier for rats [32] using the below equation:

HOMA-IR = [fasting plasma glucose (mg/dl) × fasting plasma insulin (μU/ml)/2,430].

Electroretinography (ERG): Retinal functional abnormalities were assessed by ERG at the end of the experiment using the LKC's UTAS visual electro diagnostic test system as reported earlier [33]. The pupils were dilated with atropine sulphate eye drops after the overnight dark adaptation and anaesthesia was induced with ketamine and xylazine (90 and 5 mg/kg, respectively) under dim red illumination.
A standard ERG was recorded from one (left) eye of the each animal through an ERG-jet gold foil positive electrode placed on the cornea of the eye, reference electrode was placed on the ear and a ground electrode on a tail of the animal. Scotopic and photopic responses were recorded using a series of Ganzfeld (BigShot) flashes from 10-5 to 25cd-s/mm (-50 dB to +10 dB) using dim white LED. Recordings were amplified with UBA-4204 amplifier and analyzed with EM. Oscillatory potentials (OPs) were calculated using same photopic and scotopic amplitudes between 10-5 to 25cd-s/mm (-2 to 10dB). A resultant mean value for each eye was used to compute the group means of b-wave amplitudes and OPs.

Histology: At the end of the experiment, rats were sacrificed using CO2 asphyxiation and eye balls were collected and few eye balls were kept in 4% para-formaldehyde prepared in phosphate-buffer (pH 7.2). These eyeballs were embedded in paraffin blocks and 5μ thick sections were made and subsequently mounted onto slides. These sections were then used for staining with Hematoxylin (H) & Eosin (E).

Immunohistochemistry: Paraffin was removed from eye sections followed by antigen retrieval and blocking. Incubation with rat polyclonal primary antibody raised in rabbit/mouse/goat (VEGF, CML, rhodopsin or GFAP) was done. After washing, the slides were incubated with secondary (anti goat/ anti rabbit/anti mouse) antibody conjugated with Alexafluor 488 (green)/594 (red). Excess secondary antibody was removed by washing with PBS three times (5 min each). It was then mounted with DAPI antifade mounting medium (Vecta shield). Sections were screened for specific staining using fluorescent microscope (Leica Microsystems). Merged Images were taken with DAPI as counter stain.

Western blot: Equal amounts of retinal protein lysates from each group were loaded on 12% SDS–PAGE and blotted on to PVDF membrane (PALL, USA).The membrane was incubated with anti-human recombinant VEGF (Biomeda Corp., Foster City, CA; dilution1:1000) or anti-carboxy methyl lysine (CML) (1:500) or rhodopsin (Rho) (1:1000) or Glial fibrillary acidic protein (GFAP) (1:25000) or Aldose reductase (AR) (1:500). Finally it was incubated with secondary anti rabbit/anti goat/anti mouse IgG conjugated to peroxidase after thorough washing. Immunoreactive bands were developed and visualized by chemiluminescence kit (Bio-Rad) and quantified using Image J software.

Gene expression studies by Quantitative Real-Time PCR: Total RNA extraction was done from whole frozen retina by Trizol method and quantified by Nanodrop spectrophotometer (ND1000) and the integrity of RNA was checked on denaturing MOPS-Formaldehyde gel. 5μg of RNA was reverse transcribed using Transcript or First Strand cDNA Synthesis Kit (Roche). The real time PCR was performed with the use of Applied Biosystems 7500 instrument with SYBR green (Applied Biosystems) on cDNA samples using the primers listed in [Table-2] according to the manufacturer's instructions. At the end of the run, specific products generated for each set of primers was examined by melting curve analysis. The relative expression levels of each gene of interest were normalized by subtracting the corresponding beta-actin threshold cycle (CT) values by using the DDCT comparative method. From each group three samples were used and each sample was run in triplicate.

Statistical analysis: By using SPSS 19.0 software all statistical analyses were performed. All quantitative data were presented as mean ± standard deviation (SD). Differences among means were analyzed by one-way ANOVA test, followed by Tukey HSD and student test. Statistical significance was set at $p<0.05$.

Results

OGTT after 2nd and 10th month and pre-diabetes: As reported earlier, nSTZ rats developed IGT associated pre-diabetes by two months [31]. In the present study, after 2nd and 10th month of STZ injection, OGTT graph showed higher plasma glucose levels in all the time points except 0 min in nSTZ injected rats when compared to control [Fig-1A and 1B] indicating development of IGT or pre-diabetes by two months and maintained pre-diabetes upto 11 months. Moreover after 10 months of STZ injection, BG treated PD rats showed a slightly lower plasma glucose levels when compared to PD rats in all the time points except at 0 min fasting plasma glucose [Fig-1B].

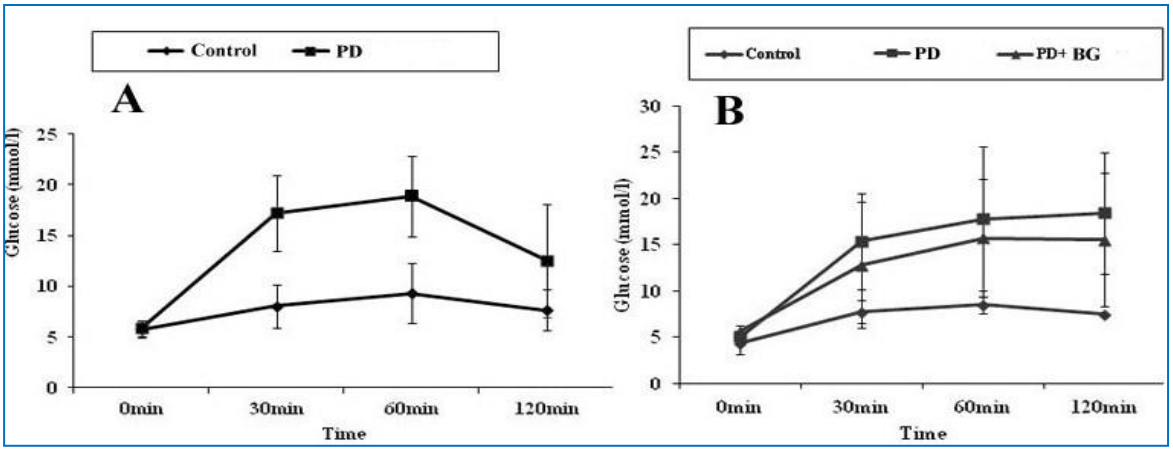
Food intake, body weights and blood glucose levels: All untreated PD and BG treated PD rats showed a marginal increase in fasting blood glucose, but there was a significant ($p<0.01$) increase in post prandial blood glucose (11.92±3.98 mmol/l) levels of untreated PD rats when compared to control (6.32±0.31 mmol/l). There was a slight decrease in body weights in both PD and BG treated PD rats when compared to control. There was a marginal increase in mean food intake in PD rats when compared to control rats. However, a significant ($p<0.01$) decrease in food intake was observed in BG treated PD rats when compared to PD rats. There was a slight decrease in body weights of PD rats and BG treated PD rats when compared to control rats by the end of the experiment [Table-1].

HOMA-IR and HbA1C: There was a decrease in HOMA-IR index in PD rats when compared to control rats which was restored in BG treated PD rats [Table-1]. Further, HbA1C levels were slightly increased in PD rats when compared to control rats, which were restored to a maximum extent when PD rats fed with BG [Table-1]

Table-1 *General characteristics of control, PD, PD+BG group rats*

Parameters	Control	PD	PD+BG
Food intake (g/day/rat)	19.62±2.83	20.26±3.07	18.66±3.32**
Body weight (g/rat)	470±29	440±48	433±36
Fasting blood glucose (mmol/L)	4.45±0.57	5.17±0.73	5.00±0.42
2h postprandial blood glucose (mmol/L)	6.32±0.31	11.92±3.98**	10.47±1.79
HOMA-IR	0.85±0.14	0.70±0.24	0.80±0.27
HbA1C	7.23±0.28	8.30±0.80	7.30±0.44

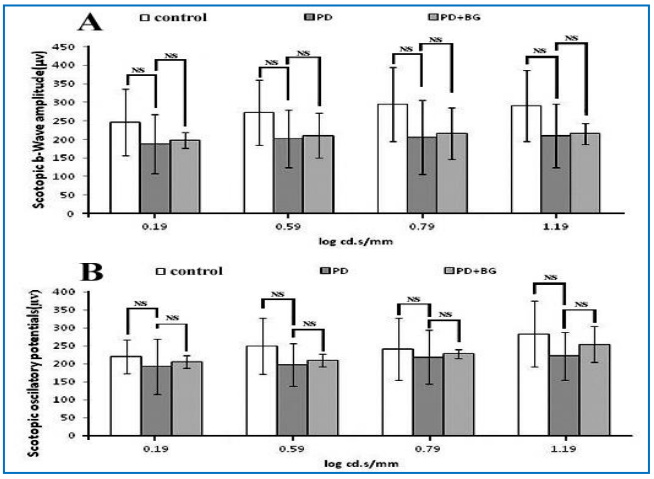
Pre-diabetic (PD); PD+BG: Pre-diabetic + Bitter gourd; Values are mean ±SD, n=9-10 animals; **p<0.01.



OGTT was conducted on overnight fasted rats at two and ten months after STZ injection. Glucose levels at various time points (0, 30, 60, and 120 min) during OGTT in control, PD and PD+BG group animals. Panel A: Glucose response at two months and Panel B: Glucose response at ten months. Values are mean ± SD, (n=9-10).

Fig-1 Glucose response during OGTT at 2nd month (1A) and 10th month (1B)

Altered retinal light responses in PD rats: We have examined the overall retina light responses to study development of functional abnormalities in these PD and BG treated PD rats along with control rats by using ERG. Rats were dark adapted overnight before scotopic testing. ERG responses in PD rats showed a decreased scotopic b-wave amplitudes and OPs compared to control rats indicating development of retinal functional abnormalities in pre-diabetic state. Though BG had prevented loss of scotopic b-wave amplitudes and OPs indicating its protective effect on the retinal function, but it was not statistically significant [Fig-2A and 2B].



ERG was recorded in control, PD and PD+BG group animals at the end of the experiment. Panel A: Scotopic b-wave and Panel B: Scotopic OPs. Values are mean ± SD, (n=4-6).

Fig-2 Pre-diabetes induced retinal functional abnormalities by ERG: Scotopic b-wave amplitude (2A) and OPs (2B)

Retinal morphology: Retinal morphology was examined in H&E stained retinas, where there was a decrease in outer and inner nuclear layer thickness in the retinas of PD rats when compared to control indicating progressive degeneration of the photoreceptor cells in the central retina. Feeding of BG to PD rats marginally prevented these changes [Fig-3A].

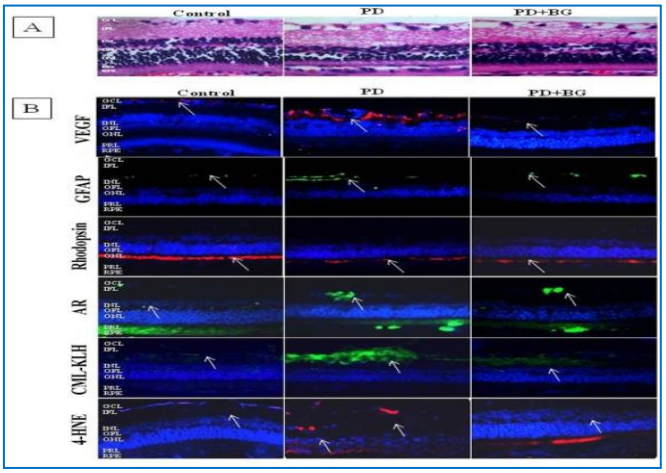
Table-2 *List of primers used in this study.*

Gene	Primer sequence
VEGF	Forward 5'GGA GTA CCC CGA TGA GAT AGA GTA3' Reverse 5'TAT CTT TCT TTG GTC TGC ATT CAC3'
GFAP	Forward 5'TTT CTC CAA CCT CCA GAT CC3' Reverse 5'AGC TTT AGG CCC TCA CAC TG3'
Rho	Forward 5'CTT CCT GAT CTG CTG GCT TC3' Reverse 5'ACA GTG TCT GGC CAG GCT TA3'
AR	Forward 5'ACT GCC ATT GCA AAG GCA TCG TGG3' Reverse 5'CCC CCA TAG GAC TGG AGT TCT AAG3'
β-Actin	Forward: 5'GAG AAG AGC TAT GAG CTG CC3' Reverse: 5'CTC AGG AGG AGC AAT GAT CT 3'

VEGF: Vascular endothelia growth factor; Rho: Rhodopsin; GFAP: Glial fibrillary acidic protein; AR; Aldose reductase; *β-Actin*: beta actin

Alterations of retinal proteins expression: We used immunohistochemical and western blot methods to study expressions of key protein molecules, which are involved in retinal abnormalities/retinopathy/retinal degeneration. Immunostaining with VEGF, GFAP, AR, CML-KLH, 4-HNE antibodies showed increased immuno-reactivity in PD rat retinas indicating increased angiogenesis, glial cell activation, polyolpathway activation, glycation and oxidative stress respectively, which are commonly associated with retinopathy. A decrease in rhodopsin-specific signal in PD rat retinas when compared to control indicates association of retinal degeneration with pre-diabetic state. Interestingly, feeding of BG to PD rats had

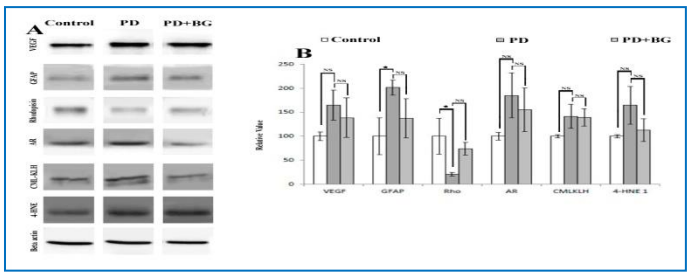
prevented these alterations [Fig-3B]. Immunofluorescence data was further confirmed by immune blot methods to study expression of VEGF, GFAP, rhodopsin, AR, CML and 4-HNE molecules in control, PD and BG treated PD rat retinal samples. As expected there was slight increase in the relative expression of VEGF, AR, CML-KLH, 4-HNE proteins and there was a significant (P<0.05) increase in GFAP protein in PD rat retinas when compared to control. Rhodopsin expression was significantly (P<0.05) decreased in PD rats when compared to control rats. Feeding of BG to PD rats marginally prevented these altered protein expressions [Fig-4A&B].

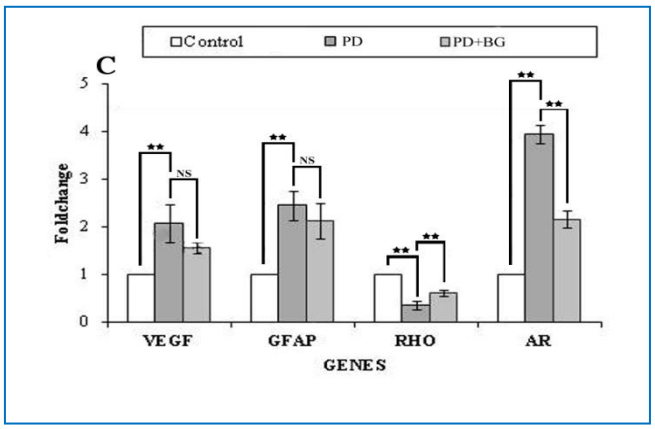


Retinal sections of control, PD and PD+BG group animals were stained with H&E. The stained retinal sections were examined under light microscope and images were captured (Panel A)

Expression of VEGF, GFAP, Rho, AR, CML-KLH, 4-HNE were studied in control, PD and PD+BG rats retinas using Immunohistochemistry (Panel B).

Fig-3 Pre-diabetes induced retinal morphology by H&E (A) and protein expressions by Immunoflourescence (B)





Expression of VEGF, GFAP, Rho, AR, CML-KLH, 4-HNE were studied in control, PD and PD+BG rats retinas using western blot (Panel A) and concentration of these proteins was measured by densitometric analysis using Image J software (Panel B). Expression of VEGF, GFAP, Rho and AR genes in control, PD and PD+BG rat retinas were analyzed by qRT-PCR (Panel C).

Fig-4 Pre-diabetes induced altered protein expressions in the retina by western blot (A); Desitometric analysis (B) and qRT-PCR (C)

Retinal gene expression: Real time analysis showed a significant (P<0.01) up-regulated expressions of VEGF, GFAP, AR, and a significant (P<0.01) down-regulated expression of rhodopsin in PD rats when compared to the control, indicating the retinal abnormalities [Fig-4C] which supports IHC and immunoblot results. Feeding of BG to PD had significantly (P<0.01) down-regulated AR and up-regulated rhodopsin expressions. VEGF and GFAP expressions are marginally down-regulated in BG treated PD rats when compared to PD rats [Fig-4].

Discussion

Diabetic retinopathy is an important microvasular complication of diabetes also occurs in IGT/IFG or pre-diabetic individuals, but the pathophysiology of this complication is not fully understood. Understanding the pathophysiology of retinopathy in IGT/IFG or pre-diabetic state will help to undertake appropriate interventions at early stage of disease. Though the pathogenesis of diabetic retinopathy has been extensively investigated using number of animal models of diabetes [34], such studies are limited with pre-diabetic animal models especially with IGT/IFG state. In this way, earlier we developed nSTZ induced pre-diabetic model using inbred SD rat pups which develops only IGT associated pre-diabetes but not insulin resistance by two months [31] and same model was used in the present study. Since many studies had shown the health-promoting benefits of bitter gourd, including glucose lowering and antioxidant effects, in the present study we used BG for its protective effect in PD induced retinal abnormalities. In diabetic retinopathy, functional abnormality develops earlier than the morphological alterations. The most commonly observed ERG abnormalities in subjects with diabetes without retinopathy or at early diabetic retinopathy include a reduction in the scotopic b-wave amplitude and OPs [35,36]. Further, b-wave reflects the functions of inner retinal layers, which include outer nuclear layer, bipolar cells and muller cells or glial cells [37]. In the present study, we also observed a decrease in the scotopic b-wave and OPs in the pre-diabetic rat retinas when compared to control indicating altered retinal functional abnormalities at pre-diabetic state and BG marginally prevented these changes in pre-diabetic rats. This functional abnormality observed with these IGT rats was well correlated with previously published ERG data of early diabetic retinopathy in humans (50) as well as experimental animals with non-fasting hyperglycemia [38]. Though we observed retinal functional abnormalities in these IGT/IFG rats for six months (data not shown), we maintained these rats for longer duration (eleven months) to study pre-diabetes induced structural and molecular alterations related to retinopathy. There are several morphological, molecular and cellular events that contribute to initiation of diabetic retinopathy. In general, morphological changes are also associated with severity of retinopathy. In this study, a decrease in the thickness of outer and inner nuclear layer of PD rats when compared to control indicate progressive degeneration of the photoreceptor layer and these morphological changes were well correlated with functional alterations as assessed by ERG in PD rats. VEGF and GFAP are two molecules, which play an important role in the pathogenesis of diabetic retinopathy [39; 40]. Several investigators had reported high level of VEGF in vitreous, which was significantly correlated with the severity, or progression of diabetic retinopathy in human subjects [39, 41]. In experimental animals, increased expression of VEGF, GFAP was also observed in early stage of diabetes [42] and before observable retinal proliferative changes [43]. These molecular changes occur after initiation of functional abnormalities in the retina of muller cells [44]. In the present study elevated expression of VEGF and GFAP in PD rats after development of function abnormalities were well correlated with previous studies [38, 44]. Expression of VEGF and GFAP is not only associated with hypoxia condition but also increases in oxidative stress condition [40, 45].In the present investigation, elevated expression of VEGF and GFAP along with 4-HNE in PD rats indicated association of these molecules (VEGF & GFAP) with oxidative stress condition. Photoreceptors of the outer retina have been implicated in the pathogenesis of early diabetic retinopathy mainly through hypoxia and oxidative stress conditions [46]. Rhodopsin was decreased in the present study in PD rat retina when compared to control indicating association of retinal degeneration in this model. These results were comparable with some of the studies at early stage of diabetes [47], as well as in obesity associated retina degeneration in WNIN-Ob rats [48]. In

general, activation of polyolpathway and glycation associated with various long-term diabetic complications. However, the present study results showed increased expressions of AR and CML-KLH in PD rats revealed that these pathways activated at pre-diabetic state.

Conclusions

In conclusion, STZ injection to neonatal SD rat pups had developed IGT/pre-diabetes at two months. Prolonged exposure of these rats to pre-diabetic state had developed retinal abnormalities. Feeding of BG to pre-diabetic rats prevented retinal abnormalities probably through its hypoglycemic and antioxidant nature. This nSTZ induced IGT/pre-diabetic models can be useful for IGT/pre-diabetes associated complications.

Acknowledgements: K.S.K. Rao received a research fellowship from the Indian Council of Medical Research, Government of India.

Funding Sources: P.S.N. received grants from the Department of Biotechnology, Government of India (Grant No**:** BT/PR3446/BRB/10/969/2011) and National Institute of Nutrition (Indian Council of Medical research, Government of India) for intramural funding (#12-BS11).

Conflict of Interest: None declared

References

[1] Tabak A.G., Herder C., Rathmann W., Brunner E.J. and Kivimäki M. (2012) *Lancet.,* 16 379(9833), 2279-90.
[2] Forouhi N.G., Luan J., Hennings S. and et al. (1990–2000) *Diabet Med.,* 24 (2007), 200–7.
[3] Nathan D.M., Davidson M.B., DeFronzo R.A. and et al. (2007) *Diabetes Care.,* 30, 753–9.
[4] Knowler W.C., Fowler S.E., Hamman R.F. et al. (2009) *Lancet.*, 374, 1677-86.
[5] Rhee S.Y. and Woo J.T. (2011) *Diabetes Metab J.,* 35, 107–16.
[6] Nyenwe E.A. and Dagogo-Jack S. (2011) *Minerva Endocrinol.,* 36, 129–45.
[7] Kolb H. and Eizirik D.L. (2011) *Nat Rev Endocrinol.,* 8(3), 183-92.
[8] Berenson G.S., Agirbasli M., Nguyen Q.M., Chen W. and Srinivasan S.R.(2011) *Glycemic status, Med Clin North Am.,* 95, 409–17.
[9] Dhingra R. and Vasan R.S. (2012) *Heart Fail Clin.,* 8, 125–33.
[10] Milman S. and Crandall J.P. (2011) *Med Clin North Am.,* 95, 309–25.
[11] Colagiuri S. (2011) *Med. ZClin North Am.,* 95, 299–307.
[12] Chen L., Magliano D.J. and Zimmet P.Z. (2011) *Nat Rev Endocrinol.,* 8(4), 228-36.
[13] Ho J., Leung A.K. and Rabi D. (2011) Recent Pat Endocr Metab Immune Drug Discov. 5, 66–73.
[14] Levetan C. (2007) *Curr Med Res Opin.,* 23, 945–52.
[15] Plutzky J. (2011) *Am J Cardiol.,* 108, 25B–32B.
[16] Madsbad S., Kielgast U., Asmar M., Deacon CF., Torekov S.S. and Holst JJ. (2011) *Diabetes Obes Metab.,* 13, 394–407.
[17] Hollander P., Raslova K., Skjøth T.V., Råstam J. and Liutkus J.F. (2011) *Diabetes Obes Metab.,* 13, 268–75.
[18] Starner C.I., Schafer J.A., Heaton A.H. and Gleason P.P. (2008) *J Manag Care Pharm.,* 14, 523–31.
[19] Home P. (2003) *The Diabetes Metab.,* 29, 101–9.
[20] Lee T. and Dugoua J.J. (2011) *J. Curr Diab Rep.,* 11, 142–8.
[21] Davì G., Santilli F. and Patrono C. (2010) *Cardiovasc Ther.,* 28, 216–26.
[22] Najm W. and Lie D. (2010) *Prim Care.,* 37, 237–54.
[23] Bartlett H.E. and Eperjesi F. (2008) *Ophthalmic Physiol Opt.,* 28, 503–23.
[24] Karunanayake E.H., Tennekoon K.H.A., Sharma K. (ed.). (1993) Diabetes Mellitus and Its Complications. An Update, Macmillan India Ltd, New Delhi, India,
[25] Day C., Cartwright T., Provost J. and Bailey CJ. (1990) *Planta Med.,* 56, 426–429.
[26] Platel K. and Srinivasan K.(1997) *Nahrung,* 41, 68–74.
[27] Ahmed I. (1999) University of Central Lancashire
[28] Sharma A.K., Ahmed I., Tadayyon M., Ponery A.S., Aloamaka P., Absood. G. and Pallot D.J. (1996) *Int J Diabetes,* 4, 29–38.
[29] Welihinda J., Karunanayake E.H., Sheriff M.H.R. and Jayasinghe (1986) *KSAJ Ethnopharmacol,* 17, 277–282.
[30] Welihinda J., Arvidson G., Gylfe E., Hellman B. and Karlesson E. (1982) *Acta Biol et Med Germ.,* 41, 1229–1240.
[31] Patil M.A., Suryanarayana P., Putcha U.K., Srinivas M. and Reddy G.B (2014) Oxid Med Cell Longev.
[32] Mather K. (2009) *Am J Physiol Endocrinol Metab,* 296 (2), E 398-9.
[33] Deshpande J., Jekodi S., Reddy G.B., Sreenivas Reddy and Vijaya Jaturu (2015) *Advances in Ophthalmology & Visual System*, 3(1), 00077.
[34] Robinson R,, Barathi V.A., Chaurasia S.S., Wong T.Y. and Kern T.S (2012) *Dis Model Mech.,* 5(4). 444-56
[35] Holopigian K., Seiple W., Lorenzo M. and Carr R. (1992) *Vis Neurosci.,* 14(3), 533-44.
[36] Juen S. and Kieselbach G. F. (1990) *Archives of Ophthalmology*, 108(3), 372-375.
[37] Miller R. F. and Dowling J. E. (1970) *J. neurophysiology,* 33(3), 323-341.
[38] Salido E.M., de Zavalía N., Schreier L., De Laurentiis A., Rettori V., Chianelli M., Keller Sarmiento M.I., Arias P. and Rosenstein R.E. (2012) *Exp Neurol.,* 236 (1), 151-60.
[39] Adamis A.P., Miller J.W., Bernal M.T, D'Amico D.J., Folkman J., Yeo T.K. and Yeo K.T. (1994) *Am J Ophthalmol.*, 118(4), 445-50.
[40] Rungger–Brändle E., Dosso A. A. and Leuenberger P.M. (2000) *Investigative ophthalmology & visual science,* 41(7), 1971-1980.
[41] Funatsu H., Yamashita H., Noma H., Mimura T., Nakamura S., Sakata K., & Hori S. (2005) *Graefe's Archive for Clinical and Experimental Ophthalmology,* 243(1), 3-8.
[42] Zuo Z.F., Zhang Q., & Liu X.Z. (2013) *International journal of ophthalmology,* 6(4), 422.
[43] Sone H., Kawakami Y., Okuda Y. A., Sekine Y., Honmura S., Matsuo K et al. (1997) *Diabetologia*, *40*(6), 726-730.
[44] Zeng, Kaihong, Hongxia Xu, Mantian Mi, Qianyong Zhang, Yajie Zhang, Ka Chen, Fang Chen, Jundong Zhu and Xiaoping Yu. (2009) *Neurochemical research,* 34(2), pp.-244-254.
[45] Obrosova, I. G., Minchenko, A. G., Vasupuram, R., White, L., Abatan, O. I., Kumagai, A. K. et al. (2003). Diabetes, 52(3), 864-871.
[46] Kern., Berkowitz B.A., Kern T.S., Bissing D., Datel. P., Bhatia A., Kefalov V.J. and Roberts R. (2015) *Invest ophthal mol vis sci.,* 50 (7), 34562-63.
[47] Park S.H., Park J. W., Park S. J., Kim K. Y., Chung J. W., Chun M. H. & Oh S. J. (2003) *Diabetologia,* 46(9), 1260-1268.
[48] Reddy G.B., Vasi Reddy V., Mondal M.N., Tiruvalluru M., Wang X.F., Jabolski M.M., Nappanveetti G. and Ayyapagari R. (2009) *Invest ophthal mol vis sci.,* 50 (7), 34562-63.