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Influence of *Punica granatum* L. on region specific responses in rat brain during Alloxan-Induced diabetes

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ABSTRACT

Objective: The present study was carried out to investigate the effects of *Punica granatum* peel methanolic extract (*PGPE*) on cerebral cortex (CC) and Hippocampus (HC) brain antioxidant defense system and markers of lipid and protein oxidation in alloxan induced diabetic rats. **Methods:** Oral administration of *PGPE* (75 and 150 mg of kg body weight) for 45 days resulted in significant reduction in blood glucose levels. **Results:** Supplementation of diabetic rats with *PGPE* showed increased activities of SOD and GPx with concomitant decrease in MDA and PC content. Region-specific changes were more evident in the HC when compared to CC. **Conclusions:** The present study indicated that *PGPE* can ameliorate brain oxidative stress in alloxan induced diabetic rats by up regulating antioxidant defense mechanism by attenuating lipid and protein oxidation. *PGPE* thus may be used as a potential therapeutic agent in preventing diabetic complications in the brain.

1. Introduction

Diabetes mellitus is a multi-faceted disease characterized by alterations and multiple complications resulting in retinopathy, nephropathy and neuropathy. Oxidative stress is believed to play a central role in the development of diabetic complications in many tissues.[1] It has been clearly demonstrated that diabetes mellitus is associated with an increased production of free radicals and lipid peroxidation[2] produced by glucose oxidation.[3]

Insulin and synthetic drugs are mainly used for diabetes treatment, are helpful in controlling hyperglycemia, but these drugs also have prominent side effects, such as hypoglycemia, dropsy, drug-resistance, weight gain and so on. On the other hand, plant products are generally considered to be less toxic with fewer side effects than synthetic products.[4]

Punica granatum L. (Punicaceae), commonly called pomegranate, is a large shrub or small tree which grows well in the warm valleys and outer hills of the Himalayas and is cultivated throughout the India. *P. granatum* is a rich source of bioactive compounds and is used in traditional indian medicine for the treatment of various diseases. The biological activities viz. antibacterial, antifungal, anthelmintic, antifertility, antioxidant, antidiabetic, and antiulcer of the various extracts of different parts of this plant have already been documented.[5]

Diabetes of types I and II induces serious complications related to functional and structural changes in the central nervous system.[6] Oxidative damage to various brain regions result into the long term complications such as morphological abnormalities and memory impairments.[7] Distinct regional distribution of antioxidant biochemical defense and variation in metabolic rates responsible for differential oxidative damage in brain region has been reported.[8] Therefore the present study was initiated to understand the protective effects of *P. granatum* methanolic peel extract (*PGPE*) on different regions of the brain such as cerebral cortex (CC) and hippocampus (HC) in alloxan-induced diabetic rats.

2. Materials and Methods

2.1 Chemicals

Epinephrine, thiobarbituric acid, pyridine, t-butyl hydrogen peroxide, reduced glutathione, 2,4- dinitro phenyl hydrazine, glutathione reductase, , nicotinamide adenine dinulcloetide phosphate, 1,1,3,3-tetra methoxy propane, bovine serum albumin and alloxan were obtained from

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Sigma (St. Louis, MO,USA). Insulin was obtained from Biocon Pvt. Lmt., Bangalore, India. All organic solvents were of spectral grade and general chemicals were of analytical grade and were purchased from local companies.

2.2 Preparation of plant extract

Fresh *P. granatum* L. was collected from the local market of Bangalore, India during the month of August. The plant material was identified and authenticated at the Regional research centre, Bangalore, where a voucher specimen has been deposited in the herbarium (RRCBI Acc. No. 2137). The air dried peel (110gm) was coarsely powdered and extracted with 70% methanol (1:10) by soxhlation for 18 hrs.[9] The extract was centrifuged at 1500 x g for 10 minutes and was vacuum dried with the help of rotary vacuum evaporator and stored in an air tight container (Yield, 14.28% wt/wt).

2.3 Animal Maintenance

Male Wistar albino rats of 4 months of age were obtained from the Central Animal Facility, IISc, Bangalore, INDIA and maintained in a clean rodent room. Animals were housed three per cage in polypropylene fitted steel mesh-bottom cages and were maintained at a temperature of 23± 2 ℃, relative humidity of 55-65 % and daily exposure of a 12 h-light and 12 h dark cycle. Animals had free access to standard food (Amruth feeds, India) and tap water ad libitum. The present study was approved by the Institutional Animal Ethics Committee (IAEC), Bangalore University, India.

2.4 Induction of Diabetes

The animals were fasted for 24 h prior to the induction of diabetes. Diabetes was induced by a single intraperitoneal injection of freshly prepared alloxan monohydrate in normal saline (150 mg per kg body weight). Development of diabetes was confirmed by polydipsia, polyuria and by measuring blood glucose concentrations 72 h after injection of alloxan. Rats with blood glucose level of 250 mg/dl or higher were considered to be diabetic.

2.5 Experimental Design

The rats were randomized into six groups comprising of six animals in each group as given below. *PGPE* was administered orally in normal saline once per day.

Group 1: Normal Control rats, received 1ml saline

Group 2: Control Diabetic rats, received 1 ml saline

Group 3: Diabetic rats administered with low *PGPE* (75 mg/kg body weight) for 45 days

Group 4: Diabetic rats administered with high *PGPE* (150 mg/kg body weight) for 45 days

Group 5: Diabetic rats given glibenclamide (600 μg /kg body weight) in saline solution daily using an intragastric tube for 45 days.

Group 6: Diabetic rats given daily protamine zinc insulin suspension intraperitoneally (2 units/ kg body weight) (Insugen , Biocon, Bangalore, India) for 45 days.

2.6 Tissue Preparation

At the end of experimental period, rats were anesthetized with di-ethyl ether and sacrificed by cervical dislocation. The brain tissue was removed, cleaned and washed in ice cold saline. The different regions of the brain such as CC and HC were separated, weighed and homogenized in 50 mM phosphate buffer pH (7.0). The homogenate was centrifuged at 600 × g for 15 min at 40C (RV/FM, superspin, plastocraft , India). The supernatant was collected and used for enzymatic antioxidant analysis.

2.7 Biochemical Analysis

2.7.1 Blood glucose

Blood samples were collected from the tail vein by end cutting method [10] and blood glucose level was estimated by Ames One Touch Glucometer (Accu Check, Roche, Germany).

2.7.2 Glutathione peroxidase (GPx 1.11.1.9)

GPx activity was measured at 37 ℃ by the method of Flohe and Gunzler [11] (1984). The reaction mixture consisted of 500 μl of phosphate buffer, 100 μl of 0.01 M reduced glutathione (GSH), 100 μl of 1.5 mM NADPH and 100 μl of glutathione reductase (GR) (0.24 U). 100 μl of tissue extract was added to the reaction mixture and incubated at 37 ℃ for 10 min. 50 μl of 12 mM t-butyl hydro-peroxide was added to 450 μl of reaction mixture and absorbance was measured at 340 nm for 180 s in a spectrophotometer (ELICO, BL 192, INDIA). A molar absorptivity of 6.22×10^3 M/cm was used to determine enzyme activity. One unit of activity is equal to mM NADPH oxidized per min per mg protein.

2.7.3 Superoxide dismutase (SOD, EC 1.15.1.1)

SOD activity was determined at room temperature (RT) according to the method of Misra & Fridovich[12] (1972). Tissue extract (100 μl) was added to 880 μl carbonate buffer of 0.05M, pH (10.2), 0.1mM EDTA. 20 μl of 30mM epinephrine in 0.05% acetic acid was added to the mixture and the change in absorbance was recorded for 4 min at 480nm in a spectrophotometer. The amount of enzyme that results in 50% inhibition of epinephrine auto-oxidation is defined as one unit.

2.7.4 Lipid Peroxidation (LP)

Malondialdehyde (MDA) content was analyzed by the method of Ohkawa *et al*.[13] (1979) using 1, 1, 3, 3-tetramethoxypropane (TMP) as the standard. Briefly, 0.1 ml of tissue homeganate was taken and 50 μl of 8.1% sodium dodecyl sulphate (SDS) was added and incubated for 10 min at RT. 375 μl of 20% acetic acid (pH 3.5) and 375 μl of 0.6% TBA were added and then placed in boiling water bath for 60 min. And then sample was allowed to cool and 1.25 ml of Butanol: Pyridine (15:1v/v) mixture was added, vortexed and centrifuged at 1000 rpm for 5 min. Absorbance of the organic layer was measured at 532 nm and the concentration was expressed in terms of n moles MDA per mg protein.

2.7.5 Protein Oxidation

Protein carbonyl (PC) content was measured according to the procedure of Levine *et al*[14] (1994). Briefly, 10 mmol dinitro phenylhydrazine (DNPH) in 2.5 M HCl was added to the tissue homogenate and incubated in dark for 60 min at RT. This was followed by vortex mixing, addition of 20% tri-chloro acetic acid (TCA) and subsequently washing thrice with ethanol: ethyl acetate (1:1v/v) mixture. Precipitated protein was then re-dissolved in 6 M homogenate guanidine

HCl in 20 mM phosphate buffer pH (6.5). Insoluble substances were removed by centrifugation and absorbance of the supernatant was read at 370 nM. An extinction coefficient of 22,000 M−1cm−1 was used to determine the protein carbonyl content and was expressed as μ moles per mg protein.

2.7.6 Protein Estimation

Protein concentration was estimated by the method of Lowry *et al* [15] (1951) using BSA as a standard.

2.8 Statistical analysis

All data was expressed as mean ± S.E and were analyzed by one way analysis of variance for blood glucose and by two way analysis of variance between different groups and regions. When significant F−ratios were found, Duncan's multiple range test were used to assess the mean difference between groups and regions. Probability values (P< 0.05) were considered significant.

3. Results

3.1 Blood glucose

Table 1 shows the levels of blood glucose in normal and experimental groups. The alloxan diabetic rats showed a significant (P<0.05) increase in the blood glucose levels compared to the normal control rats. The administration of *PGPE* at 75 and 150 mg / kg body weight and glibenclamide significantly (P<0.05) reduced the glucose levels by 48%, 64% and 57% as compared with diabetic control rats. However, the changes are insignificant between these groups.

Table 1

Changes in Glucose levels of normal and experimental diabetic rats

Groups	Initial	Final
NC	93.2±1.27	95.83±1.64 a
DC	344.2±10.88	358.3±10.71 b
LP	304.2±32.73	185.8±6.41ce
HP	340.2±6.45	127.8±9.47 de
DG	342.8±1.25	154.0±12.75 e
DI	327.8±1.33	98.0±2.75 f

NC, Normal control; DC, Diabetic control; LP, Low *P. granatum* (75mg/kg body weight); HP, High *P. granatum* (150mg/kg body weight); DG, Diabetic rats treated with glibenclamide; DI, Diabetic rats treated with insulin. Values are mean±S.E of 6 animals/ group .Significance between the groups means is represented in lower case (P<0.05). Those not sharing the same letters are significantly different.

3.2 Enzymatic antioxidants

The SOD activity was significantly (P<0.05) decreased in diabetic rats compared to the normal rats (Fig. 1). Treatment of diabetic rats with low and high *PGPE* significantly (P<0.05) up regulated SOD activity by (27% and 57%) in CC and (32% and 25%) in HC respectively. A similar trend was noticed in glibenclamide treated animals. The activity was restored to normal levels in insulin treated animals. The changes between the two regions were found to be statistical significant (P<0.05).

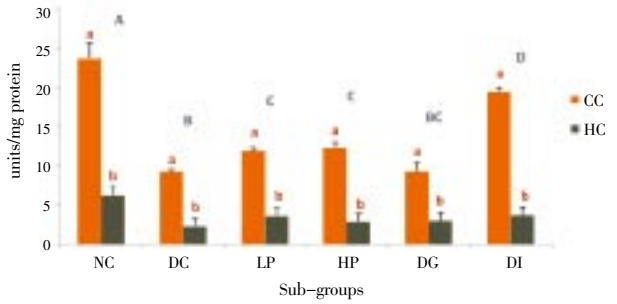

Figure 1. Superoxide dismutase activity in Cerebral cortex and Hippocampus of normal and experimental diabetic rats

NC, Normal control; DC, Diabetic control; LP, Low *P. granatum* (75mg/kg body weight); HP, High *P. granatum* (150mg/kg body weight); DG, Diabetic rats treated with glibenclamide; DI, Diabetic rats treated with insulin. Values are mean±S.E of 6 animals/group. Significance between the group means is represented in upper case and between regions in lower case. Those not sharing the same letters are significantly different at P<0.05.

In diabetic rats, the GPx activity was reduced significantly (P<0.05) with respect to the control rats. Rats supplemented with *PGPE* at 75 mg /kg body weight showed significant (P<0.05) elevation in the activity by (41% and 31%) in CC and HC. Whereas significant (P<0.05) increases of (27% and 13%) were observed in the animals supplemented with *PGPE* at 150 mg/kg body weight (Fig. 2). However, the changes were insignificant between high *PGPE* supplemented, glibenclamide and insulin treated animals.

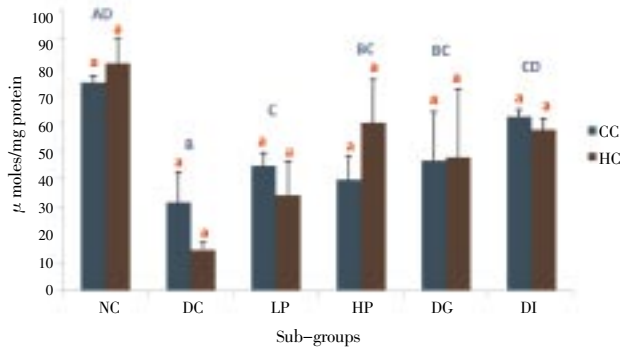

Figure 2. Glutathione Peroxidase activity in Cerebral cortex and Hippocampus of normal and experimental diabetic rats.

NC, Normal control; DC, Diabetic control; LP, Low *P. granatum* (75mg/kg body weight); HP, High *P. granatum* (150mg/kg body weight); DG, Diabetic rats treated with glibenclamide; DI, Diabetic rats treated with insulin. Values are mean±S.E of 6 animals/group. Significance between the group means is represented in upper case and between regions in lower case. Those not sharing the same letters are significantly different at P<0.05

3.3 Lipid peroxidation and protein carbonyl content

The levels of the MDA significantly (P<0.05) increased in diabetic rats with respect to the normal control rats as shown in the Fig. 3. Supplementation of low *PGPE*

significantly (P<0.05) decreased MDA levels by 57% and 66% in CC and HC. Whereas 49% and 53% reduction is observed in the animals fed with high *PGPE*. The optimum effect of *PGPE* was to found to be superior to that of the reference antidiabetic drug glibenclamide used in the study. The MDA levels in the insulin treated animals were similar to the normal rats.

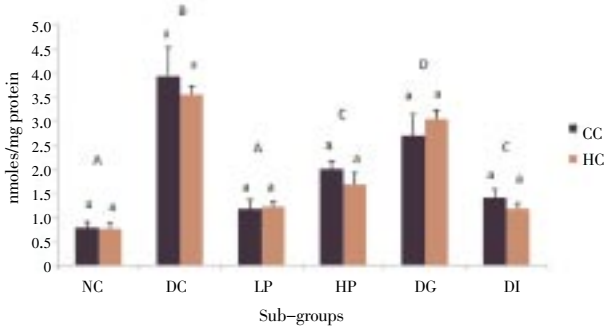

Figure 3. Changes in MDA content in cerebral cortex and Hippocampus of normal and experimental diabetic rats.
NC, Normal control; DC, Diabetic control; LP, Low *P. granatum* (75mg/kg body weight); HP, High *P. granatum* (150mg/kg body weight); DG, Diabetic rats treated with glibenclamide; DI, Diabetic rats treated with insulin. Values are mean±S.E of 6 animals/group. Significance between the group means is represented in upper case and between regions in lower case. Those not sharing the same letters are significantly different at P<0.05

PC contents, the marker of protein oxidation showed significant (P<0.05) changes (Fig. 4) between the regions. Significant (P<0.05) decreases (63% and 80%) were noted in the CC and HC of the animals supplemented with high *PGPE* extract of 150 mg/ kg body weight. The animals fed with low dose of *PGPE* showed a significant (P<0.05) reduction (50%) in both the regions. A similar trend was observed in the glibenclamide and insulin treated animals.

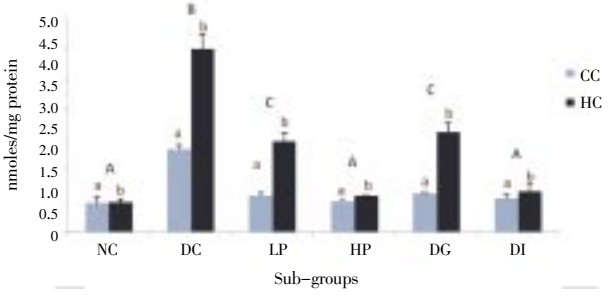

Figure 4. Changes in Protein carbonyl content in cerebral cortex and Hippocampus of normal and experimental diabetic rats
NC, Normal control; DC, Diabetic control; LP, Low *P. granatum* (75mg/kg body weight); HP, High *P. granatum* (150mg/kg body weight); DG, Diabetic rats treated with glibenclamide; DI, Diabetic rats treated with insulin. Values are mean±S.E of 6 animals/group. Significance between the group means is represented in upper case and between regions in lower case. Those not sharing the same letters are significantly different at P<0.05

4. Discussion

In the present study, the administration of *PGPE* showed anti-hyperglycemic effect in alloxan-induced diabetic rats. The possible mechanism by which *PGPE* brings about its hypoglycemic action may be by potentiation of the insulin effect of plasma by increasing either the pancreatic secretion of insulin from β-cells of islets of Langerhans or its release from the bound form.[16]

Brain is susceptible to oxidative damage due to its high oxygen consumption rate and high levels of polyunsaturated fatty acids, high concentration of iron and paradoxically low levels of defense mechanisms [17]. Several studies have clearly demonstrated that diabetes is associated with increased oxidative stress in brain.[18]

In the present study, the activity of endogenous enzymatic antioxidants such as SOD and GPx has been reduced in the different regions of brain of the diabetic rats. SOD is an important antioxidant defence enzyme, which catalyzes the dismutation of superoxide radicals. GPx plays an important role in detoxifying H_2O_2 and protects the membrane from lipid peroxidation. Therefore a reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide. Earlier studies also reported decrease in the activity of SOD[19] and GPx in the brain of diabetic rats. [20]

Administration of low and high *PGPE* up regulated SOD and GPx activity in both the regions of the brain. The results obtained were in agreement with the studies of Althunibat *et al*.[21] *P. granatum* extract has been reported to be rich in terpenoids such as ursolic acid and oleanolic acid[22], which may help to scavenge the free radicals generated during diabetes.

There is neurochemical evidence that brain structures have different levels of vulnerability to oxidative stress[1]. In the present study, the cerebral cortex has shown higher SOD levels compared to the hippocampus, suggesting more oxidative stress in the hippocampus which may be due to the differences in their iron content and also in their oxygen consumption rate, which influence the production of ROS.

Lipid peroxide mediated tissue damage has been observed in the development of both type I and II diabetes mellitus [23]. Increased concentration of MDA has been observed in the CC and HC of the alloxan induced diabetic animals. Kuhad *et al* [7] (2008) also reported increased lipid peroxides in CC and HC of diabetic brain. Significant decrease in the levels of MDA in CC and HC of *PGPE* treated rats and increase in the activities of antioxidant enzymes indicates an adaptive mechanism in response to oxidative stress by quenching the free radicals. Our results are in agreement with Althunibat *et al* [21], wherein supplementation of *PGPE* restored the MDA levels in different tissues.

Protein carbonyl groups are products of oxidative damage to proteins and are sensitive markers for oxidative stress evaluation [24]. Our results shows a significant increase in the formation of PC groups in rats treated with alloxan, possibly because of the depletion in the antioxidant enzymes. Induction of protein carbonyls in rats exposed to alloxan

has been reported earlier. [25] Particularly, the HC showed a significant increase in protein carbonyl levels compared to the CC, suggesting that this region is more vulnerable to oxidative stress. The supplementation of high *PGPE* was more effective than glibenclamide in attenuating the protein oxidation in both the regions in diabetic rats and confirms its antioxidant property.

In conclusion, the present study showed decreased antioxidant enzyme activities with concomitant increase in MDA and PC in alloxan-induced diabetic rats. Supplementation of *PGPE* prevented or reversed the changes associated with diabetes induced oxidative stress. These findings support the role of dietary antioxidant supplementation in the prevention of diabetes-induced oxidative stress, and suggest a potential therapeutic benefit for the reduction of diabetic complications associated with oxidative stress. However, further research is needed, for the better understanding of the mechanism of action by which it modulates the oxidative stress in central nervous system due to diabetes.

Conflict of interest

The authors declare that there are no conflicts of interest.

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