Chronic Oral Pelargonidin Alleviates Streptozotocin-Induced Diabetic Neuropathic Hyperalgesia in Rat: Involvement of Oxidative Stress

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ABSTRACT

Background: Diabetes mellitus in some clinical cases is accompanied with hyperalgesia. In this study, we evaluated the possible beneficial effect of chronic pelargonidin (PG) treatment on hyperalgesia in streptozotocin (STZ)-diabetic neuropathic rat. **Methods:** Male Wistar rats (n = 56) were divided into seven groups, i.e. control, diabetic, PG-treated control, PG (single- and multiple-dose)-treated diabetic, and sodium salicylate-treated control and diabetics. For induction of diabetes, STZ was injected i.p. at a single dose of 60 mg/kg. PG was orally administered at a dose of 10 mg/kg once and/or on alternate days for 8 weeks; 1 week after diabetes induction. After two months, hyperalgesia was assessed using standard formalin and hot tail immersion tests. Meanwhile, markers of oxidative stress in brain were measured. One-way analysis of variance was used for statistical analysis of the data. **Results:** Diabetic rats showed a marked chemical and thermal hyperalgesia, indicating that development of diabetic neuropathy and PG treatment (especially multiple-doses) significantly ameliorated the alteration in hyperalgesia (P<0.05-0.01) in diabetic rats as compared to untreated diabetics. PG (multiple doses) also significantly decreased diabetes-induced thiobarbituric acid reactive substances formation and non-significantly reversed elevation of nitrite level and reduction of antioxidant defensive enzyme superoxide dismutase. **Conclusion:** These results clearly suggest that PG prevents diabetic neuropathic hyperalgesia through attenuation of oxidative stress. *Iran. Biomed. J. 14 (1 & 2): 33-39, 2010*

INTRODUCTION

Diabetes mellitus is one of the serious problems worldwide and the number of diabetic people is estimated to be increased markedly by the year 2030 [1]. Uncontrolled chronic hyperglycemia in diabetes leads to severe complications including neuropathy, retinopathy, and autonomic dysfunctions. Diabetic neuropathy as observed in some deranged conditions of nociception (i.e. hyperalgesia) is the most common complication with an incidence of more than 50% [2]. Diabetes-induced deficits in motor and sensory nerve conduction velocities and other manifestations of peripheral diabetic neuropathy have been well correlated with chronic hyperglycemia. Hyperglycemia could also lead to an increased oxidative stress (enhanced free radical formation and/or a defect in antioxidant defenses), advanced glycation end product formation, nerve hypoxia/ischemia, and impaired nerve growth factor support [3-5].

Several studies suggest that an oxidative stress may be one of the major pathways in the development of diabetic neuropathy and antioxidant therapy can prevent or reverse hyperglycemia-

induced nerve dysfunctions [6, 7]. Recent interests are focusing on the use of non-vitamin antioxidants such as flavonoids in reducing the devastating complications of diabetes in experimental animals and patients [8]. Plant-based pharmaceuticals including flavonoids have been employed in the management of various mankind diseases [8]. They are as an essential part of human diet and are present in plant extracts that have been used for centuries in oriental medicine. Antioxidant properties, reactive oxygen species (ROS) scavenging, and cell function modulation of flavonoids could account for the large part of their pharmacological activity [8, 9]. Since diabetes mellitus is considered as a free radical mediated disease, there has been renewed interest in the use of flavonoids in diabetes research. Anthocyanins and their aglycone derivatives anthocyanidins are important groups of flavonoids. Of these groups, pelargonidin (PG) has been reported to exhibit an anti-diabetic activity in diabetic rats [10, 11].

Recent reports have also demonstrated multiple benefits associated with the consumption of pelargonidin-rich fruits including decreased vulnerability to oxidative stress, reduced ischemic brain damage, protection of neurons from stroke-induced damage and the reversal of age-related changes in brain and behavior [12]. Berry fruits contain high amounts of anthocyanins, which play a major role as free radical scavengers which could inhibit H_2O_2-induced lipid peroxidation in the rat brain homogenates [13]. Therefore, we designed this study to investigate, for the first time, the effect of chronic PG treatment, a free radical scavenger on hyperalgesia in streptozotocin (STZ)-diabetic neuropathic rat, using standard formalin and hot tail immersion tests and also to evaluate the role of oxidative stress.

MATERIALS AND METHODS

Animals. Male Albino Wistar rats, weighing 215-285 g and 10-12 weeks old, were procured from Pasteur Institute of Iran, Tehran. The animals were housed in an air-conditioned colony room on a 12/12 cycle at 21-23°C with 30-40% humidity and supplied with a standard pelleted diet and tap water ad libitum. Procedures involving animals and their care were conducted in the conformity with NIH Guidelines for the Care and Use of Laboratory Animals.

Experimental protocol. The rats (n = 56) were randomly allocated and similarly grouped into seven groups: normal vehicle-treated control, PG (multiple doses)- and sodium salicylate (SS)-treated control, vehicle-treated diabetic, PG-treated diabetics (single dose), PG-treated diabetics (multiple dose), and SS-treated diabetics. SS (200 mg/kg, i.p.) was administered 1 h before conducting the formalin test as positive control. The rats were rendered diabetic by a single i.p. injection of 60 mg kg^{-1} STZ, freshly dissolved in a cold normal saline. Control animals received an injection of an equivalent volume of normal saline and vehicle. Diabetes was confirmed by the presence of hyperglycemia, polyphagia, polydipsia, polyuria and weight loss. One week after STZ injection, the blood samples were collected and serum glucose concentrations were measured using glucose oxidation method (Zistshimi, Tehran). Only those animals with serum glucose higher than 250 mg dl^{-1} were selected as diabetics for the following experiments. The day on which hyperglycemia had been confirmed was designated as day 0. PG was either administered once p.o. (using gavage needle) one week after STZ injection at a dosage of 10 mg/kg body weight (single dose) or on alternate days for a period of 8 weeks at the same dose (multiple doses). Dose of PG was selected according to the Roy *et al.* [11] on the basis of our pilot study. PG was dissolved in 10% cremophor with further dilution in distilled water before use. Changes in body weight, food consumption and water intake were regularly observed during the experimental period.

Formalin test. The previously described method was applied [14]. Briefly, each animal was acclimatized to the observation box before any testing began. Then, it was given a subcutaneous injection of 50 μl of 2.5% formalin into the plantar surface of one hind paw. It was then immediately placed in a Plexiglas box. Observations continued for the next 60 min. A nociceptive score was determined for 5 min blocks by measuring the amount of time spent in each of the four behavioral categories: 0, the position and posture of the injected hind paw is indistinguishable from the contralateral paw; 1, the injected paw has little or no weight placed on it; 2, the injected paw is elevated and is not in contact with any surface; 3, the injected paw is licked, bitten, or shaken. Then, a weighted nociceptive score, ranging from 0 to 3 was calculated by multiplying the time spent in each category by the category weight, summing these

products and dividing by the total time for each 5 min block of time. The first 10 min post-injection was considered as the early (first) phase and the time interval 15-60 as the late (second) phase.

Hot tail immersion test. Diabetic thermal hyperalgesia was assessed using tail immersion test [15]. After adaptation, rat tail was immersed in warm water (49°C) and the tail-flick response latency (withdrawal response of tail) was observed as the endpoint response. Each experiment was repeated 4 times for each animal with an interval of 2 min and its average was reported. Meanwhile, a cut-off time of 30 s was also considered.

Determination of brain malondialdehyde (MDA) concentration. The rats were anesthetized with ketamine (100 mg/kg), decapitated, the brains were removed, blotted dry and weighed. Then, they were made into 10% tissue homogenate in ice-cold 0.9% saline solution and centrifuged (1000 ×g, 4ºC, 10 min) to remove particulates. The obtained supernatant was aliquoted, then stored at -80°C until assay. The MDA concentration (thiobarbituric acid reactive substances, TBARS) in the supernatant was measured as described before [16]. Briefly, trichloroacetic acid and TBARS reagent were added to the supernatant, then mixed and incubated at 100ºC for 80 min. After cooling on ice, the samples were centrifuged at 1000 ×g for 20 min and the absorbance of the supernatant was read at 532 nm. TBARS results were expressed as MDA equivalents using tetraethoxypropane as standard.

Measurement of brain* superoxide dismutase *(SOD) activity. The supernatant of brain homogenate was obtained as described earlier. SOD activity measurement was according to the previous works [16]. Briefly, supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer (pH 7.8 at 37ºC) for 40 min and nitro blue tetrazolium was added. Blue formazan was then monitored spectrophotometrically at 550 nm. The amount of protein that inhibited nitro blue tetrazolium reduction by 50% of maximum was defined as 1 nitrite unit of SOD activity.

Assay of brain nitrite concentration. Supernatant nitrite content was assayed by the Griess method. Because NO is a compound with a short half life and is rapidly converted to the stable end products nitrate (NO_3^-) and nitrite (NO_2^-), the principle of the assay is the conversion of nitrate into nitrite by cadmium and followed by color development with Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) in acidic medium. The total nitrite was measured by Griess reaction. The absorbance was determined at 540 nm with a spectrophotometer.

Protein assay. The protein content of the supernatant was measured with Bradford method using bovine serum albumin (Sigma Chemical, St. Louis, MO) as the standard [17].

Chemicals. PG, cremephor and reagents for oxidative stress assessment were purchased from Sigma Chemical and Fluka (St. Louis, Mo., USA). SS was obtained from Darupakhsh (Tehran, Iran) and STZ from Pharmacia and Upjohn (USA). All other chemicals were purchased from Merck (Germany) and Temad (Tehran).

Statistical analysis. All results are expressed as means ± S.E.M. For multiple comparisons, one-way analysis of variance (ANOVA) was used. When ANOVA showed a significant difference, Tukey's post hoc test was applied. Statistical significance was regarded as $P<0.05$.

RESULTS

General considerations. Two rats died in diabetic groups at 2nd week after STZ injection due to possibly cytotoxic effect of the drug and one rat was excluded at final weeks of the study due to severe weight loss and immotility. Meanwhile, 77.5% of rats were made diabetic following STZ injection and had a serum glucose level higher than 250 mg/dl. After 8 weeks, the weight of the vehicle-treated diabetic rats was found to be significantly decreased as compared to control rats ($P<0.01$) and PG treatment (single and multiple doses) caused a less significant decrease in diabetic rats as compared to diabetics ($P<0.05$-0.01). In this respect, PG administration for 8 weeks (multiple doses) was more effective in preventing weight loss than its single dose. In addition, diabetic rats had also an elevated serum glucose level over those of control rats ($P<0.001$) and treatment of diabetic rats with PG (single and multiple doses) caused a significant decrease in the serum glucose ($P<0.01$-0.005) relative to vehicle-treated diabetics. Again, PG administration for 8 weeks was more effective in serum glucose reduction than its single dose. Meanwhile, PG treatment of control rats did not produce any significant change regarding serum glucose level (Fig. 1).

Formalin test. Hind limb formalin injection produced a marked biphasic response in the rats of all groups (Fig. 2). Hyperalgesia was significantly ($P<0.05$) greater in untreated diabetics than in control rats in early and late phases of the test. Pre-treatment of control and diabetic rats with SS (200 mg/kg, i.p.) 1 hour before the test caused a significant reduction ($P<0.01$) in nociceptive score only in the second phase of the formalin test. In addition, treatment of diabetic rats with PG (10 mg/kg), especially with its multiple-dose caused lower nociceptive scores in both phases of the formalin test as compared to vehicle-treated diabetic rats ($P<0.05$-0.01). No such response was observed for PG-treated controls.
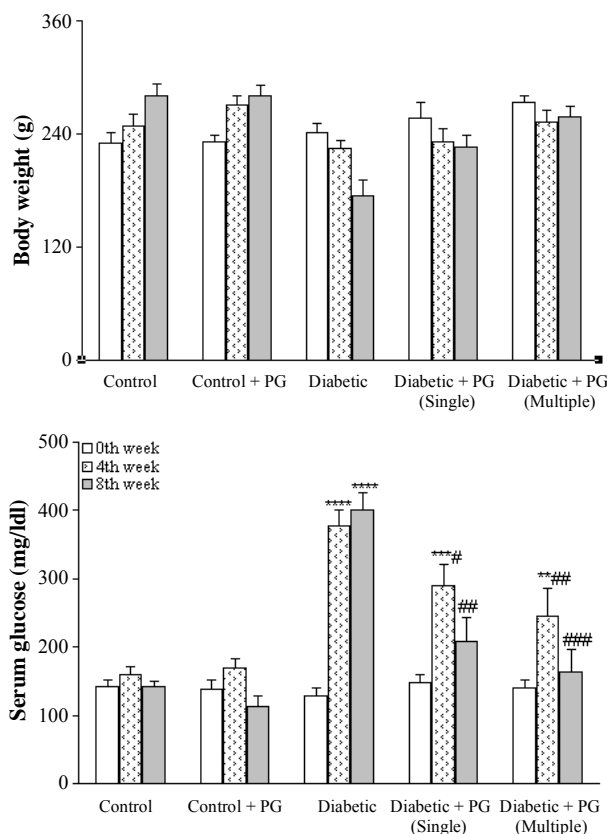

Fig. 2. The effect of pelargonidin and sodium salicylate on nociceptive scores in the first (early) and second (late) phases of the formalin test. All data represent mean ± S.E.M. *$P<0.05$, **$P<0.01$ (as compared to control); #$P<0.05$, ##$P<0.01$ (as compared to diabetic).

significantly reversed on treatment with PG, especially again with its multiple dose ($P<0.05$).

Markers of oxidative stress. Regarding brain lipid peroxidation and oxidative stress markers (Figs. 4-6), STZ-induced diabetes resulted in significant elevation of MDA and nitric oxide content ($P<0.05$) and non-significant reduction of SOD activity and treatment of diabetic rats with PG (multiple doses) significantly attenuated the increased MDA content ($P<0.05$). However, level of SOD was non-significantly higher and level of nitrite was lower in PG-treated diabetics as compared to diabetic group.

Fig. 1. Body weight and serum glucose concentration in different weeks (means ± S.E.M). **$P<0.01$, ***$P<0.005$, **** $P<0.0001$ (as compared to week 0 in the same group); #$P<0.05$, ##$P<0.01$, ###$P<0.005$ (as compared to diabetics in the same week). PG stands for pelargonidin.

Thermal hyperalgesia. A significant decrease in tail-flick latency was observed after two months of diabetes in hot tail immersion test ($P<0.05$) (Fig. 3). This deficit in tail-flick response latency was
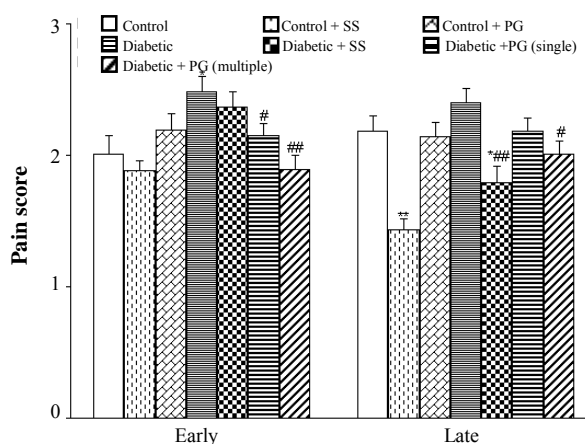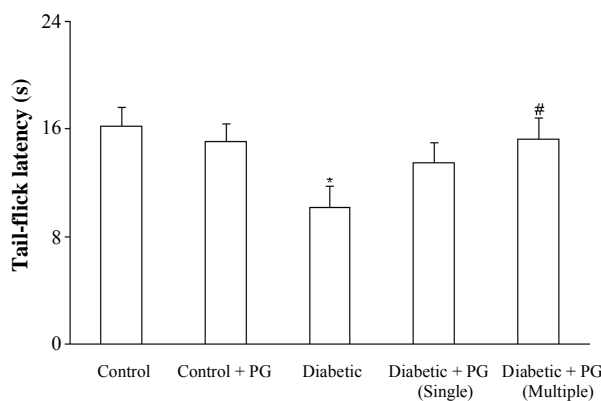

Fig. 3. The effect of pelargonidin (PG) treatment on hyperalgesia in hot tail immersion test. All data represent mean ± S.E.M. *$P<0.05$ (as compared to control); # $P<0.05$ (as compared to diabetic).
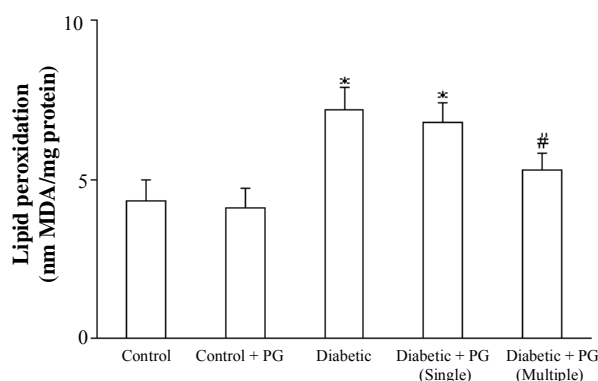
Fig. 4. MDA concentration in whole brain homogenate from different groups. $^*P<0.05$ (as compared to controls); $^{\#}P<0.05$ (as compared to diabetics). PG stands for pelargonidin.

DISCUSSION

In this study, development of diabetic neuropathy in STZ-induced diabetic rats was confirmed after two months, which was consistent with previous reports [18]. It has been well established that oxidative stress finally leads to nerve deficits in diabetic rats [7]. Some studies indicated that a hypoxic mechanism is involved in peripheral diabetic neuropathy. Decreased nerve blood flow and ensuring endoneurial hypoxia may cause functional and morphological abnormalities of nervous system [4]. In addition, increased free radicals due to hyperglycemia may affect central and peripheral nervous system [19]. Furthermore, there is some evidence for involvement of sorbitol pathway related to glucose metabolism in the pathogenesis of diabetic neuropathy [3].

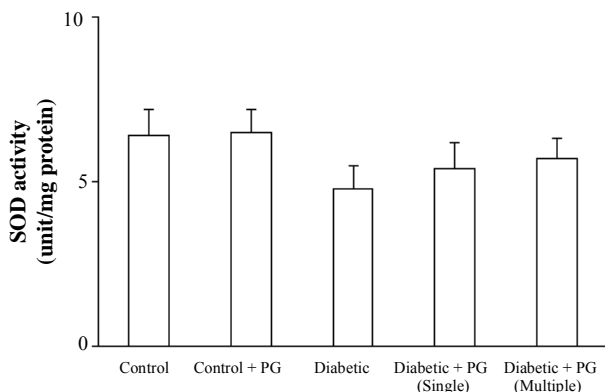In this study, we observed a reduction in tail-flick latencies in hot immersion test in diabetic rats,

Fig. 5. Superoxide dismutase activity in whole brain homogenate from different groups. PG stands for pelargonidin.

which indicates thermal hyperalgesia. Several researchers have reported hyperalgesia in diabetic rats [20, 21]. Such mechanisms including tissue injury due to ischemia, sensitization of peripheral receptors and ectopic activity in sprouting fibers and alterations in dorsal root ganglia cells have been reported to contribute to changes in nociception [22]. It is a well-established fact that diabetic rats display exaggerated hyperalgesic behavior in response to noxious stimuli that may model aspects of painful diabetic neuropathy [23]. For this reason, STZ-diabetic rats have been increasingly used as a model of painful diabetic neuropathy to assess the efficacy of potential analgesic agents [24].
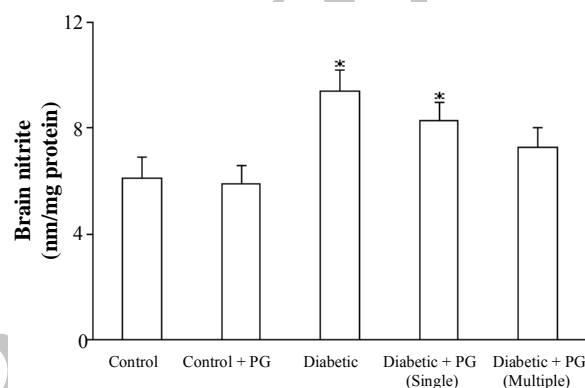

Fig. 6. Nitrite content in whole brain homogenate from different groups. $^*P<0.05$ (as compared to controls). PG stands for pelargonidin.

Although mechanisms causing these symptoms are complicated, direct toxicity of hyperglycemia in the peripheral nervous system [25], an increased activity of primary afferent fibers, increased release of glutamate, reduced activity of both opioidergic and GABAergic inhibitory systems [26], altered both sensitivity of the dopaminergic receptors and responsiveness of the dopaminergic system (possibly through endogenous enkephalinergic system) [27] and also alterations in L-type Ca^{2+} channels [28] are involved in the modulation of nociception in diabetic rats.

Increased free radical mediated toxicity has also been well documented in clinical diabetes [29] and STZ-diabetic rats [30]. The elevated level of toxic oxidants in diabetic state may be due to the processes such as glucose oxidation and lipid peroxidation [29]. STZ-diabetes is also characterized by several derangements in endogenous antioxidant enzymes [29] and induction of antioxidant enzymes is a critical approach for protecting cells against a variety of endogenous and exogenous toxic

compounds such as reactive oxygen species [30]. Increased level of MDA and a reduction in the activity of SOD were observed in diabetic rats in our study which might be due to the increased lipid peroxidation and overproduction of ROS, which is in agreement with the previous report [29].

Oral administration of PG for two months in our study also produced a significant analgesic effect at both phases of the formalin test only in diabetic rats and SS significantly reduced the nociceptive score only in the second phase of the formalin test in control and diabetic rats. It has been known that central-acting drugs like narcotics inhibit both phases of the formalin test equally [31], while peripheral-acting drugs like aspirin only inhibit the late phase [32]. Therefore, the effect of PG in this study could be mediated possibly through a central and/or a peripheral mechanism. One of the possible mechanisms that could partially explain the beneficial analgesic property of PG may be attributed to its hypoglycemic and antioxidant effects. Since hyperglycemia in diabetic state could induce some functional alterations in the nervous system [21], PG may have attenuated the hyperalgesic condition in formalin test.

The results of the present study also showed that PG administration could exert an anti-hyperglycemic effect in diabetic rats and not in control normoglycemic rats. Blood glucose level is mainly controlled by insulin secretion from pancreatic β cells and insulin action on liver, muscle and other target tissues. STZ destroys pancreatic β cells by different mechanisms such as DNA damage through alkylation, depletion of NAD$^+$, and generation of ROS species to induce type 1 diabetes. Decrease in elevated blood glucose and normalization of glucose tolerance curve in diabetics following PG have been attributed to the stimulatory activity of PG by protecting β cells of islets of Langerhans and enhancing their functions in diabetic rats [11, 33]. PG may have protected and stimulated pancreatic β cells by its antioxidant activity and/or by other activities like modulation of gene expression and interaction with signal transduction pathways, leading to insulin release in diabetic rats [11]. However, PG does not change insulin level and other biochemical parameters in control rats to maintain their physiological balance. A similar result has been obtained with other flavonoids, which had almost no effects on glycemic status and oxidative stress markers in normal rats, but showed significant effects on these parameters in STZ-treated rats [34].

Overall, PG showed an anti-hyperglycemic and protective effect on diabetic neuropathy as it was evident from a reduction in chemical and thermal hyperalgesia in treated diabetic rats and it seems that PG may be a good adjuvant in the present armamentarium of anti-diabetic drugs to prevent the development of some diabetic complications.

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