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Research Article
Neuroprotective Effects of Protocatechuic Acid in Diabetes Induced Neuropathic Pain

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Abstract

Background and Objective: Chronic hyperglycemia leads to the secondary complications in diabetic condition which are difficult to control. Supplementations of compounds having strong antioxidant property are reported to prevent the progression of various diseases and disorders. So, the present study was designed to assess the effects of protocatechuic acid (PCA) an antioxidant compound in streptozotocin (STZ) induced neuropathic pain. **Materials and Methods:** Male albino rats of wistar strain were selected in the study. Diabetes was induced in the rats by a single intraperitoneal dose of streptozotocin (60 mg kg^{-1}). Protocatechuic acid (40 mg kg^{-1}, p.o) was administered for 21 days after the confirmation of neuropathic pain. Animals were tested for behavioural changes (von Fray hair test, hot plate test, cold allodynia and rota rod test) and biochemical alteration (serum glucose, lipid peroxidation and reduced glutathione). Data was analyzed by one-way analysis of variance (ANOVA) followed by the Dunnet test and GraphPad prism. **Results:** Treatment with protocatechuic acid for 21 days in neuropathic pain induced rats showed a significant prevention of altered body weight and glucose level. Protocatechuic acid supplementation showed a significant reduction in paw withdrawal threshold in Von frey filament test, hot plate test and cold allodynia. The level of lipid peroxidation and nitric oxide was found to be significantly reduced whereas the level of reduced glutathione was significantly increased in treatment group as compared to diseased group. **Conclusion:** The observed neuroprotective effect of protocatechuic acid might be due to its strong antioxidant activity.

Key words: Neuropathic pain, protocatechuic acid, von fray filament test, nitric oxide, biomarkers of oxidative stress

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Neuropathy is one of the most common complications of diabetes affecting more than 50% patient with diabetes[1]. Streptozotocin (STZ) induced diabetic rat model has been widely used to mimics insulin-dependent diabetes mellitus and a number of abnormalities. A single dose of STZ leads to the development of hyperglycemia, after three weeks pain was developed in rats which are similar to those observed in patients with painful diabetic neuropathy[2]. 50% of diabetic patients will develop neuropathic pain after 25 year of the disease and is the major causes of mortality and morbidity. The role of oxidative stress in the pathogenesis and progression of diabetic neuropathy has been considered as one of the major cause of secondary complications[3]. Protocatechuic acid (PCA)is a dihydroxybenzoic acid, a type of phenolic acid. It is a major metabolite of antioxidants found in green tea. It is reported to have anti-diabetic[4], anti-cancer[5], anti-oxidant[6], anti-ulcer[7], anti-hyperlipidemic[8] and neuro protective activity[9,10]. Study showed that the supplementation of antioxidant compounds in chronic illness prevents or control the progression of the disease[3,9,10]. As there is no report regarding the usefulness of protocatechuic acid in neuropathic pain, the present study was design to study its effects on diabetes induced neuropathic pain.

MATERIALS AND METHODS

Procurement and preparation of drug solution: This study was carried out in 2014. PCA and STZ were purchased from Sigma Aldrich USA. Pregabaline as standard drug procured from Micro Lab. India. All other chemicals used in the study were procured from reputed supplier. PCA and pregabalin were suspended in distilled water, prepared freshly before administration. STZ was dissolved in freshly prepared, ice chilled citrate buffer (pH 4.4). All the drug solutions were freshly prepared before starting experiment.

Experimental animals: Male wistar rats (200-300 g) were used in the study. The animals were procured from Lachmi biotech, Pvt. Ltd, Pune and were placed separately in polypropylene cages. The rats were maintained under standard laboratory conditions with 12 h light and 12 h dark cycles throughout the experiment. Animals had free access of water and standard laboratory feed *ad libitum*. The experimental protocol was approved by Institutional animal ethics committee (IAEC).

Induction of diabetes: Diabetes was induced in the rat by intraperitoneal injection of STZ (60 mg kg^{-1}) prepared in citrate buffer (pH 4.4,0.1M). The control rats received an equal volume of citrate buffer[11,12]. Diabetes was confirmed after 72 h of STZ injection, the blood sample was collected via retro orbital plexus using capillary glass tubes and serum glucose level was estimated by using standard diagnostic kit (Span Diagnostic, India)[13]. The rats having serum glucose level more than 250 mg dL^{-1} were selected and used for the present study. Body weight, food intake and the fluid intake was also monitored in the study[14].

Experimental design: Rats were divided in five groups, each group contain 6 animals. Group I: Served as normal control and received equal volume of citrate buffer as vehicle for, Group II: Served as diabetic rats (injected with STZ 60 mg kg^{-1}, i.p), Group III: Served as treatment group and received PCA (40 mg kg^{-1}, p.o) in diabetic rats, Group IV: Rat received only PCA (40 mg kg^{-1} p.o) and Group V: Served as standard group and received pregabalin (10 mg kg^{-1}, p.o) for seven weeks.

Assessment of biochemical parameters: At the end of experiment period body weight of animals and serum glucose level was monitored.

Assessment of neuropathic pain

Mechanical hyperalgesia (von Frey test): The mechanical hyperalgesia was assessed by the von Frey test[15]. Rats were individually placed in suspended acrylic chamber on a mesh floor. After the acclimatization period for 30 min, planter surface of hind paw was tested with von Frey hair. The latency of paw withdrawal was recorded. The paw withdrawal time was measured weekly after confirmation of diabetes to confirm neuropathic pain[11].

Cold allodynia: The cold allodynia was assessed by acetone test, rats were individually placed in suspended acrylic chamber on a mesh floor. After the acclimatization period for 30 min, cotton buds dipped in acetone were applied to the planter surface of hind paw with sufficient force and paw withdrawal latency was recorded[11,12].

Hot plate method: The responses like jumping, withdrawal of the paws and licking of the paws were recorded using hot plate test. The temperature of the hot plate was regulated to 55±2°C. Hyperalgesia is an early symptom occurs in neuropathic pain in an experimental animals[13].

Grip strength using rotarod test: The test is used to evaluate the activity of drugs interfering with motor coordination. In brief rats were placed individually for 1 min on the rotating drum (20 rpm). The number of animals falling from the roller during in 1 min was recorded[14].

Assessment of tissue parameters: At the end of treatment period, rats were sacrificed and isolated sciatic nerve was quickly transferred to ice-cold Tris hydrochloric buffered saline (pH 7.4), after centrifugation the supernatant was used for the estimation of lipid peroxidation[16] and reduced glutathione[17].

Statistical analysis: Data was expressed as Mean±SEM. Multiple group comparisons were carried out using one-way analysis of variance (ANOVA) followed by the Dunnet test. Statistical significance was acceptable to a level of $p<0.05$. All statistical analysis was performed using statistical software (GraphPad Prism, version 0.5).

RESULTS

Effect of PCA on glucose level and body weights: Diabetic rats showed a significant elevation in glucose level and reduction in body weight as compared to control rats. Treatment with PCA (40 mg kg^{-1}) and PG (10 mg kg^{-1}) showed the significant increase ($p<0.05$) in the body weight and reduction in blood glucose level which was not significant as compared to STZ diabetic control (Table 1).

Effect of PCA on mechanical hyperalgesia and cold allodynia: Paw withdrawal threshold of STZ diabetic control rat was significantly decreased ($p<0.05$) as compared to normal non-diabetic control rats. PCA administration (40 mg kg^{-1}) resulted in a significant increase ($p<0.05$) in paw withdrawal threshold as compared to STZ diabetic control rats. Rats treated with PG (10 mg kg^{-1}) also showed the significant increase ($p<0.05$) in the mean paw withdrawal threshold as compared to STZ diabetic control rat. However, this inhibition in decrease in the mean paw withdrawal threshold by PG (10 mg kg^{-1}) treatment was more significant ($p<0.05$) than PCA (40 mg kg^{-1}) treatment (Fig. 1 and 2).

Effect of PCA on thermal hyperalgesia and muscle grip strength: Administration of STZ resulted in a significant decrease ($p<0.05$) in the mean paw withdrawal latency in the STZ diabetic control rats as compared to normal rats. This decrease in mean paw withdrawal latency after induction of DN was significantly inhibited by the PCA (40 mg kg^{-1}) treatment as compared to STZ diabetic control rats. Rats treated with PG (10 mg kg^{-1}) significantly ($p<0.05$) decreased mean paw withdrawal latency as compared to STZ diabetic control rats (Fig. 3). Administration of STZ (i.p.) resulted in a significant decrease ($p<0.05$) in the muscle strength in STZ diabetic control rat as compared to normal rats after 60 days.

Table 1: Effect of PCA on glucose level and body weight

Groups	Body weight (g)	Blood glucose (mg dL^{-1})
Normal control	262.2±1.990	89.72±3.281
STZ diseased control	167.8±4.402***	237.5±18.16***
STZ+PCA	221.6±4.913###	190.7±2.642#
PCA	258.3±3.658###	200.3±6.19
STZ+PG	248.3±1.869###	180.7±3.728#

All values are presented as Mean±SEM, (n = 6). $p<0.001$*** compared to control group. $p<0.05$#, $p<0.001$### compared to disease group

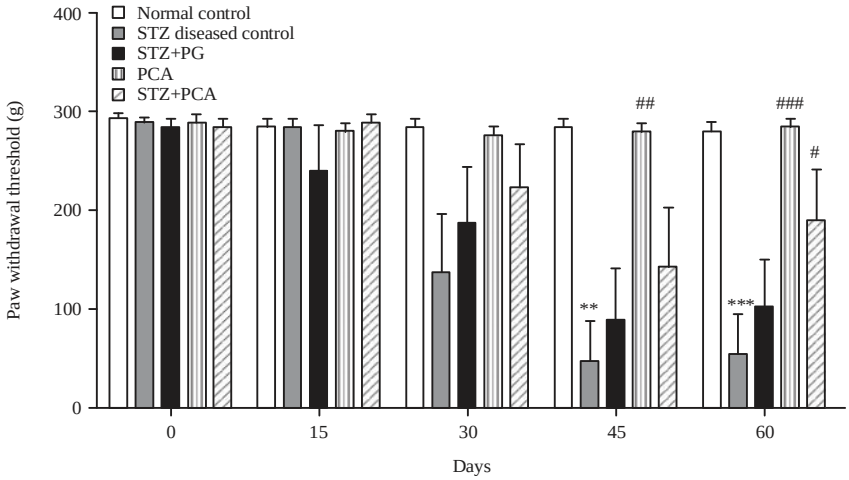

Fig. 1: Effect of PCA on mechanical hyperalgesia

All values are presented as Mean±SEM, (n = 6). $p<0.01$**, $p<0.001$*** compared to control group. $p<0.05$#, $p<0.01$##, $p<0.001$### compared to disease group

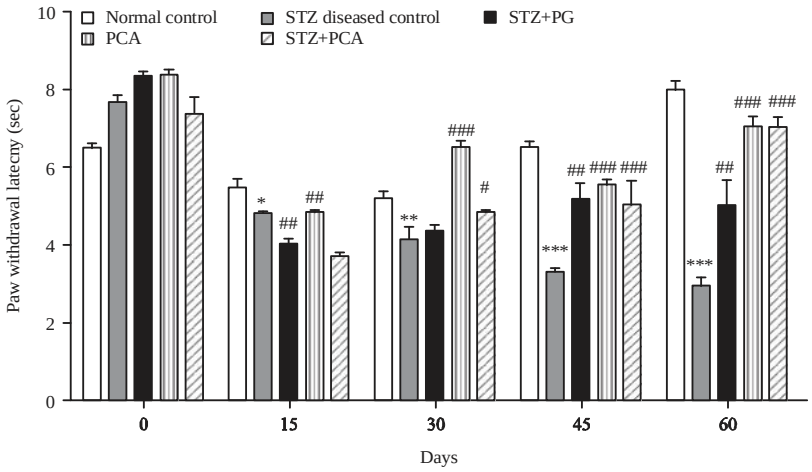

Fig. 2: Effect of PCA on cold allodynia

All values are presented as Mean±SEM, (n = 6). p<0.05*, p<0.01**, p<0.001*** compared to control group. p<0.05#, p<0.01##, p<0.001### compared to disease group

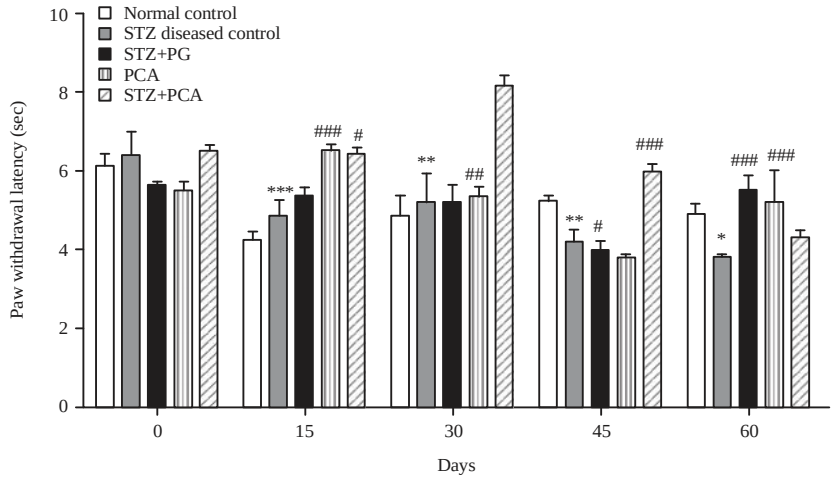

Fig. 3: Effect of PCA on thermal hyperalgesia

All values are presented as Mean±SEM, (n = 6). p<0.05*, p<0.01**, p<0.001*** compared to control group. p<0.05#, p<0.01##, p<0.001### compared to disease group

Rats treated with PCA (40 mg kg^{-1}) showed the significant and dose dependant increment ($p<0.05$) in this reduced level as compared to STZ diabetic control rat. When compared with STZ diabetic control rat, treatment with PG (10 mg kg^{-1}) showed the significant increase ($p<0.05$) in the muscle grip strength (Fig. 4).

Effect of PCA on LPO and GSH levels in diabetic neuropathic pain: Administration of STZ resulted in a significant increase ($p<0.05$) in the level of neural MDA in STZ diabetic control rat as compared to normal rats after 60 days. Rats treated with PCA (40 mg kg^{-1}) showed the significant attenuation ($p<0.05$) in this elevated level of MDA as compared to STZ diabetic control rat. When compared with STZ diabetic control rat, treatment with PG (10 mg kg^{-1}) showed the significant inhibition ($p<0.05$) in the elevated levels of MDA (Fig. 5). GSH level in sciatic nerve of STZ diabetic control rat was significantly decreased ($p<0.05$) after 60 days induction of diabetes as compared to normal rats. When compared with STZ diabetic control rat, PCA (40 mg kg^{-1}) treated rats showed the significant ($p<0.05$) increased in GSH level. Rats treated alone with PCA significantly shows ($p<0.05$) normal GSH level as compared to STZ diabetic control rat. However, this increase in the GSH level by PG (10 mg kg^{-1}) treatment was

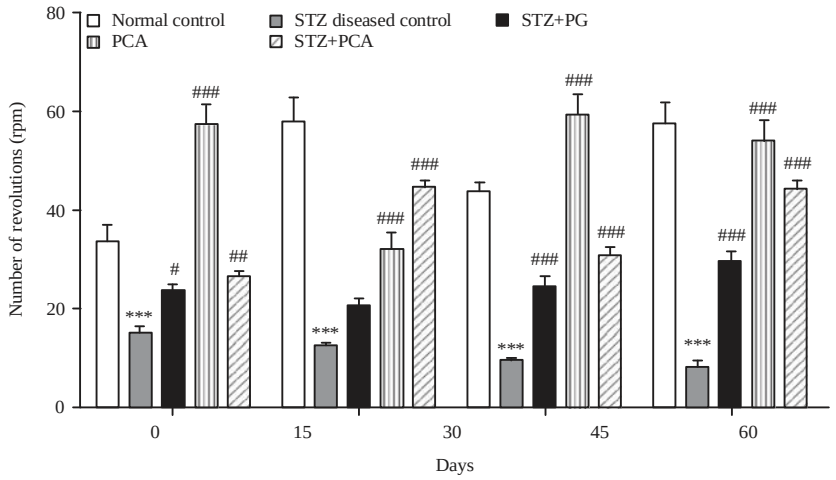

Fig. 4: Effect of PCA on muscle strength

All values are presented as Mean±SEM, (n = 6). p<0.05*, p<0.01**, p<0.001*** compared to control group. p<0.05#, p<0.01##, p<0.001### compared to disease group

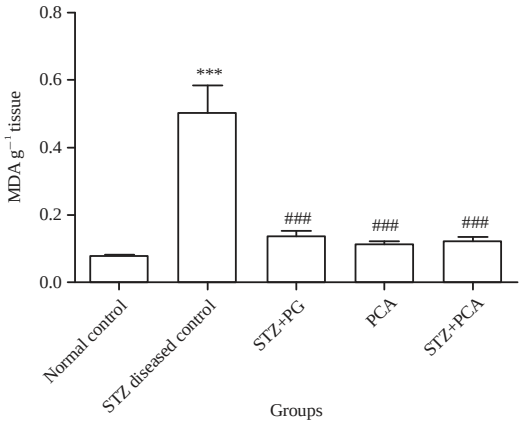

Fig. 5: Effect of PCA on lipid peroxidation level

All values are presented as Mean±SEM, (n = 6). p<0.001*** compared to control group. p<0.001### compared to disease group

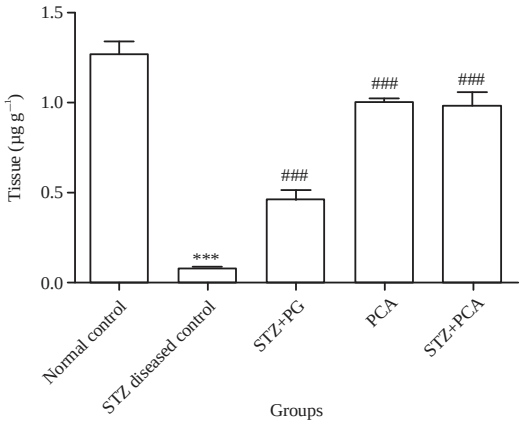

Fig. 6: Effect of PCA on reduced glutathione level

All values are presented as Mean±SEM, (n = 6). p<0.001*** compared to control group. p<0.001### compared to disease group

more significant (p<0.05) as compared to PCA (40 mg kg^{-1}) treatment. There was no significant change in the GSH level in normal rats (Fig. 6).

DISCUSSION

The present study was design to focus on the role of hyperglycemia and ROS in diabetic complication of neuropathy and the exclusive potential of simultaneous targeting above mentioned pathways in the reversal of diabetic complication of neuropathy. Diabetes was induced by administration of single dose STZ (60 mg kg^{-1}, i.p.). DN was developed after four weeks of diabetes induction and marked by various parameters. There are many hypotheses which support the hyperglycemia induced damage in nerves resulting in diabetic neuropathy. These include aldosereductase pathway, increased advanced glycation end products pathway, PARP overactivation, activation of PKC, increased hexosamine pathway, MAPK activation and inflammatory damage[3]. All this pathways directly promotes oxidative stress by decreasing endogenous antioxidant (α tocoferol, ascorbate and Vitamin E. etc) defence mechanism[18,19].

Several workers have reported induction of diabetes with STZ is associated with the characteristic loss of body which is due to increased muscle wasting and loss of tissue proteins in diabetes[20,21]. Administration of PCA to diabetic rats caused a significant increase in the body weight. This protective effect

might be due to its ability to reduced hyperglycemia. The similar incidence on body weight on STZ induced diabetes is in line with previous studies[2,3]. Rats treated with PCA (40 mg kg^{-1}) attenuated the elevated food intake and water consumption as compared to STZ diabetic control rats. Serum glucose level of STZ diabetic control rats was increased as compared to normal non-diabetic control rats. Administration of PCA (40 mg kg^{-1}) for 60 days reduces didn't show any significant reduction in glucose level.

DN is associated with decrease in paw withdrawal latency and which can be assessed by behavioural nociceptive tests like mechanical hyperalgesia, von Frey filament testing, cold allodynia and hot plate method. A change in nociception was reported in earlier studies[22,23].

Curative treatment of PCA attenuated the response of pain in diabetic neuropathic condition. It might be due to tight control of glucose level, decreased the over production of ROS which regulates the expression of gene involved in promoting inflammatory reaction and neuronal dysfunction to the formation of diabetic neuropathic pain[18]. The paw withdrawal threshold of diabetic control rat was decreased as compared to normal nondiabetic control rats in mechanical hyperalgesia. Chronic administration of PCA (40 mg kg^{-1}) resulted in increase in paw withdrawal threshold as compared to STZ diabetic control rats. This similar incidence by mechanical hyperalgesia on STZ induced diabetes is in line with previous studies[2].

Administration of STZ resulted in decrease in the mean paw withdrawal latency in the STZ diabetic control rats as compared to normal rats in thermal hyperalgesia. This decrease in mean paw withdrawal latency after induction of DN was inhibited by the PCA (40 mg kg^{-1}) treatment. This similar incidence is reported in previous studies[23].

Mean paw withdrawal threshold of STZ diabetic control rats was decreased as compared to normal non diabetic control rats in cold allodynia. Treatment with PCA (40 mg kg^{-1}) resulted in increase paw withdrawal threshold as compared to STZ diabetic control rats. However, rats treated with PCA alone (40 mg kg^{-1}) showed similar paw withdrawal threshold in normal control group rats. This similar incidence by cold allodynia on STZ induced diabetes is in line with previous studies[2].There was decrease in the muscle strength in STZ diabetic control rat as compared to normal rats after 60 days. Rats treated with PCA (40 mg kg^{-1}) showed increment in this reduced level as compared to STZ diabetic control rat. When compared with STZ diabetic control rat, treatment with PG (10 mg kg^{-1}) showed increase in the muscle grip strength.

ROS are critically involved in the development and maintenance of neuropathic pain. In diabetes condition production of oxidative damage in sciatic nerve is indicated by rise in lipid peroxidation (LPO) and decreases the reduced glutathione (GSH) level. The elevated level of lipid peroxidation has been concerned with an array of disease. It has been proved clinically as well as preclinically that the level of lipid peroxidation was significantly increased in plasma of diabetic patients and diabetic rats[18,24]. The increase in production of oxidative stress in diabetic condition leading to structural destruction of unsaturated fatty acids in lipid membrane resulted in the elevated levels of MDA[11]. Treatment with PCA significantly attenuated this elevated level of MDA in STZ diabetic rats. In the present investigation, the level of endogenous antioxidant especially GSH was decreased in the STZ control rats. Administration of STZ resulted in deprivation in level of GSH which caused cell death and thus give rise to hyperalgesia[11,12]. GSH level in sciatic nerve of STZ diabetic control rat was decreased as compared to normal rats. When compared with STZ diabetic control rat, PCA (40 mg kg^{-1}) treated rats showed increased in GSH level. Rats treated alone with PCA showed normal GSH level as compared to STZ diabetic control rat. However, this increase in the GSH level by PG (10 mg kg^{-1}) treatment was more significant as compared to PCA (40 mg kg^{-1}) treatment. There was no significant change in the GSH level in normal rats over the same period of time. PCA was able to increase the level of GSH reiterating its antioxidant profile[2]. PCA is chemically 3,4-Dihydroxybenzoic acid and it has been reported to possess anti-diabetic activity[4] and much more activities. In the present study PCA showed the significant alteration in various biochemical as well as behavioural parameters in STZ induced neuropathic rats. This alteration in various parameters and protection of rats from diabetic induced neuropathic pain might be due to strong antioxidant activity of PCA[6,19,25]. Further in depth study is required to confirm the mechanism of action of PCA in neuropathic pain using different animal models of neuropathic pain. Study also required at molecular level to confirm the protective effects of PCA in neuropathic pain.

CONCLUSION

In conclusion, the present study demonstrated the neuroprotective effects of PCA in neuropathic pain. The study focused mostly on behavioural and biochemical alteration during diabetes induced neuropathic pain. PCA significantly prevents the changes in pain threshold, markers of oxidative stress such as reduced glutathione and lipid peroxidation which reflected its potential protective effects in diabetic neuropathy.

SIGNIFICANCE STATEMENTS

This study discover that protocatechuic acid a natural product having strong antioxidant activity can be useful in reducing oxidative stress in diabetic neuropathy that can be beneficial for preventing the propagation of neuropathic pain in diabetic condition. This study helps the researchers to uncover the critical area of oxidative stress and pain management that many researchers were not able to explore. Thus a new theory on pain management with antioxidants may be arrived at.

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