

Effect of Vanillic Acid in Streptozotocin Induced Diabetic Neuropathy

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Submission Date: 20-08-2020; Revision Date: 12-10-2020; Accepted Date: 05-11-2020

ABSTRACT

Diabetic neuropathy is a one of the usual complications of both type 1 and 2 diabetes mellitus. Lesion to or diseases of somatosensory system may lead to neuropathic pain which are severally painful. Apart from other etiological factors, the oxidative stress has vital role in the pathogenesis and continuation of diabetic neuropathy. Vanillic acid is the phenolic compound and aromatic secondary plant metabolite. Phenolic acids are proved to have antioxidant and neuroprotective role. So, this study was undertaken to evaluate the effects of vanillic acid on STZ induced diabetic neuropathy by assessing behavioural, biochemical, electrophysiological and histological changes. Diabetes was induced in Wistar rats by using single injection of STZ (55 mg/kg, i.p.). After confirmation of diabetes (blood glucose level >200mg/dl), animals treated with Gabapentin (300 mg/kg, p.o.) and Vanillic acid (25, 50 and 100 mg/kg, p.o) for next 4 weeks. Vanillic acid (50 and 100 mg/kg) treated rats showed significant ($p < 0.05$) behavioral changes, decrease in blood glucose levels, significant ($p < 0.05$) increase in reduced glutathione (RGS) level. Treatment with vanillic acid has also reversed histopathological and electrophysiological changes. In conclusion, the present study suggested anti-hyperglycemic, antioxidant and neuroprotective effect of vanillic acid in diabetic neuropathy.

Key words: Hyperglycemia, Hyperalgesia, Allodynia, Diabetes, Vanillic acid, Antioxidants.

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INTRODUCTION

Neuropathic Pain (NP) are chronic pain caused due to damage to nervous system either by injury or diseases. NP is distinguished by the sensory abnormalities viz., dysesthesia (unpleasant abnormal sensation), hyperalgesia (an elevated response to painful stimuli) and allodynia (pain to stimuli that normally does not provoke pain).^[1] NP is complication of both types of diabetes. It occurs at about 8% in new patients and more than 50% in patients with long-standing disease.^[2] Oxidative stress

raised due to chronic hyperglycemia is responsible for diabetic complications like neuropathy. Apoptosis in neurons and supporting glial cells is also developed by this oxidative stress and could be the mechanism causing nervous system damage in diabetes.^[3] Reduction in hyperglycemia mediated mitochondrial ROS by certain agents prevent production of advanced glycation end products, glucose-induced activation of protein kinase C, accumulation of sorbitol and activation of NF- κ B (nuclear factor B) and thus, prevent development of diabetic complications.^[4] As most of pain producing stimuli produces neural injury, human experimentation to evaluate of NP is complex. So, animal experimentation is required to understand various mechanisms involved with NP.^[5] STZ (Streptozotocin) induced neuropathy is widely accepted model that mimics the diabetic neuropathy. STZ is an anticancer antibiotic and chemically nitrosoureas analogue. STZ

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DOI: 10.5530/ajbls.2020.9.46

at dose of 45 to 60 mg/kg administered either i.v or i.p. selectively causes pancreatic beta cells destruction and in rats induces diabetes after three to four days.^[5] This is one type of peripheral neuropathies identified by hyperalgesia, hyperesthesia and cold or hot allodynia, hyperglycemia induced nitrosative and oxidative stress which is a major mechanism in diabetic neuropathy. ROS can lead to afferent and efferent nerve conduction defects.^[6] Consistent control on hyperglycemia is a challenge in many cases and patients with good glucose control can experience neuropathy. Therefore, therapies that additionally target various pathways causing hyperglycemia-mediated complications are important to maintain long-term quality of life for diabetic patients.^[7] Also, hyperglycemia induced oxidative stress can mediate microvascular and neuronal deficits which are major contributors for diabetic complications. Until we can fully control blood glucose levels, antioxidants might therefore be helpful for treating diabetes and its complications.^[8] Drugs from natural sources are safer therapeutic option to treat neuropathy instead of modern medicines as these modern medicines are with several adverse effects. Various plants phytoconstituents have been studied for the management of neuropathy in rats. Many flavonoids and polyphenols with promising antioxidant and anti-inflammatory activity have been evaluated in the treatment of neuropathic pain. Isolated plant bioactive moieties are promising free radical scavengers and play important role in management of neuropathy in animals.^[9] Besides antioxidant activity and ability of phenolic acids to scavenge free radicals, recently many phenolic acids are studied for their neuroprotective role as those are protecting glial cells along with neurons.^[10] Currently, many research studies have shown the role of various phenolic acids e.g. Chlorogenic acid, CAPE (Caffeic acid phenethyl ester), Ferulic acid, Protocatechuic acid in treatment of neuropathic pain and other neurological disorders.^[10] Vanillic acid (VA) (4-hydroxyl-3-methoxy benzoic acid) is possessing antimicrobial, anti-cancer, anti-DNA oxidation activities,^[11] hepato-protective activity,^[12] antihypertensive and antioxidant potential.^[13] It is also found to be an inhibitor of 50-nucleotidase, snake venom^[14] and having anti-nociceptive activity.^[15] Vanillic acid has exhibited neuroprotection by reducing AChE, TNF- α and corticosterone and improved antioxidants and could be effective to treat neurodegenerative disorders.^[16] In view of the above facts, the present study was aimed to evaluate the effect of vanillic acid against STZ induced diabetic neuropathy.

MATERIALS AND METHODS

All chemicals and reagents used in the study were of analytical grade and were procured from Rankem, Mumbai and Himedia Laboratories Ltd., Mumbai. Streptozotocin (STZ), Vanillic acid (VA) and gabapentin was procured from Sigma-Aldrich, Germany. Commercial reagent kits were used for determination of biochemical parameters and enzymatic assays.

Test animals

Healthy Wistar female albino rats (150-200 g) were maintained under standard environmental conditions (temperature $25\pm 2^\circ\text{C}$, relative humidity $50\pm 5\%$) with a 12 h light/dark cycle. They were fed on with normal laboratory chow pellet diet and drinking water was given *ad libitum*. Animals were allowed to acclimatize for 7 days before commencement of the experiment. The study protocol was approved by the IAEC of MGV's Pharmacy College, Nashik (Letter number MGV/PC/CPCSEA/XXXIV/01/2018/05) with CPCSEA registration number CPCSEA-212/PO/Ere/S/2000/CPCSEA, Date: 16/09/2016.

Antidiabetic activity

Wistar rats were grouped as follows ($n=6$, either sex, 150-200 g) and treated for 4 weeks. Group I: Normal control received vehicle as saline only.

Group II: Streptozotocin (55 mg/kg; i.p) once

Group III: Diabetes + Gabapentin (300 mg/kg; p.o.)

Group IV: Diabetes + VA (25 mg/kg; p.o.)

Group V: Diabetes + VA (50 mg/kg; p.o.)

Group VI: Diabetes + VA (100 mg/kg; p.o.)

STZ (55 mg/kg, i.p.) dissolved in freshly prepared 0.001 M Citrate buffer, pH 4.5. After 72 hr, animals with distinguished hyperglycemia (fasting blood glucose ≥ 200 mg/dl)^[17] were selected and used for study.

Blood samples were withdrawn from tail vein under mild isoflurane anesthesia. Blood glucose was monitored by using glucometer (Accusure) to confirm hyperglycemia. Blood glucose was measured at 24, 48, 72 hr and at end of treatment.

Behavioral study

Mechanical hyperalgesia (Von frey test)

Rats placed individually on elevated maze in acrylic cage and adopted for test environment for at least 15 min. Filament (Von frey hairs) was applied from below the mesh floor to the planter surface of left hind paw. Sufficient force of filament was applied against paw (causing slight bending) and hold for sec. Application

repeated 5 times at interval of 4-5 sec. Withdrawal of paw was considered as a positive response.^[9] Cold allodynia (Cold plate test).

This is simple test to determine behavioral responses to cold temperature. Here, the rodent was placed on the cooled plate at desired temperature (4°C) and the time to induce nociceptive behavior indicated by paw licking, shaking and jumping and was recorded as the response time.^[18] Motor co-ordination (Rota rod test).

This test was conducted using rotarod apparatus. Rats placed on rotating spindle with 15 rpm. The fall latency of each rat from rotating spindle was recorded during 5 min period.^[19] Heat hyperalgesia (Hot plate test).

Eddy's hot-plate was used to study the thermal nociceptive threshold by keeping the temperature at 55± 2°C. Animal individually tested by placing on the hot plate and paw licking latency (sec.) was recorded. The cut-off time of 20 sec was maintained.^[20] Evaluation of motor nerve conduction velocity (MNCV).

MNCV recording were carried out 4 weeks after induction of diabetes on day 28th. Animals were anesthetized by intraperitoneal injection of Ketamine (90 mg/kg i.p) and Xylazine (10mg/kg i.p). MNCV assessment was done by using 8 channel powerlab (AD Instruments) with animal nerve stimulating electrode (MLA0320) and needle electrodes (MLA1204) of AD Instruments. Action Potential was generated by applying stimulating electrode at proximal end and recording done from distal end. The distance between the stimulating electrode and recording electrodes divided by latent period is calculated as conduction velocity. Latent period is the time, elapsed between applications of stimulus until the peak of the maximum compound action potential.^[21]

Antioxidant activity

Preparation of tissue homogenate

After scarification sciatic nerve isolated and quickly transferred to ice-cold Tris HCl buffer (10 mM, P^H 7.4). The tissues were then minced and homogenized in ice cold Tris HCl buffer (10% w/v). Centrifugation (using Remi C-24 high speed cooling centrifuge) carried out at 10,000 rpm for 15 min. To determine reduced glutathione, clear supernatant was used^[22] Estimation of RGS (Reduced glutathione).

Reduced glutathione was determined by adding equal volumes of tissue homogenate (supernatant) and 20% TCA. The precipitate was centrifuged and to 0.25 ml of supernatant, 2ml of DTNB reagent was added. The final volume was made up to 3ml with phosphate buffer. The colour intensity developed was measured at 412nm against reagent blank^[23] and results were expressed % inhibition RGS activity.^[24]

Histopathology

Isolated sciatic nerve was kept in the 10% formalin. Histopathological study was done at Histopathological lab (Dr.Vasantarao Pawar Medical College and Research Center, Nashik). Staining was done by using hematoxyline and eosin. Sections observed under light microscope (40×).^[25]

RESULTS

Antidiabetic activity

After 72 hrs of STZ treatment, blood glucose levels found to be increased significantly ($p < 0.05$) in all treatment groups compared to normal group. Gabapentin and vanillic acid treatment have shown significant antihyperglycemic effect ($p < 0.05$) in comparison with positive control group (Figure 1).

Behavioral activity

Mechanical hyperalgesia

Mechanical hyperalgesia was observed in the positive control rats at the 2nd week of induction of diabetes (STZ induced diabetic neuropathy). Hyperalgesia was indicated by attenuation of paw withdrawal threshold as compare to normal control rats. Diseased rats treated with Gabapentin and Vanillic acid showed significant ($p < 0.05$) improvement in paw withdrawal threshold after 3rd week of treatment schedule than positive control rats (Figure 2).

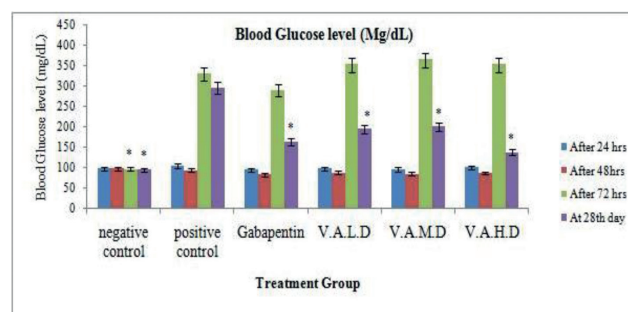


Figure 1: Effect of VA (25, 50 and 100 mg/kg, p.o.) and Gabapentin (300 mg/kg, p.o.) on blood glucose level.

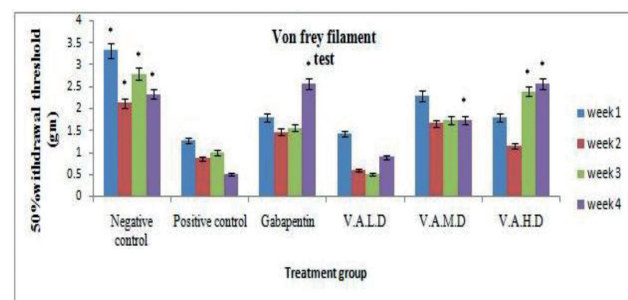


Figure 2: Effect of VA (25, 50 and 100 mg/kg, p.o.) and Gabapentin (300 mg/kg, p.o.) on mechanical hyperalgesia.

Heat Hyperalgesia

Diabetic rats shown heat hyperalgesia at the 3rd week after induction of diabetes. Heat hyperalgesia was indicated by significant reduction in paw lick latency. Treatment with Gabapentin and vanillic acid showed significant ($p < 0.05$) improvement in paw lick latency at 3rd and 4th week of treatment schedule than positive control rats (Figure 3).

Cold allodynia

Diabetic rats have shown cold allodynia at the 2nd week of induction of diabetes. Cold allodynia was indicated by decrease of paw withdrawal latency (sec) as compare to negative control rats. Treatment with Gabapentin and Vanillic acid (50 and 100mg/kg, p.o.) showed significant ($p < 0.05$) improvement in paw withdrawal latency (sec) after 3rd week of treatment schedule than positive control rat (Figure 4).

Motor in-coordination

Diabetic rats showed motor in-coordination as indicated by significant ($p < 0.05$) decrease in fall latency (sec). Treatment with Gabapentin and vanillic acid showed significant ($p < 0.05$) improvement in motor coordination, indicated by increased fall latency time(sec) than diabetic control rats after 3rd week of treatment schedule (Figure 5).

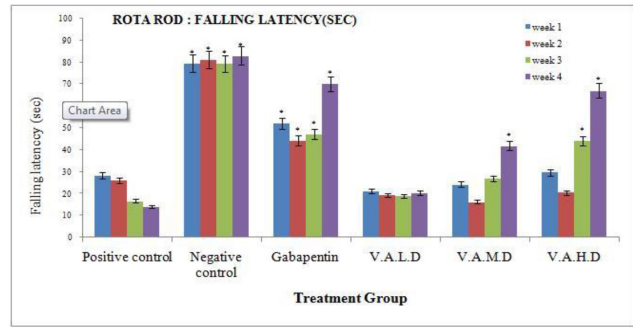


Figure 5: Effect of VA (25, 50 and 100 mg/kg) and Gabapentin (300 mg/kg) on motor in-coordination.

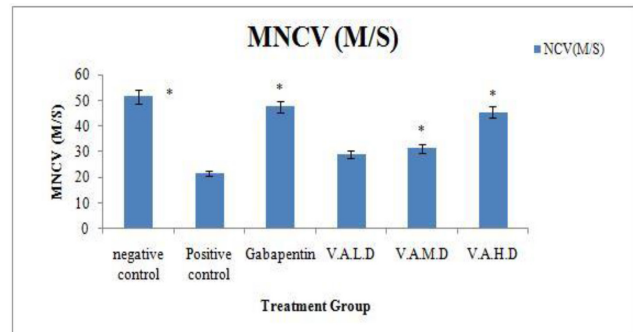


Figure 6: Comparative increase MNCV due to the treatment of VA (25, 50 and 100 mg/kg) and Gabapentin (300 mg/kg, p.o.) at 28th day.

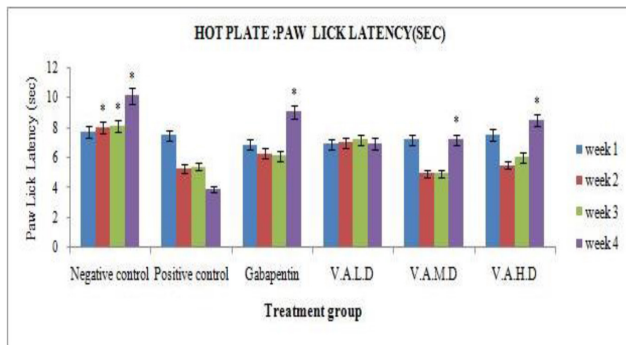


Figure 3: Effect of VA (25, 50 and 100 mg/kg) and Gabapentine (300 mg/kg) on heat hyperalgesia.

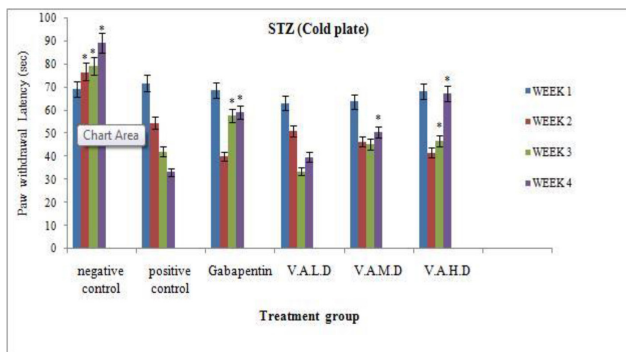


Figure 4: Effect of VA (25, 50 and 100 mg/kg p.o) and Gabapentin (300 mg/kg p.o) on cold allodynia.

Nerve Conduction Velocity

Reduction in NCV observed in positive control rats after 28th days indicating nerve damage compared to negative control group. Diabetic rats which are treated with the Gabapentin and Vanillic acid showed significant ($p < 0.05$) improvement in NCV as compared to positive control rats indicating neuroprotective effect (Figure 6).

Antioxidant activity

In vivo antioxidant RGS (reduced glutathione) levels were estimated from tissue homogenate of sciatic nerve by using Ellman’s reagent procedures. RGS is primary antioxidant in the cell. In positive control animals RGS level was found to be decreased as compared to negative control animals. Significant increase in % inhibition of RGS was observed in positive control group compared with negative control group, while treatment with Gabapentin and Vanillic acid showed significant decrease ($p < 0.05$) in % inhibition by increasing availability RGS as compared with Positive control group (Figure 7).

Statistical analysis

The observations are mean \pm SEM. Data was analyzed by ANOVA followed by Dunnett’s test. The *P* value less

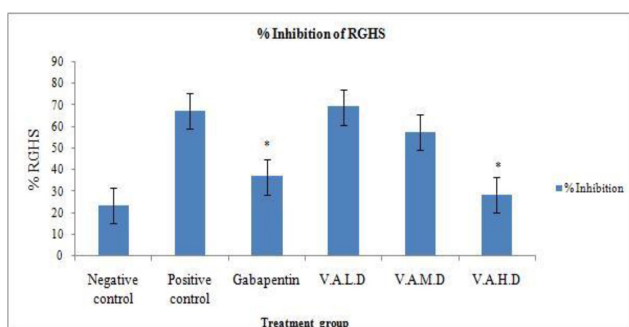


Figure 7: Effect of VA (25, 50 and 100 mg/kg) and Gabapentin (300 mg/kg) on reduced glutathione in sciatic nerve tissue homogenate of STZ induced diabetic rats.

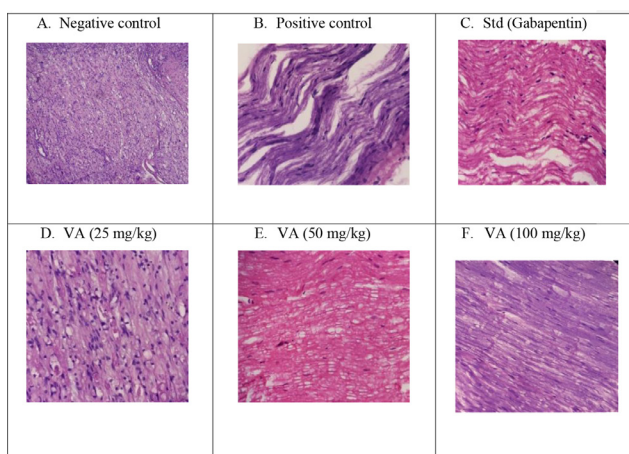


Figure 8: Histopathology Section of H&E stained sciatic nerve of diabetic control and treated animals.

than 0.05 considered significant as compared to positive control group.

Histopathology

Histopathology Section of H&E stained sciatic nerve of diabetic control rats showed epineuronal edema and infiltration of neutrophils around blood vessels and swelling of nerve fibers (100x) compared to negative control group (Figure 8). Whereas, swelling of nerve fibres and demyelination of nerve fibres and accumulation of monocytes and macropages around schwann cells are observed in VA (25 mg/kg) treated group. Treatment with VA (50 mg/kg) rats showed mild swelling of nerve fibres, VA (100 mg/kg) showed mild edema and well organized myelin sheath around axons absence of infiltrating cells and Gabapentin (300 mg/kg) showed mild edema and few infiltrating neutrophils around blood vessels and minor swelling of nerve fibers (40x).

DISCUSSION

Approximately, worldwide 6.9% to 10% general population suffering from NP which is resulting from

neuronal dysfunction or injury in nervous system. This neuronal insult is also associated with many disorders viz. diabetes and disc prolapsed.^[26] Imbalance between generation of free radicals and natural antioxidants is significantly contributing to diabetes and related complications. Many research studies have focused role of oxidative stress in abnormal secretion of pancreatic β -cells and abnormal glucose utilization in peripheral tissues that further results into long-term complications.^[27] As modern medicines have prominent adverse effects, natural drugs, due to their safety, are considered as better therapeutic option to treat neuropathy. Many research of neuropathic pain have evaluated polyphenols considering their promising antioxidant and anti-inflammatory activity.^[9] So, the present study is undertaken to evaluate effect of vanillic acid in STZ induced diabetic neuropathy, assessed by behavioral, biochemical, electrophysiological and histopathological parameters.

Various biochemical and physiological processes can lead formation of ROS (Reactive Oxygen Species), which further damage cellular lipids, proteins and also can cause DNA mutation resulting in cell death^[4]. There are many evidences indicating role of ROS in onset and progression of diabetes to its complications. ROS activate signaling pathways leading to apoptosis which is potential mechanism to develop chronic diabetic complications. This mechanism can be counteracted by natural antioxidants. Major soluble antioxidant present in cell compartments is Glutathione (GSH). In diabetes hyperglycemia induces GSH depletion and impaired regeneration which links to diabetic neuropathy like complications.^[7] In the present study, amount of reduced glutathione (RGS) was found to be decreased significantly in diabetic control group compared with normal control group, while in animals treated with Gabapentin (300 mg/kg) and vanillic acid (50 and 100 mg/kg) showed significant increase in RGS levels than diabetic control group indicating the antioxidant activity of vanillic acid. In this study vanillic acid exhibited good neuroprotective and antioxidant activity when administered at a dose 50 and 100 mg/kg orally. Hyperglycemia was observed after 72 hr of injection of streptozotocin (55 mg/kg i.p). The onset of behavioral changes was observed after 2nd week of induction of diabetes. Peripheral nerve injury in diabetic neuropathy is characterized by behavioral biomarkers such as dysesthesia, hyperalgesia, allodynia and with motor in co-ordination.^[1,28] The behavioral parameters such as cold allodynia, thermal and mechanical hyperalgesia and motor coordination, were assessed by using hot plate test, von frey filament test, cold plate test, rota

rod test respectively. Diabetic rats showed significant reduction in paw withdrawal latencies in tests for cold plate and hot plate method. In case of cold allodynia and thermal hyperalgesia, treatment with Gabapentine (300 mg/kg) and vanillic acid (50 and 100 mg/kg) showed significant improvement in paw withdrawal latency in 3rd, 4th week of treatment schedule than diabetic control rats. Mechanical hyperalgesia and motor incoordination is shown to be improved by treatment of vanillic acid as there was significant increase in reaction time i.e. 50% threshold of paw withdrawal by von frey filament method and significant decrease in fall latency (sec) for rota rod test compared to positive control rats.

Results of the present study have indicated that four week treatment with vanillic acid (50 and 100 mg/kg) improved cold allodynia, thermal hyperalgesia and mechanical hyperalgesia and motor in-cordination in experimental animals.

In the study of neuropathic pain, determination of electrophysiological changes in nerve impairment is considered as gold biomarker. A nerve conduction velocity (NVC) is an electrical test used to determine nerve impulse conduction down to nerve that detects nerve injury. In diabetic animals sensory NCV and motor NCV is slowed down. Etiology of this type of nerve dysfunction induced by diabetes is considered to involve both non-vascular and vascular mechanisms.^[28] These abnormalities in motor nerve conduction may be due to blood flow reduction induced by hyperglycemia and such resultant endoneural hypoxia which lead to development of diabetic neuropathy.^[29] Hyperglycemia induced oxidative stress may have significant role in microvascular and neuronal deficits leading to neuropathy. Therefore, until we can fully control blood glucose levels, therapies such as antioxidants that are targeted against oxidative stress remain our most promising approach to prevent neuropathy and other diabetic complications.^[3] In this study hyperglycemia is found to be significantly decreased after treatment with Gabapentin (300 mg/kg) and vanillic acid (50 and 100 mg/kg) than diabetic control rats. Also, MNCV is found to be increased significantly in diabetic animals treated with Gabapentin (300 mg/kg) and vanillic acid (50 and 100 mg/kg). Vanillic acid 25mg/kg dose has shown protective effect in all parameters but statistically non-significant.

It is reported that sciatic nerve of diabetic rats produces severe pathological changes. In the present study, treatment with Gabapentin (300 mg/kg) and vanillic acid (50 and 100 mg/kg) have shown reversal of histopathological changes. Diabetic control group showed edema round the epineurium and infiltration

of neutrophils around blood vessels and showed swelling of nerve. Vanillic acid (25 mg/kg) showed swelling of nerve fibers and demyelination of nerve fibers. Less macrophages and monocytes were observed around the schwann cells of diabetic rats treated with Gabapentin (300 mg/kg), while treatment with vanillic acid (50 and 100 mg/kg) showed mild epineuronal edema, few infiltrating neutrophils around blood vessels and minor swelling of nerve fibers. Thus, vanillic acid has shown sciatic nerve stability in treated animals than diabetic control animals indicating its neuroprotective activity in STZ-induced diabetic rats. Thus, vanillic acid ameliorates STZ- induced diabetic neuropathy in rats possibly by its antioxidant and neuroprotective effect.

CONCLUSION

The present study has shown the effect of vanillic acid on STZ induced diabetic neuropathy assessed by its behavioral parameters, antioxidant, MNCV and histopathological studies. Treatment with Vanillic acid (50 and 100 mg/kg) have showed significant improvement in blood glucose level, cold allodynia, thermal and mechanical hyperalgesia and motor in-cordination. Antioxidant RGS levels was found to be increased significantly in vanillic acid (50 and 100 mg/kg) treated groups. These doses have also reversed histopathological changes compared to those observed in diabetic control animals. Thus in conclusion, Vanillic acid treatment has shown significant protective effect in STZ induced diabetic neuropathy, possibly which may be due its antioxidant, anti-hyperglycemic and neuroprotective effect.

ACKNOWLEDGEMENT

The authors are grateful to Orchid Scientifics Pvt. Ltd., Ambad, Nashik for providing laboratory facilities.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Cite this article: Khairnar S, Pawar S, Patil V, Rudrapal M. Effect of Vanillic Acid in Streptozotocin Induced Diabetic Neuropathy. *Asian J Biol Life Sci*. 2020;9(3):306-12.