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Research Article

Vitamin C Attenuates Hyperalgesia, Peripheral Nerve Degeneration and Reversed Paw Numbness in a Wistar Rat Model of Diabetic Neuropathy

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ABSTRACT

Painful diabetic peripheral neuropathy (DPN) is prevalent amidst diabetic patients. It develops with a background of long-standing hyperglycaemia, metabolic derangements and oxidative stress. Vitamin C an aqueous phase antioxidant which may help in combating oxidative stress. Vitamin C depletion occurs in diabetes and this has been reported to be associated with spinal and musculoskeletal pain. Therefore, this study was conducted to investigate the effect of vitamin C administration on neuropathic pain and peripheral nerve in DPN. Thirty wistar rats (180-200g) were grouped (n=10) into control (C), diabetic and vitamin C treated diabetic. Diabetic neuropathy was induced using 20% fructose and 150mg/kg.ip alloxan. Vitamin C (1g/kg.p.o) treatment via oral-canula lasted for two weeks. Neuropathy was assessed using tail flick and formalin paw lick tests. Peripheral nerve histology and sympathetic activity were assessed using light microscopy and tyrosine hydroxylase (TH) immunohistochemistry respectively. Oxidative and nitrosative stress markers (SOD, MDA, GSH, VC & NO) were evaluated using spectrophotometry. Hyperalgesia, paw numbness, sciatic nerve degeneration and reduced sympathetic activity were observed in diabetic rats showing peripheral neuropathy. However, these were reversed in vitamin C treated diabetic rats via mechanism related to upregulation of tyrosine hydroxylase activities and downregulation of oxidative and nitrosative stress, as SOD, NO and MDA were significantly ($p<0.01$) reduced, while GSH, VC and TH were increased ($p<0.05$) in Vitamin C treated rats. Thus, vitamin C may be an effective adjunct therapy for neuropathic pain relief, restoration of normal sensation in lower limbs and amelioration of diabetic peripheral neuropathy.

Keywords: *Diabetic peripheral neuropathy, oxidative stress, hyperalgesia, Sciatic nerve, neuropathic pain*

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INTRODUCTION

Diabetic neuropathy is a heterogenous set of both clinical and sub-clinical manifestations of diseases connected with the peripheral nervous system, which arises as a complication of diabetes mellitus. This can affect both the somatic and autonomous nervous systems (Haslbeck *et al*. 2004). Diabetic neuropathies are proven to be heterogenous, not only by their symptoms, but also their pattern of neurologic involvement, underlying mechanisms, pathologic alterations, course, and risk co-variates. Neuropathy in diabetes can be classified into (multi)focal and generalized (or symmetric) varieties of neuropathy. Multifocal neuropathies comprise of lumbosacral, cervical radiculoplexus, mononeuropathy and thoracic neuropathies. On the other hand, classified under generalised/symmetric neuropathies are: Autonomic and peripheral neuropathies. Peripheral neuropathy is regarded the commonest kind of diabetic neuropathy that is often associated with pain and peripheral nerve dysfunction.

About thirty percent of persons living with type 1 or type 2 diabetes are reported to have the painful symptoms of peripheral neuropathy. Diabetic peripheral neuropathy (DPN) can be symptomatic or asymptomatic with nearly 50% of patients having symptoms in the feet, lower limbs and sometimes in the hands. These include burning, tingling, sharp, shooting, lancinating, stabbing or electrical sensations. In addition, there could be hyperaesthesia, paraesthesia, prickling, severe aching pain and unresponsiveness (numbness) all of which develops proximally from the feet and hands (Dyck *et al*. 2006, Tesfaye *et al*. 2013). These symptoms are generally accompanied with pain, hyperalgesia and allodynia (Abbott *et al* 2011).

Neuropathic pain reduces the quality of life of patients, their physical mobility, and general wellbeing. Diabetic neuropathy usually originates and progresses with a history of established hyperglycaemia, oxidative stress, metabolic derangements, lipid alterations, accumulated advanced glycation end products, and other metabolic abnormalities (Dyck *et al*. 2006, Rajan *et al*. 2014). Evidently, anti-oxidant enzymes are found to be reduced in diabetic nerves compared with non-diabetic nerves. This, coupled with increased production of oxidative radicals, contribute to nerve blood flow and transmission deficits, diminished neurotrophic support and morphological abnormalities characteristic of DPN (Zherebitskaya *et al* 2009). In the light of these, inhibition of oxidative stress may attenuate the neuropathic complications of diabetes. Studies have shown that antioxidants reduced neuropathy pain scores without the side effects of opioids or anticonvulsants often used in the treatment of pain associated with neuropathy (Ziegler *et al*. 2004). Thus, antioxidants might be a better alternative or adjunct in the management of diabetic neuropathy. Vitamin C is an aqueous phase antioxidant reported to be deficient in diabetic subjects and its depletion has been linked with both spinal and musculoskeletal pains (Dionne *et al*. 2016). It is a co-factor in the fusion of catecholamine neurotransmitters and, as a result, is implicated in neuromodulation (May *et al*. 2013). Furthermore, it is a co-factor in the fusion of opioid peptides and amidated neuropeptide such as endomorphine with potent opioid activity (Carr and McCall, 2017). Thus, vitamin C may possess potential analgesic and neuroprotective properties in addition to its anti-oxidative properties. However, the use of vitamin C alone in the treatment of peripheral neuropathic pain symptoms is yet to be validated scientifically thus the need for this work. This study, therefore, investigates the effect of vitamin C on neuropathic pain and peripheral nerve in a rat model of diabetic neuropathy.

MATERIALS AND METHODS

Animal grouping and Diabetic neuropathy induction: Thirty Wistar rats weighting 180-200g were randomly allotted into control (C), diabetic neuropathy (DN) and VC-treated-DN (DNVC) groups with n=10. The Control group received water, while other groups took 20% w/v fructose enriched water, alongside their normal feeding, for two weeks. Thereafter, DN and DNVC rats received an injection of 150mg/kg alloxan (5% w/v) in normal saline solution while control rats received the normal saline vehicle intraperitoneally (Fabiyi-Edebor and Fasanmade 2019). Twenty-four hours after, rats having fasting blood glucose greater than 250mg/dl were regarded diabetic. Diabetic rats are considered as a prototype of diabetic neuropathy (Lee-Kubli *et al*. 2014, Mehta *et al*. 2017). Next, DNVC group received 1g/kg/day of vitamin C (ascorbic acid) via oral cannula for two weeks (Fabiyi-Edebor and Fasanmade, 2019). On the last day of the treatment period, experiments were conducted on the rats to research into the effects of vitamin C on DPN pain. The animals were cared for according to the handbook on the care and use of laboratory animals (National Academies Press 2011) and the experiments approval of the animal ethical committee of Afe Babalola University.

Hyperalgesia Assessment: Thermal and mechanical hyperalgesia response tests were used to assess peripheral neuropathic pain in the rats. (Lee-Kubli *et al* 2014). Response to thermal hyperalgesia was done using the tail flick test. Five centimetres of the rats' tail from the tip was dipped in hot water maintained at 55±1°c. The tail withdrawal latencies were taken at 0, 15, 30, 60, and 90 minutes intervals (Onasanwo *et al*. 2012).

Mechanical hyperalgesia was done using formalin paw lick test. Twenty microliters of formalin were injected into the planter surface of the left hind paw of the rats. The formalin paw lick response test is bi-phasic. Following formalin injection, the early phase lasted for the first fifteen minutes, but the late phase occurred between fifteen- and sixty-minutes following formalin injection. The time spent by the rats in licking the injected paws and the number of times the rats licked the injected paws were observed and measured as an index of mechanical hyperalgesia (Onasanwo *et al* 2012). The experiment was carried out in a translucent plastic chamber (30x30x30) cm), with a mirror placed at the bottom for an unhindered view of the animals.

Oxidative stress studies: Markers of oxidative stress such as superoxide dismutase (SOD), Gluthatione (GSH), lipid peroxidation or malondialdehyde (MDA), Nitric oxide (NO) and vitamin C (VC) were assayed for in sera samples of the rats using spectrophotometry.

Histological assessment of the sciatic nerve: Cross sections of the sciatic nerve tissue were fixed in 10% formaldehyde solution, rooted in paraffin wax blocks and divided into 5µm slices with the use of a microtome. The samples were respectively stained with both eosin and hematoxylin. The stained slides were mounted and observed microscopically. A microscopic report of all the samples was, thus, graded as mild, moderate or severe neuropathy for the sciatic nerves.

Immunohistochemical studies on the sciatic nerve: Tyrosine hydroxylase (TH) antibody was used to stain paraffin sections of the sciatic nerves of the rats. Dopaminergic neurons of the sciatic nerves were stained brown by TH. The density and intensity of the stain was examined microscopically.

Statistical analysis

The results presented as mean + SEM were subjected to analysis of variance (ANOVA) test at α 0.05.

RESULTS

There was no considerable difference in tail flick latency between the vitamin C treated diabetic group and control ($p>0.05$) at 0,15 and 30 minutes after vitamin C administration. However, at 60minutes, DN+VC tail flick latency was considerably ($p>0.05$) greater than control. The untreated diabetic group showed significant ($p<0.001$) decrease in tail flick latencies at t=0,15,30,60 minutes

compared with ascorbic acid treated diabetic and control groups. The diabetic rats did not show significant paw lick response to the formalin test; however, significant response was observed in vitamin C treated rats (Figure 2). The number and duration of paw licks were considerably ($p<0.01$) reduced in DNVC rats unlike control but greater ($p<0.05$) compared with diabetic rats.

Biochemical analysis revealed that MDA, SOD and NO were significantly($P<0.001$) increased while GSH and VC were significantly ($p<0.01$) decreased in diabetic rats. In contrast, these variables in VC treated rats were similar to control values except for GSH that was greater ($P<0.05$) in DNVC than control (Figure 3).

Plate 1 shows severe histopathological changes in the sciatic nerves of diabetic rats with mild alteration in vitamin C treated rats while Plate 2 shows tyrosine hydroxylase activity in the sciatic nerves of the rats.

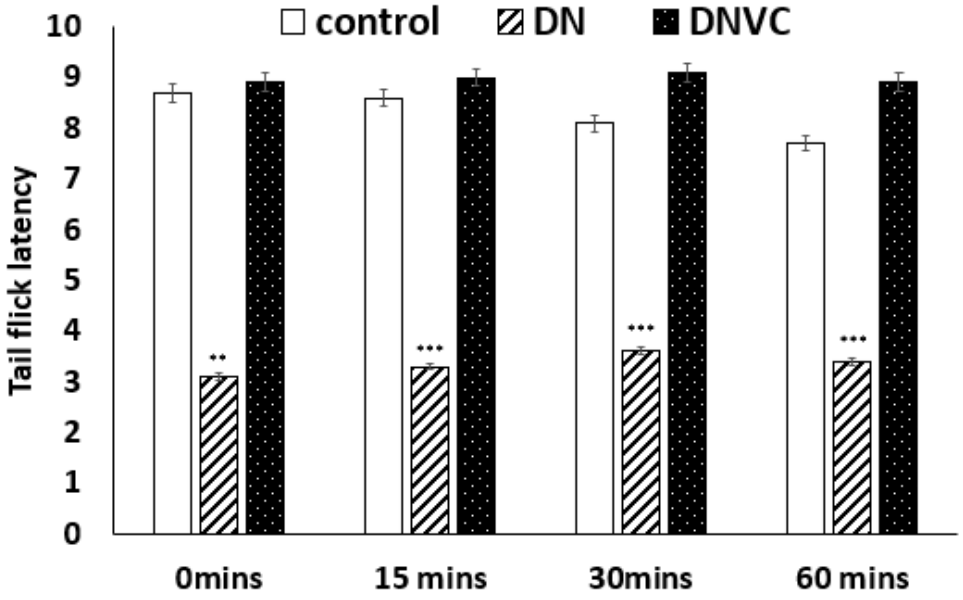


Figure 1:
Tail flick latency of control, diabetic (DN) and vitamin C treated diabetic (DNVC) rats at (a) 0 minutes, (b) 15 minutes, (c) 30 minutes, and (d) 60 minutes after oral administration of vitamin C. *The results are shown as mean ± SEM, n=10, ***p<0.001, *p<0.05 significantly different from control. $^{++++}$ p< 0.001 significantly different from DNVC.*

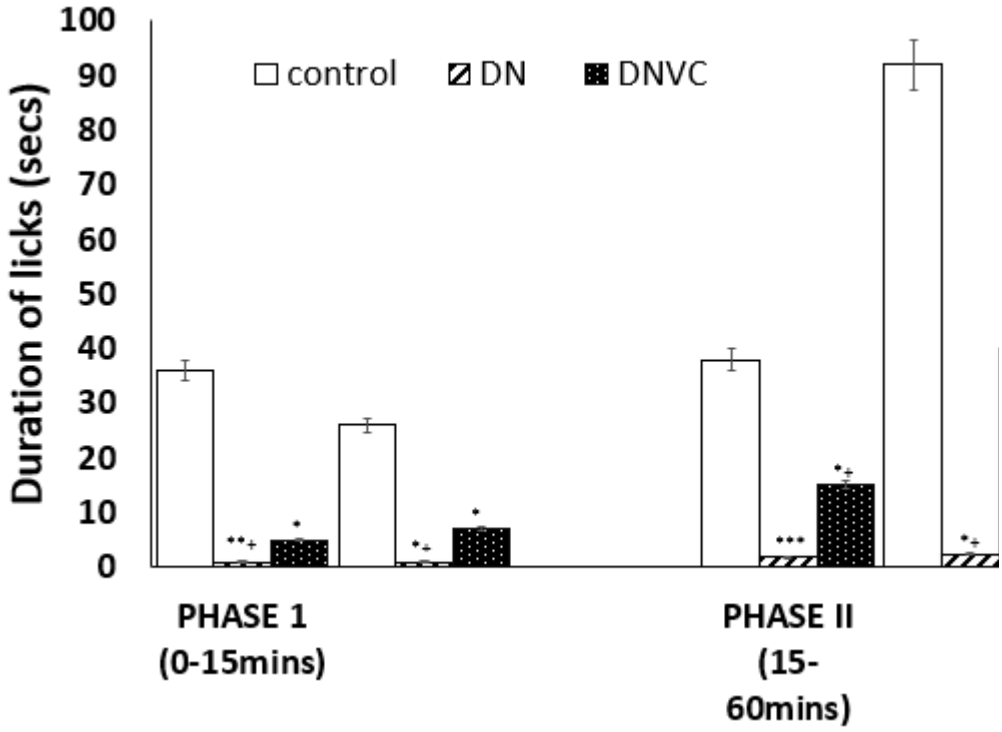


Figure 2:
Mechanical hyperalgesia using the formalin paw lick test in diabetic and DNVC rats. *The Results are shown as mean ± SEM, n=10, ***p<0.001, *p<0.05 significantly different from control. $^{+}$ p< 0.05 significantly different from VC treated.*

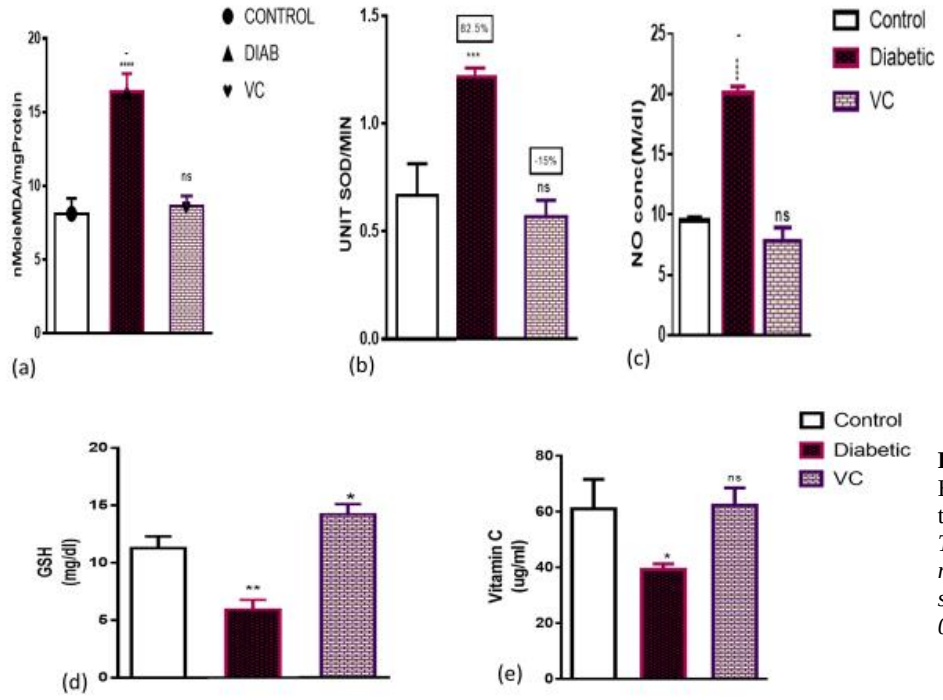


Figure 3:
Biochemical analysis of diabetic and VC treated rats.
*The results are shown as mean ± SEM, n=10, ***p<0.001, ** p< 0.01, *p<0.05 significantly different from control, $^{+}$ p< 0.001 significantly different from DNVC.*

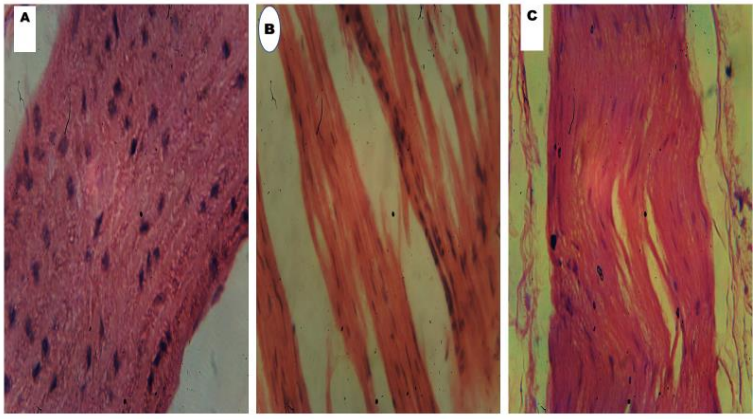
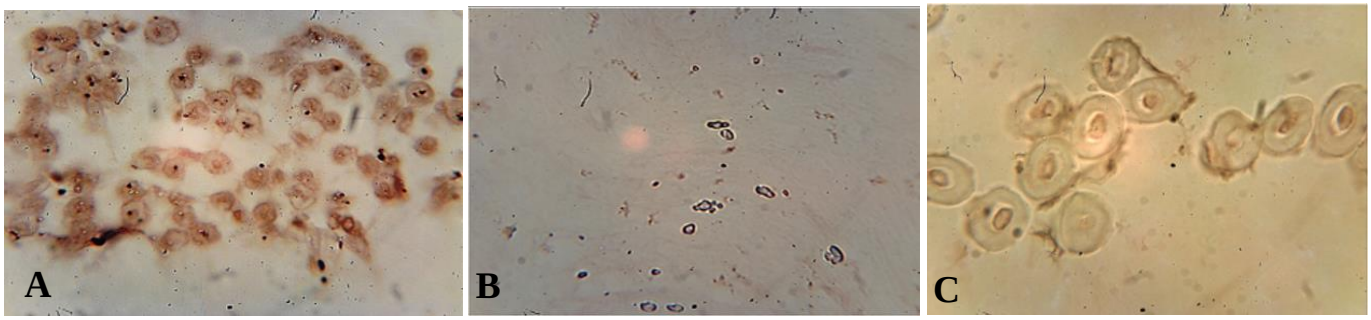


Plate 1:
Photomicrograph showing the longitudinal section of the sciatic nerves of control (A), diabetic (B) and vitamin C treated (C) rats, stained with H&E. Magnification: x400.



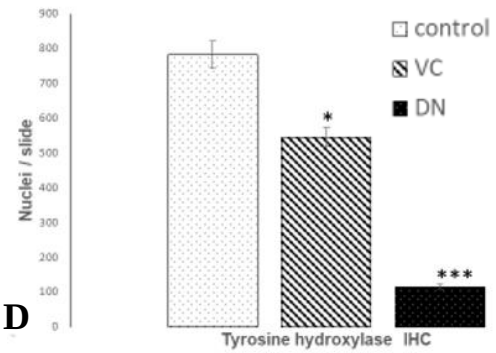


Plate 2:
Photomicrograph showing tyrosine hydroxylase activity in the sciatic nerves of A-control, B-diabetic and C- vitamin C treated rats immune-reactively stained with anti-TH antibody. (Magnification = 400x). D – nuclei count of the slides

DISCUSSION

Diabetic peripheral neuropathy has been established in animal models of diabetes including type II diabetes induced using a combination of diet and diabetogenic agents (Mehta *et al* 2017). It is usually associated with symptoms similar to that observed in patients with diabetic neuropathy. These symptoms include allodynia, slow nerve conduction velocity (NCV), progressive sensory deficits and hyperalgesia and may be accompanied with neuropathic pain and/or sensory or motor loss in the extremities (Mehta *et al*. 2017, Ziegler 1999). Neuropathic pain in diabetic animals can be assessed using thermal, chemical or mechanical hyperalgesia and/or allodynia. Hyperalgesia have been reported in diabetic animals within a week or more of diabetes (Mehta *et al* 2017). In this study, thermal hyperalgesia was observed in the second week of diabetes induction using tail flick test. Hyperalgesia suggest sensitization of central and peripheral nociceptive neurons which engender a cascade of effects experienced as pain. However, tail flick latency was increased in vitamin C treated rats, thus revealing the anti-nociceptive /analgesic effect of vitamin C. The mechanism may be by inhibiting the central mechanism of pain through prevention of oxidation since enhanced (Campbell & Meyer 2006). Oxidative damage to peripheral nerves causes hyperexcitability in the afferent nociceptors and central neurons leading to the generation of spontaneity within the axons and dorsal root ganglions of the nerves contributing to the neuropathic pain associated with diabetic neuropathy (Yorek 2003).

The behavioral response to formalin paw lick test consists of a severe phase (Phase I) of a short-lasting response, which is believed to indicate the activity of C-fiber afferent nociceptors. The severe phase is followed by a continuous prolonged response (Phase II) after a short quiescent period. This is believed to be due to central sensitization of the spinal dorsal horn neurons because of the initial input from C-fiber nociceptive afferents during the early phase (Coderra *et al* 1993; Gong *et al* 2014). In the two phases of the formalin paw licking test conducted in this study, diabetic rats showed numbness (very little or no paw licking response) in their foot when 'injected with formalin. However, sensation was restored to the foot of diabetic rats treated with vitamin C. This was demonstrated as an increase in paw licking activity of the vitamin C treated rats compared with the almost absent paw lick response in diabetic rats. Thu, vitamin C may have effect on both central and peripheral nervous system. Vitamin C has also been shown to concentrate in neurons in both central and peripheral nervous system. Its functions therein functions include peptide amidation, myelin formation, antioxidant function, synaptic potentiation, neuronal function, nerve regeneration and maturation among others (Rosa *et al*. 2005; May 2012). In addition, vitamin C deficiency has been reported to cause peripheral neuropathy and hypomyelination (Gess *et al*. 2011, Staff & Windebank 2014). Thus, hyperglycemia induced oxidative stress coupled with vitamin C deficiency in diabetic condition could bring about peripheral nerve damage via auto-destruction of nerves (Staff & Windebank 2014). This was evident in this study by the increases in oxidative stress markers; decrease in antioxidants, for example glutathione and vitamin C; and structural damage to the sciatic nerve in diabetic rats. The structural damage in the diabetic sciatic nerve was shown by the disarray in the normal parallel arrangement of the nerve fibres, degeneration of the nerve fibres, reduced tyrosine hydroxylase synthesis and reduced nuclei count. Tyrosine hydroxylase activity which was greatly reduced in diabetic sciatic nerve shows abnormally reduced sympathetic activities however, upon vitamin C administration, tyrosine hydroxylase secretion and thus sympathetic activities was elevated. In addition, vitamin C treatment showed nerve repair and regeneration. The mechanism through which vitamin C wields this neuroprotective effect may be by reducing oxidative stress.

Oxidative stress has been shown to cause nerve damage in diabetes and is the most understood process of development of diabetes to diabetic neuropathy (Yorek 2003, Staff & Windebank 2014). The mechanisms that are implicated in oxidative stress-induced neural dysfunctions comprise increased reactive nitrogen species, reduction in cellular antioxidants, DNA damage, lipid peroxidation, and generation of reactive oxygen species (Ho *et al*. 2006; (Obrosova *et al*. 2007). Increased nitrogen and reactive oxygen species are able to damage lipids existing in the myelinated structures of nerves, thereby resulting in the loss of axons as well as the disruption of the microvasculature in the nervous system (Casellini & Vinik 2006). However, vitamin C administration reduced increased reactive nitrogen species, reactive oxygen species, lipid peroxidation by reducing the levels of superoxide dismutase (SOD), nitric oxide (NO) and malondialdehyde (MDA) respectively. In addition, antioxidant status was improved by increasing glutathione and replenishing depleted vitamin C.

In conclusion, Vitamin C seems to be an efficacious adjunctive therapy for neuropathic pain relief, preventing foot numbness and ameliorating diabetic peripheral neuropathy due to its analgesic and neuroprotective effects through mechanisms that may be related to downregulation of oxidative stress and upregulation of tyrosine hydroxylase activities. However, human studies may be required to validate this effect of vitamin C on diabetic peripheral neuropathy and its associated symptoms.

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