EFFECTS OF VITAMIN C ON REGENERATION OF SCIATIC NERVE CRUSH-INJURY IN ADULT RATS- A LIGHT MICROSCOPIC STUDY

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ABSTRACT

To ameliorate secondary nerve injury many antioxidants like vitamin c have been tried but information on the histopathological aspects of its regenerative potential remains scanty and therefore, the present study was planned to assess its role in the regeneration of crush-nerve injury in animal model with special attention on histopathology. Under general anaesthesia and aseptic condition the rat sciatic nerve crush-injury was induced in the mid-thigh region. Vitamin C was given both topically and systematically. Sciatic nerve function was assessed both before induction of injury and at weekly intervals postoperatively. At the end of the study period animals were sacrificed, nerves were fixed in Karnovsky’s fixative and tissue samples were stained with Osmic acid, H& E, and Masson’s trichrome stain. The sciatic nerve function index ranged from -70 to -55 at 1 and 6 weeks post-operatively respectively. Microscopy showed fragmentation and marked clearance of myelin debris by six weeks. Many nerve fibres were seen to progress into the vacant endoneurial tubes at the site of nerve disruption. It was concluded that the use of vitamin C to either reduce the post-injury nerve dysfunction or improve nerve regeneration seems to have promising future in clinical practice.


INTRODUCTION

It is a common observation that after peripheral nerve trauma and subsequent repair, clinically the sensory outcome remains poor (Dagum, 1998) which adversely affects the fine manipulative movements (Westling and Johansson, 1984). The secondary injury which follows the primary one is believed to be caused by reactive oxygen species which is
promoted by lactic acidosis (Sies & Cadenas, 1983; Braughler & Hall, 1992; Phillis, 1994; Marin et al, 1998; Bagdatoglu et al, 2002) while antioxidant like ascorbic acid (vitamin-C), has been shown to promote recovery by protecting it from oxidative injury (Frei et al, 1989; Shokouhi et al, 2004). In fact most of the vitamins either alone or in combination with other vitamins or corticosteroids have been used to evaluate their neuroprotective effects in peripheral nerve injury animal models. For example Sevuk et al (2014) studied the effects of methyl prednisolon and vitamin A on the healing of traumatic peripheral nerve paralysis by EM & IHC concluded that there was noticeable vitamin A effect but similar finding were not established by electrophysiology. Besalti et al (2007) evaluated the regenerative effect of combined administration of B1+B2 (each 33 mg/kg) +B6 (0.5 mg/kg-IM for 3 weeks) on the injured sciatic nerve and concluded that the beneficial effect of thiamine, pyridoxine and cyanocobalamine combination was not found in regard to electrophysiological parameters. However, clinical improvement was superior in the experimental group than the control. Stratos et al (2013) have shown in a 42day study that the vitamin D increases cellular turnover and functionally restores the skeletal muscle after crush injury in rats. Muthal et al (2008) evaluated the neuroprotective effect of ultra-high dose (500 µ/kg) of Methyl cobalamine (B12) by using partial sciatic nerve ligation and sciatic nerve crush injury models in wistar rats and concluded that it has promising neuroprotective action in both circumstances. Sun et al (2012) studied the synergistic beneficial effect of dexamethasone and vitamin B12 on regeneration of peripheral nerve in sciatic nerve injury model and concluded that dexamethasone and vitamin B12 promote peripheral nerve repair through up regulation of BDNF expression. They claim that their findings provide new insight into the neurotrophic effects of dexamethasone and B12 and support the application of these agents in clinical treatment of peripheral nerve injury. Morani and Bodhankar (2010) studied the effect of vitamin E and B12 in sciatic nerve crush injury in rats concluded that early co-administration of vitamin E acetate and methyl cobalamine improves thermal hyperalgesia and motor nerve conduction velocity following sciatic nerve crush injury. Morani (2012) showed that early co-administration of vitamin E (50 mg/kg) and methyl cobalamin (500 mg/kg) prevent progression of neuropathic complication in diabetic rats. Thus, in most of the research works showing perceptible nerve regeneration are either based on biochemical or functional parameters and lack concomitant histopathological support in favour of structural basis of functional recovery and therefore, the present study was planned to assess the role of
vitamin C in regeneration of experimentally induced peripheral nerve injury in adult rat using both structural and functional parameters.

**Material and Methods**

After IAEC-clearance a total number of 30 albino rats were obtained from the central animal facility of JN Medical College, AMU, Aligarh and were divided into one control and four experimental groups of 6 rats each. Control without crush, crush without vitamin C (3 week & 6 weeks), crush with vitamin C (3 weeks & 6 weeks). Walking track analysis for sciatic function index (Bain et al, 1989) of each experimental animal was performed before induction of injury and subsequently at weekly interval. From the hind limb footprints, print length factor (PLF), toe spread factor (TSF) and intermediary toe spread factor (ITF) were calculated. Incorporating these factors in the following equation, the Sciatic Function Index (SFI) was calculated \[ SFI = -38.3 \times PLF + 109.5 \times TSF + 13.3 \times ITF - 8.8 \]. Under general anaesthesia and aseptic condition the sciatic nerve crush-injury was induced in the mid-thigh region [Fig. 1A] with Kocher’s forceps pressed and locked for 5 minutes. 0.5 ml of vitamin C (Ascorbic Acid Injection, IP, 100 mg/5ml) was instilled locally at the site of injury and additional dose of 100 mg/Kg:1.25 ml, IP was also given and wounds were closed with 3-0 Vicryl (2 metric – NW2401)-absorbable sterilizable surgical needle suture USP (synthetic; braided coated polyglactin 910 violet; from Ethicon, manufactured in India by Johnson Johnson Ltd, Aurangabad). Animals were given 0.5 ml injection of analgesic Voveran/Rumagesic (Diclofenac sodium containing 25 mg DS/3ml from Zee Drugs, New Delhi) and antibiotic Monocef (containing Ceftriaxone 1gm, injection, IP from Aristo Pharmaceuticals, Mumbai) – and wounds were dressed with Betadine solution (10% Providone-Iodine solution IP, from Win Medicare, Pvt. Ltd, New Delhi). The second dose of vitamin C was given after two weeks. At the end of the study period (3 to 6 weeks), animals were sacrificed and nerves were procured and immersion fixed in Karnovsky’s fixative. The whole nerve segments from some samples were treated with Osmic acid (Wei et al, 1989) for a couple of days and subsequently processed for paraffin sections. Other nerve samples were processed and 07-10 μm thick paraffin sections were stained with H&E, and Masson’s trichome stain. All sections were examined under trinocular light microscope (Olympus: BX40, Japan) both under low and high magnification objectives and representative images of relevant findings were recorded.
Observations

*Sciatic Nerve Function*

All animals from both control and experimental groups remained healthy throughout the period of study and none of the rats showed signs of infection or ulceration of the foot. The animals from experimental groups showed weakness in the hind limb, altered gait, dragged the dorsum of their foot and showed reduced response to pinch test. However, with the passage of time, they showed gradual improvement in their gait as compared to one observed on post-operative day-1. Assessment of sciatic nerve function by Walking track analysis revealed that SFI ranged from -70 to -59 & -70 to -55 at 1 and 6 weeks post-operatively in experimental group without vitamin C & with vitamin C respectively indicating thereby that though the functional recovery was taking place in both groups the one with vitamin C showed better SFI.

**Gross:** The crushed region of the nerve was commonly associated with aggregation of fibro-fatty tissue and nerve itself appeared a bit fluffy.

**Microscopic**

It was observed from the Osmic acid stained samples in control group that the sciatic nerve consists of fascicles having nerve fibres of different diameters and myelin sheath thickness [Fig. 1B]. The nerve fibres run parallel to each other where nodes and internodes can easily be identified [Fig 1C]. The contour of the myelin sheath were smooth, uniform and their overall thickness varied with the diameter of the nerve fibres. The perineurial and endoneurial connective tissue were minimal and could just be identified. In every field the number of nuclei/cells was quite less as compared to those seen in the samples from experimental groups. The experimental group showed degenerative changes in different stages. In early stage the nerve fibres showed swelling and fragmentation of myelin sheath [Fig. 1D] and by the end of six weeks complete degeneration was seen in the vicinity of injury. The debris of degeneration was marked in early stage while in later stages major portion of degeneration product was removed [Fig. 1E]. Macrophages were associated with degenerating myelin sheath and Schwann cells were prominently seen both because of their increased size and numbers and were arranged in a form of sheet – Band of Bungner [Fig. 1E]. The endoneurial fibroblasts were also increased in number and endoneurial connective tissue (blue in Masson’s Trichrome stain) defined the limits of each nerve fibre. Although, by the end of 6
weeks, the clearance of debris of degeneration was not complete, regenerating nerve fibres were seen to move in to the endoneurial tube left by the degenerated nerve fibre [Fig E].

Discussions

Peripheral nerve injuries include a variety of traumatic injuries, diseases, tumors and some iatrogenic lesions. Experimental nerve crush is an accepted model for peripheral nerve injury and regeneration. In the present study neuro protective effect of topical as well as systemic use of vitamin C which is an antioxidant has been assessed using both functional and qualitative histopathologic parameters. An antioxidant is a molecule that inhibits the oxidation of other molecules by terminating the chain reactions induced by free radical intermediates and therefore, vitamin C can also reduce and neutralize reactive oxygen species such as hydrogen peroxide (Padayatti et al, 2003). Oxidation reactions may be viewed as a double-edged sword because on one hand it is essential for life but its imbalance leads to oxidative stress. The plants and animals maintain complex systems of antioxidants. A state of oxidative stress is caused either by insufficient levels of antioxidants, or inhibition of the antioxidant enzymes which may prove harmful to cell structure and function.

In the present study, up to the 4th day post-injury, there was only mild improvement of gait without clear hind foot-print on the side of injury. This time period matches with the minimum duration believed to be required for the sprouting to begin from the proximal nerve stump. By the end of 1 week animals start taking some support on the injured side and thus it helped in SFI analysis. With apparent improvement in gait and improving SFI from -70 to -55 over the period of 6 weeks suggests an appreciable recovery which is in agreement with the observation of many previous workers who demonstrated functional improvement in crush-nerve model by using either vitamins (Stratos et al, 2013; Muthal et al, 2008 and Sun et al, 2012) or corticosteroids (Galloway et al, 2005; Sozucukler et al, 2013; Sulu et al, 2013; Galloway et al, 2000 and Khan et al, 2014). However, some workers claimed both structural and functional recovery but not the electrophysiological recovery (Sevuk et al, 2014 and Besalti et al, 2007). Microscopic examination in the present study revealed that as compared to control sciatic nerve the crushed region of nerve showed increased cellularity which was in the initial stage primarily due to influx of inflammatory cells, while in the later stages it was because of proliferation of resident cells of connective tissue, namely endoneurial fibroblasts and Schwann cells. These cells and macrophages collaborate and coordinate the clearance of myelin debris for nerve regeneration to follow.
In another related study Morani and Bodhankar (2010) studied the effect of vitamin E and B12 in rats concluded that their early co-administration improves thermal hyperalgesia and motor nerve conduction velocity while Morani (2012) showed that early co-administration of vitamin E and methyl cobalamin prevent progression of neuropathic complication in experimentally-induced diabetic rats.

The crushed sciatic nerve in the present study showed changes very similar to that described for Wallerian degeneration and in fact in the present study, subtle degenerative changes were seen as early as by just 24h post-injury, similar to one as described earlier in the optic nerve (a part of CNS) after ocular enucleation in adult rabbit (Khan, 2004). The changes seen in the sciatic nerve after crush-nerve injury were in the form of swelling, oedema, fragmentation and condensation of myelin. Many macrophages could be seen in the vicinity of myelin fragments. Endoneurial fibroblasts and Schwann cells showed both hypertrophy and hyperplasia. In longitudinal sections of nerve they were seen in the form of quite robust cellular band running on either side of the degenerating nerve fibres (Fig. 1E) giving a foamy appearance.

Generally peripheral nerve injury is followed by axonal degeneration first and then the myelin degeneration. Clearance of debris of degeneration is initiated by the Schwann cell and is finished by the macrophages. They were in fact associated with formation of endoneurial tube for the passage of future growing nerve fibres from the proximal nerve stump. In the present study, Masson’s Trichrome stained sections from 6 weeks-post injury revealed certain interesting findings e.g., Schwann cells were very prominent because of both hypertrophy and hyperplasia and were arranged in a form of sheet – Band of Bungner [Fig. 1E]. The endoneurial fibroblasts were also increased in number and endoneurial connective tissue (blue) defined the limits of each nerve fibre. Although, by the end of 6 weeks, though the clearance of debris of degeneration remained incomplete, the regenerating nerve fibres were clearly seen to pass in to the endoneurial tube left by the degenerated nerve fibre [Fig 1E]. Though, in most instances the regenerating nerve sprout had not fully crossed the actual site of injury the regenerating process appeared obvious and encouraging. Thus the histopathological findings lend an unambiguous support to the concept of structural basis of functional recovery over the study period of 6 weeks. That is to say, that by the end of 6 weeks post-injury both structural & functional recoveries have started although not yet completed.
Schwann cells and fibroblasts and macrophages help both directly and indirectly in removal of debris and regeneration in a planned and orchestrated manner. The Nerve growth factor (NGF) which is very low in healthy nerves, within 2 weeks of injury the NGF mRNA expression has been shown to increase by 5 to 7 times mainly through nerve fibroblasts and Schwann cells (Heumann et al, 1987) which get stimulated by the macrophages via macrophage-derived interleukin-1 (Lindholm et al, 1988). Apart from growth factors, Schwann cells also provide structural guidance to further enhance regeneration. During their proliferation phase, Schwann cells begin to form a line of cells called Bands of Bungner within the basal laminar tube. In the present study axons have been observed to regenerate in close association to these cells as described by Thomas & King (1974). Schwann cells up regulate the production of cell surface adhesion molecule ninjurin and thus further promoting growth (Toshiyuki & Jeffrey, 1996). Other neurotrophic molecules produced by Schwann cells and fibroblasts together include Brain-derived neurotrophic factor (BDNF), Glial cell line-derived neurotrophic factor, Ciliary neurotrophic factor, Leukemia inhibitory factor, Insulin-like growth factor, and Fibroblast growth factor (FGF). These factors together are believed to create a favorable environment for axonal growth and regeneration (Vargas and Barres, 2007).

Many other factors that either regulates Schwann cell or myelination also affect regeneration for example endogenously synthesized FGF-2 (Jungnickel et al, 2006) influences early peripheral nerve regeneration by regulating Schwann cell proliferation, axonal regrowth, and remyelination. Interestingly, recently a novel experimental immunological demyelination method (Kosin et al, 2011) has also been used to enhance nerve regeneration in the adult rat sciatic nerve.

In the present study though reactive Schwann cell proliferation is robust and growing axon/neurite could clearly be demonstrated from the proximal nerve stump to pass half way through to cross the actual site of injury. It is possible that some additional time is required to observe such thing to happen. But according to others (Sulaiman & Gordon, 2013) chronic Schwann cell denervation, chronic neuronal axotomy, and misdirection of regenerating axons into wrong endoneurial tubes are primarily responsible for poor functional recovery.

The secondary nerve injury which follows the primary one is likely to cause deterioration with increasing post-injury time. The reason for autodestruction during secondary nerve injury is believed to be caused by oxidative stress (Sies & Cadenas, 1983; Braughler & Hall, 1992; Phillis, 1994; Marin et al, 1998; Bagdatoglu et al 2002). On the other hand vitamin-C a
known antioxidant has been shown to have some neuroprotective effects (Frei et al, 1989; Shokouhi et al, 1989). In the present study though both sets of experimental groups (crush-without vitamin C and crush with vitamin C) showed functional improvement, the group receiving vitamin C showed better response. This may be explained by the fact that in the body there are inbuilt antioxidative mechanisms in place to check the possible harmful effects of secondary injury and additional vitamin C somehow appears to support this mechanism and impedes the secondary injury thus helping in better functional improvement. In the present study it is expected that the anti-inflammatory potential of vitamin C must also have played a significant role in the observed better functional recovery similar one described by Demir et al, (2014) for alpha-lipoic acid.

Thus, from the present study it was concluded that there is certainly an structural basis behind the observed faster functional recovery and therefore, the use of vitamin C to either trim down the post-injury nerve dysfunction or improve nerve regeneration finds a logical basis for its use in future clinical practice.

REFERENCES


Fig. 1 Photograph of control sciatic nerve [A] from left side. Arrow indicates the site of induction of crush-injury. Osmic acid stained control sciatic nerve in T.S. [B] and L.S. [C] showing nerve fascicles of different size consisting of myelinated nerve fibres of different diameters running parallel to each other. Red arrow [in C] indicates node of Ranvier. Osmic acid stained sciatic nerve from 3 weeks-post injury group showing black stained clumps of degenerating myelin. Connective tissue and areas devoid of debris of degeneration appear light yellow. Masson’s trichrome stained sciatic nerve from 6 week post-injury group placed horizontally [E] in such a way that proximal part lies on left and distal towards the right side. Degenerating nerve fibres appear light pink & cloudy with many degenerating myelin granules. The endoneurial connective tissue is blue demarcating the outline of nerve fibres. Nerve fibres are pink and green arrows indicate the regenerating nerve fibres coming from left towards right side to enter the endoneurial tube. Black arrow indicates Band of Bungner consisting of proliferated Schwann cells.

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