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Microscopic and electrophysiological changes on regenerating sciatic nerves of rats treated with simvastatin

Ergun Daglioglu[1], Mustafa Berker[2], Mehmet Demirci[3], Mürvet Tuncel[4], Erdem Karabulut[5], Aykut Erbengi[2]

[1]Department of Neurosurgery, Ankara Numune Education and Research Hospital, [2]Department of Neurosurgery, Hacettepe University, [3]Department of Neurology, Hacettepe University, [4]Department of Anatomy, Hacettepe University, [5]Department of Biostatistics, Hacettepe University, Ankara, Turkey

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Abstract

Simvastatin is a hypocholesterolemic agent presumed to cause peripheral neuropathy. We arranged an experimental design which focuses on the effect of simvastatin on peripheral nerves and neural regeneration. Sciatic nerve injury was performed at midthigh region of male wistar rats either by clamp compression or fine cut. Electrophysiological and electron microscopical studies were carried out to assess the effect of simvastatin on peripheral nerve and nerve regeneration. There was no difference between the groups that were given simvastatin and standard regimen in the sciatic nerve when electrophysiological measurements were concerned. However, some of the rats that were given simvastatin show reduction in axoplasm density (intensity) of myelinated nerve fibers and prominent vacuolization of myelin sheath according to light and electron microscopic studies. Sciatic nerve compound muscle activation potential measurements of the animals given simvastatin showed that this drug doesn't have a delaying effect on the peripheral nerve recovery time. Electrophysiological measurements showed that simvastatin did not influence nerve regeneration however it was found to induce severe vacuolization of myelin sheath of the sciatic nerve. It was apparent that the drug induces some form of structural dysfunction as myelin changes supported by electron microscopical studies. Conclusion: simvastatin was shown to delay regeneration as shown in microscopic studies but still there was no influence on nerve regeneration.

Key words: simvastatin, peripheral nerve, experimental, rat, sciatic nerve, peripheral neuropathy.

Introduction

Peripheral neuropathy can be initiated by various therapeutic agents however few agents were definitely proven to induce this side effect. In the past decade, hypocholesterolemic agents were hold responsible for this induction but the accumulated data was insufficient to prove this great suspicion. Simvastatin is the first hypocholesterolemic agent presumed to cause peripheral neuropathy. It was claimed to induce a clinical syndrome with features of sensorimotor neuropathy and the clinical symptoms were reported to subside with its withdrawal [7,10,11, 14]. Nevertheless, the interval between the onset of symptoms and initiation of therapy can't be precisely estimated so raising some doubts concerning this

Communicating author:

Ergun Daglioglu, MD, Department of Neurosurgery, Ankara Numune Education and Research Hospital, Kuzgun Sok. 15/14, Ayranci, 06540, Ankara, Turkey, phone (+90) 5052296838, fax (+90) 3123111131, e-mail: edaglioglu@gmail.com

unpredicted side effect. The clinical picture which ranges between mild numbness to evident neuropathy may be attributed to simvastatin use [3]. Recently, we met a patient with diabetic neuropathy who experienced rapid deterioration of his neuropathic symptoms a week after initiation of simvastatin treatment for hypercholesterolemia. This situation aroused our interest on drug-induced neuropathies and upon reviewing the literature we noticed similar patients complaining the same adverse affects related to the use of this agent. Rapid recovery after cessation of simvastatin therapy justified our observations and we decided to organize an experimental basis to evaluate the influence of this drug on neural tissues. To our knowledge, this is the first experimental study which focuses on the influence of simvastatin on peripheral nerves and nerve regeneration.

Material and methods

Experimental protocol was carefully reviewed and approved by Animal Studies Ethic Committee, Hacettepe University.

Animals and anesthesia

Male Wistar albino rats weighing 205 grams on average were utilized in the experimental setting. They were housed in plastic cages at room temperature and had free access to food and water. Rats were anaesthetized with a mixture of ketamine chlorhydrate 5% and xylocaine 2% solutions injected intraperitoneally, with a dosing of 50 mg/kg and 10 mg/kg respectively. Experimental groups and distribution of rats are summarized on Table IA.

Surgical procedures and electrophysiological recordings

Both sciatic nerves of rats were exposed at midthigh level and basal electrophysiological recordings of each side were taken from gastrocnemius muscle with surface cup electrodes. After repeated stimulations of the nerve with hook electrodes at the sciatic notch, maximal amplitude or area recording values were considered as reference measurement. A ground needle electrode was placed at the most distal site of the concerning extremity while an anode needle electrode was inserted at the tip of the tail. The compound muscle action potentials (CMAPs) were measured with cup electrodes from the belly of gastrocnemius muscle.

Simvastatin was given to animals at a dose of 2 mg/kg/day orally within their standard water at a dilution estimated in accordance with their basal requirements [4].

In the first part of the experiment (n = 30), rats were divided into two separate groups as Axonotmesis (n = 15) and control (n = 15) (Table IA). Half of each group to be fed with simvastatin was selected at random and baseline CMAP was measured for each group. Experimental site of the rats was also selected at random and the sciatic nerve was crushed for 2 minutes' duration at midthigh level just 5 mm proximal to trifurcation. A surgical clamp was specifically designed for this procedure: 2 cylindrical plastic sheets were adapted to the tips of the clamp 15 mm longitudinally so that four discrete degrees of compression pressure could be applied uniformly. A pilot study was performed before the experimental procedure to detect the exact amount of force on neural tissue which pointed out that 323 Newton/cm^2 compression pressure for 2 minutes. The procedure was tested at our Physics Laboratory and this compression effect was designed to produce an 80% of approximate reduction of amplitude measurements from the gastrocnemius muscle on average. Immediately after compression, CMAPs were measured at the 1st and 30th seconds subsequently. The midcrush site was labeled with a single 8/0 nylon suture and the wound was closed.

Table IA. Distribution of rats and experimental groups

Groups	Percentage of decrease in amplitude after neural injury	Treatment
Axonotmesis (n = 15)	66%	–
Axonotmesis + simvastatin (n = 15)	42%	simvastatin
Neurotmesis (n = 5)	100%	–
Neurotmesis + simvastatin (n = 5)	100%	simvastatin

The rats were then kept in numbered plastic cages separately under a 12 hour light/dark cycle and they were allowed free access to standard requirements. Four and 8 weeks after the procedure, CMAPs were recorded again from each rat in axonotmesis group. Histological sections were taken simultaneously from each group after 8 weeks.

In the second part of the experiment (n = 20), rats were divided into Neurotmesis (n = 10) and control (n = 10) at random (Table IA). Half of each group was fed with simvastatin and the other half with standard regimen. After measurement of baseline CMAPs, the sciatic nerve was cut with microscissors at midthigh level. Cut ends of the nerve were approximated with epineurial sutures (8/0 prolene). CMAPs were again measured from both sciatic nerves 14 weeks after the initial procedure and histological sections were taken from sciatic nerve.

All surgical measures were also performed at control sites except the experimental protocol and these nerves were regarded as sham operated groups. Additionally, these sites also contribute to basis of electrophysiological and morphometric analysis.

Histopathological studies

A segment of sciatic nerve measuring 2.5 mm in length (just at the midpoint of suture mark) was sectioned from each group including controls. Nerve segments of the opposite sites were reserved for standardization of the morphometric measurements. All the samples from the regarding group (axonotmesis, neurotmesis and simvastatin group) were taken simultaneously after 8 weeks. All these samples were fixed for 24 hours in 2.5% glutaraldehyde in phosphate buffer. Then the sections were postfixed in 2% osmium tetroxide and dehydrated in serially diluted alcohol solutions. After treating with propilene oxide they were embedded in Araldite CY 212. Transverse sections of 1-2 micron were cut with LKB NOVA ultratome and stained with Toluidine blue. These sections were examined by light microscopy. The ultrathin (60-90 nm) sections were taken using LKB NOVA ultratome. After double staining with uranyl acetate and lead citrate, these ultrathin sections were examined by transmission electron microscopy (Jeol JEM 1200EX). Following parameters were calculated for morphometric analysis: # of fibers, fiber density (# of fiber/10 000 μm^2), fiber diameter (µm), myelin thickness (µm), myelin area (μm^2), and axon diameter/fiber diameter ratio (G factor). The analysis was performed on five sections from five different animals in each experimental group.

Statistical studies

Electrophysiological data were statistically analysed by Friedman Two-way Anova. Following these analyses Mann-Whitney U and Wilcoxon rank-sum W tests were used to check for differences between individual groups. For morphometric data, statistical tests were not performed since the number of rats studied per group is insufficient.

Results

Electrophysiological data

Axonotmesis and neurotmesis were taken as two neural injury models to be used in our sciatic nerve study. Opposite site of extremities were reserved for sham operation both in axonotmesis and neurotmesis groups and only dissection of sciatic nerve was performed on regarded extremity. The difference between two groups was investigated by electrophysiological and electron microscopic methods in the recovery period of sciatic nerve injury. Baseline and after treatment measurements of sham operated group which were given simvastatin was also measured. This control group was designed to detect a potential side effect of simvastatin as well as to eliminate the surgical impact on sciatic nerves. Baseline CMAP values were measured bilaterally at each rat in all experimental groups (Table IB). In axonotmesis groups, EMG recordings immediately after the compression showed no significant difference between the CMAP baseline values of simvastatin group and its control.

In axonotmesis group, the difference between CMAPs measured at baseline and 8 weeks after clamp compression were not found to be significant (Table II) (Graphic 1). The values obtained 4 and 8 weeks after the crush were also insignificant. The va-

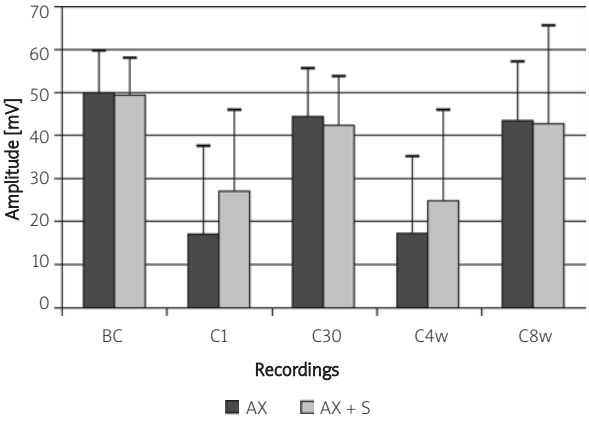
Table IB. Median amplitude values measured from experimental side and the sham side in all groups of the experiment

	Experimental side	Sham operated side	P value
Amplitude (mV)	47.1 ± 6.87	44.9 ± 12.6	0.96

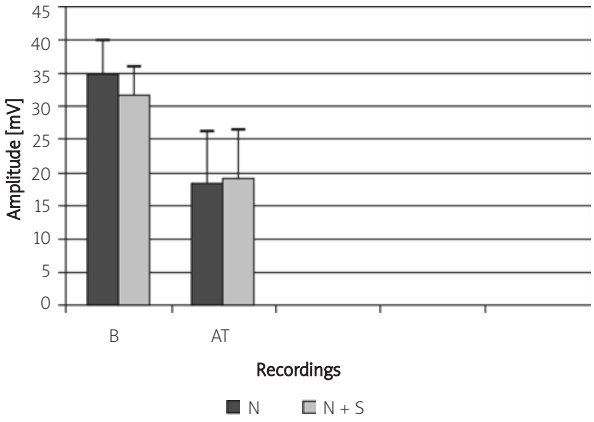
Table II. Amplitude of compound muscle action potentials recorded in axonotmesis and axonotmesis + simvastatin groups (mean ± standard deviation)

Groups	Mean amplitude values in axonotmesis group (mV)	Change in amplitude (%)	Mean amplitude values in axonotmesis + simvastatin group (mV)	Change in amplitude (%)
BC	49.9 ± 9.9		49.5 ± 8.7	
C1	16.9 ± 20.7	–66.1	27.2 ± 19.0	–45.1
C30	44.4 ± 11.4	–11.0	42.4 ± 11.4	–14.2
C4w	17.4 ± 17.8	–65,1	24.7 ± 21.5	–50.0
C8w	43.5 ± 14.0	–12.9	42.9 ± 22.9	–13.4

BC – baseline mean values before compression, C1 – values recorded 1 second after compression, C30 – values recorded 30 second after compression, C4w – values recorded 4 weeks after compression, C8w – values recorded 8 weeks after compression
Change in Amplitude: Percentage of change in mean amplitude values compared to reference measurements



Graphic 1. Graphic presentation of amplitude values recorded in axonotmesis groups.



Graphic 2. Graphic presentation of amplitude values recorded in neurotmesis groups.

lues obtained after 4 weeks were significantly lower in amplitude and latency when compared to baseline values (p = 0.014 and 0.0077 for simvastatin fed rats and rats on standard diet respectively). This finding verified axonotmesis in both groups and there was no significant difference between simvastatin and control group values (p = 0.31). The values after 8 weeks did not show any difference between simvastatin fed rats and rats on standard regimen (p = 0.063). Similarly, the values obtained after 8 weeks were quite similar to the initial values (p = 0.83) (Table II). These values obtained after 8 weeks designated successful nerve regeneration and again no difference between simvastatin and control group. Again, no significant difference was found between cMAP values of simvastatin fed rats and rats on standard regimen. These findings showed that the drug doesn't delay the recovery period in axonotmesis group.

In Neurotmesis group, EMG recordings 14 weeks after the repair showed significant difference when compared with initial values (p = 0.02 and 0.0128 for simvastatin fed rats and rats on standard diet respectively) (Table III) (Graphic 2). However no significant difference was observed in neurotmesis group between simvastatin fed rats and rats fed with standard regimen (p = 0.9168). This finding demonstrated partial nerve regeneration at 14 weeks with no statistical difference between simvastatin and control group (Table III).

Another group of measurements was performed in sham operated sites of axonotmesis and neurotmesis groups. The purpose of these measurements is to

Table III. Amplitude of compound muscle action potentials recorded in neurotmesis and neurotmesis + simvastatin groups (mean standard deviation)

Groups	Mean amplitude values in neurotmesis group (mV)	Change in amplitude (%)	Mean amplitude values in neurotmesis + simvastatin group (mV)	Change in amplitude (%)
B	34.9 ± 5.2		31.6 ± 4.4	
AT	18.3 ± 8.0	–47.7	19.1 ± 7.5	–39.5

B – baseline mean values before neural repair, AT – values recorded 14 weeks after neural repair
Change in amplitude: Percentage of change in mean amplitude values compared to reference measurements

detect possible effects of surgical procedure as well as assessment of a side effect of simvastatin treatment. The difference between cMAP values of sham operated extremities before and after the procedure both in axonotmesis and neurotmesis groups was found to be insignificant (Table IVA and IVB).

The cMAP values of control groups were further analysed in order to estimate the p values between simvastatin fed rats and the ones on standard regimen. At the beginning of the experiment we aimed to eliminate the probable effect of surgery on CMAP values by generating a sham operated group. With the analysis of this group of measurements, no statistical difference could be found between the ones on standard regimen and simvastatin fed rats (Table IVA and IVB).

Light and electron microscopic results

In axonotmesis group, rats which were fed with standard regimen showed remarkable regeneration features, particularly on the peripheral region of the nerves. There were some small myelinated axons and most of them were fully regenerated axons on the observed regions (Fig. 1a). Axonal myelin was normal in appearance. In axonotmesis group including simvastatin fed rats, little regeneration delay was observed in comparison with the former group (Fig. 1b). On electron microscopic study, vacuoles were shown between the myelin layers of the axons. This appearance was hard to differentiate from artifacts during the preparation of the sections.

Tables IVA and IVB. Amplitude of compound muscle action potentials recorded in sham operated sites in axonotmesis and neurotmesis groups (mean ± standard deviation)

A

Groups	Mean amplitude values of shams in axonotmesis group (mV)	Mean amplitude values of shams in axonotmesis + simvastatin group (mV)
BS	42.7 ± 15.4	44.9 ± 6.0
AS	47.8 ± 8.8	46.0 ± 7.3
P	1	0.8

BS – baseline mean values before sham operation, AS – values recorded after sham procedure, P – P values between simvastatin and non-simvastatin group

B

Groups	Mean amplitude values of shams in neurotmesis group (mV)	Mean amplitude values of shams in neurotmesis + simvastatin group (mV)
BS	31.7 ± 0.6	33.7 ± 3.7
AS	27.4 ± 6.8	34.1 ± 2.9
P	0.24	0.41

BS – baseline mean values before sham operation, AS – values recorded after sham procedure, P – P values between simvastatin and non-simvastatin group

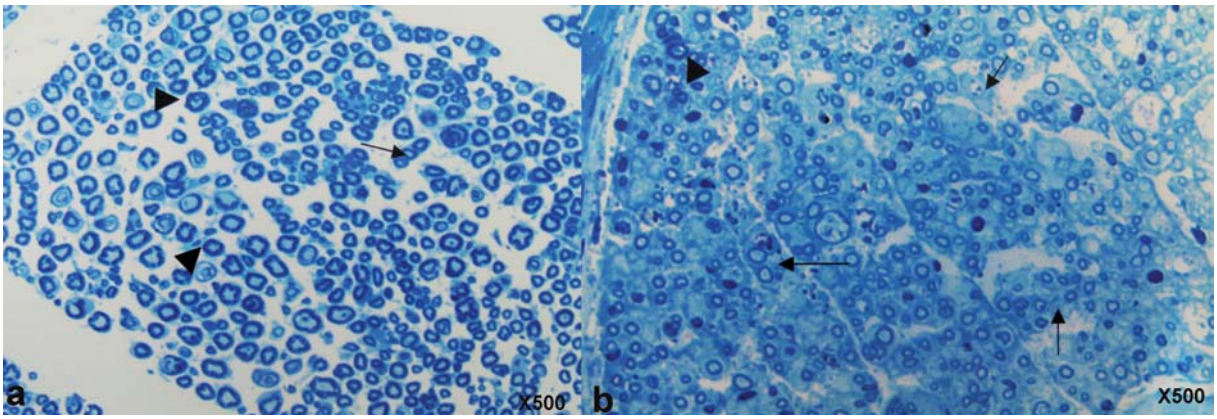


Fig. 1. (a) Regeneration of axons in axonotmesis group. Note marked regeneration of axons with thick myelinated (arrowheads) and few small axons with thin myelinated sheaths after 8 weeks (small arrow). Light microscopic section. (b) Regeneration of axons in axonotmesis + simvastatin group after 8 weeks. Regeneration of axons is less with few thick myelinated (arrowhead) and many small axons with thin myelinated sheaths (small arrows). Light microscopic section.

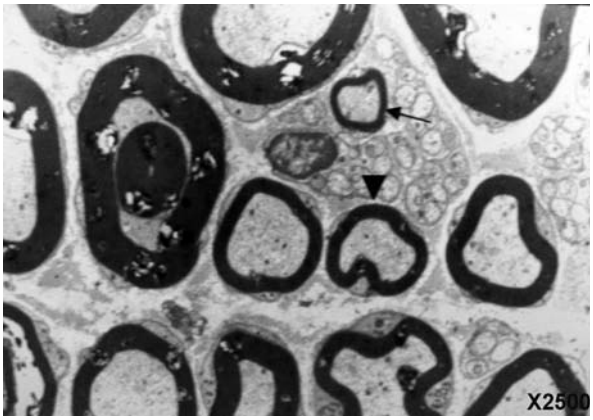


Fig. 2. Electron microscopic section of axonotmesis group shows regeneration of many thick myelinated axons (arrowhead) with few thin myelinated axons after 8 weeks (small arrow).

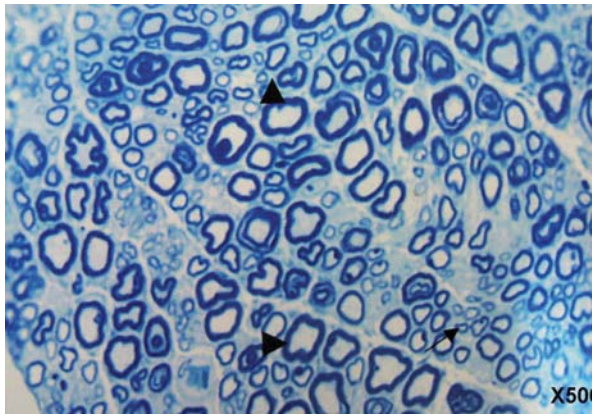


Fig. 3. Regeneration of axons in neurotmesis group. Fine regeneration with thick myelin sheath (arrowheads) is visualized with a small amount of thin myelinated axons after 14 weeks (small arrow). Light microscopic section.

Simvastatin fed rats of neurotmesis group show regenerative findings centrally as in axonotmesis group with a few large myelinated axons and increased number of small regenerating thin axons. Rats fed with standard regimen in this group, had large myelinated axons with a few unmyelinated ones on light and electron microscopic studies (Fig. 3). Separation between the layers and vacuoles of the myelin sheath is also shown on the samples of simvastatin fed rats in neurotmesis group (Fig. 4b). However, fine myelinization of axons and less separation between the layers of myelin sheath were observed in the neurotmesis group (Fig. 4a). Electron microscopic investigation of the samples in neurotmesis group show regeneration, many thick myelinated axons with few thin myelinated axons were observed (Fig. 4).

Regarding histopathological studies, signs of a regenerative process especially in the central regions of the injured nerve was observed in numerous myelinated or some unmyelinated axons, both on electron and light microscopic studies of simvastatin fed rats (Fig. 5). These findings were quite the same as simvastatin fed rats both in axonotmesis and neurotmesis groups. However large and thickly myelinated axons with fine regenerative patterns and featuring less degenerative findings was the domi-

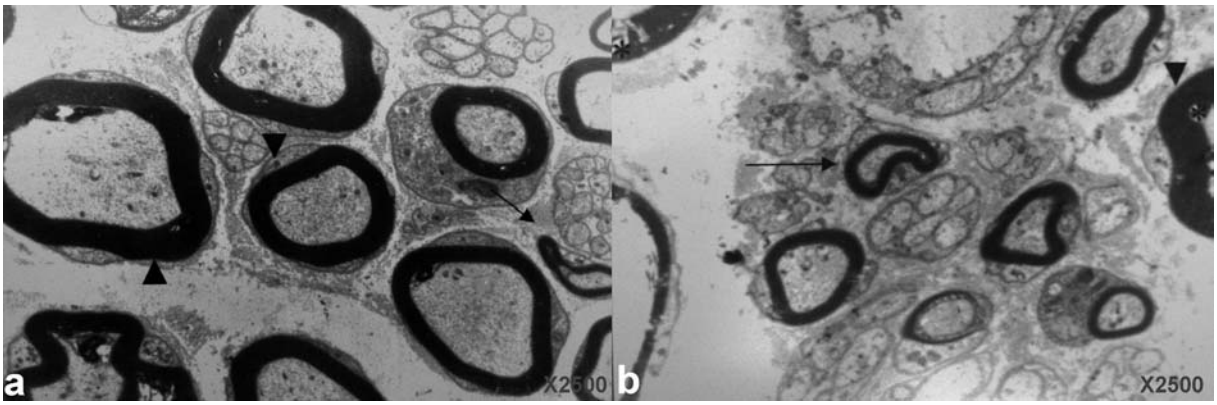


Fig. 4. (a) Electron microscopic section of neurotmesis group show regeneration of many thick myelinated axons (arrowheads) with few thin myelinated axons (arrow) after 14 weeks. (b) Electron microscopic section of neurotmesis + simvastatin group shows regeneration of few thick myelinated axons (arrowhead) and many thin myelinated axons after 14 weeks (small arrow). Asterisks show vacuoles as defects in myelin layers.

nating property when rats on standard regimen were examined.

Discussion

Simvastatin may delay regeneration of peripheral nerve axons and formation of the myelin layers even though it was shown to generate no effect on electrophysiological conduction. Although it is difficult to term this delay as a peripheral neuropathy or to differentiate from artifacts related to preparation, this finding may be considered as a form of neuropathic alteration.

Diabetes, alcohol, anemia, ischemia, nutritional disorders and toxins are well known etiological factors of peripheral neuropathy. Regarding nerve trauma, as a cause of neuropathy, several experimental methods were practiced. The purpose of these methods is to produce the same amount of injury at each trial in order to obtain a standardized set up of lesion. While generating neural injury, we should also avoid foreign body reactions and surgical trauma.

In the electrophysiological studies after clamp compression, it has been shown that the median compound muscle action potential measured values can reach medial basal amplitude and area values approximately in 34 days [1]. In the studies of Kitao, Uchio, Bridge and their colleagues, it was shown that the intensity and duration of compression applied on the sciatic nerve is the contributing factor and the lesion is harmonious with axonotmesis [2,6,13]. Clamp compression model of axonotmesis used in our experimental procedure was specifically designed to introduce a unique and graded neural injury set up to provide a graded model of axonotmesis.

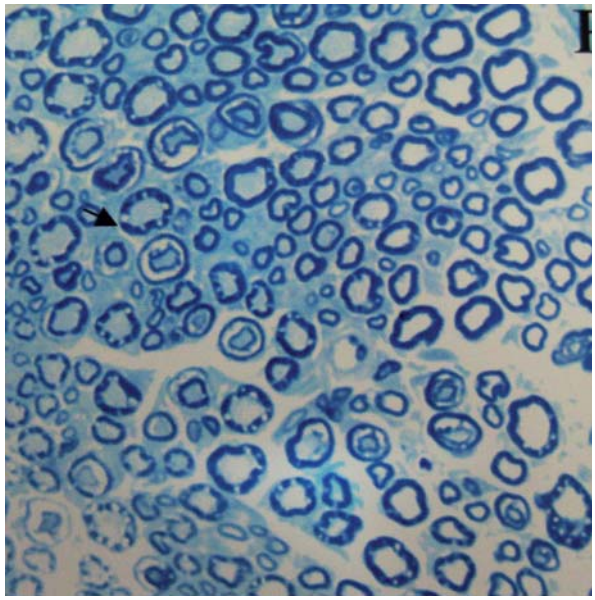


Fig. 5. Appearance of axons in simvastatin fed rats (sham-operated group). Degeneration of axons both in central and peripheral (P) axonal regions with marked vacuolar appearance in myelin sheaths after 8 weeks. Arrow points to axonal degeneration in the central region of axons. Light microscopic section.

Simvastatin, while decreasing cholesterol synthesis by inhibiting the enzyme HMG-CoA reductase in mitochondria, also decreases the synthesis of ubiquinone which is a mitochondrial respiratory

chain enzyme. It is thought that this condition is one of the causes of myopathy [4]. Nakaharo *et al.* found that the lesion occurring in the muscle cell membrane could be a cause the experimental myopathy in rats [8]. It was also found that the mitochondrial enzyme activities of rats which were given simvastatin are normal, despite lower values of ubiquinone by electrophysiological, pathological and biochemical methods [9].

With regard to the clinical studies, Phan *et al*. reported 4 patients with overt signs of peripheral neuropathy related with simvastatin treatment [4]. The association of peripheral neuropathy and simvastatin usage was also emphasized by Australian Adverse Drug Reactions Advisory Committee with a report of 38 similar patients. Recently, a population based epidemiological study revealed that long term treatment with statins may increase the risk of polyneuropathy [5]. We consider that an experimental study regarding statins and peripheral neuropathy is lacking despite several reports on experimental simvastatin induced myopathy.

There was no difference between the groups that were given simvastatin and standard regimen in the sciatic nerve when electrophysiological measurements were concerned. However, some of the rats who were given simvastatin show reduced regeneration of myelinated nerve fibers and slight vacuolization. Sciatic nerve compound muscle action potential measurements of the animals given simvastatin showed that this drug doesn't have a delaying effect on the peripheral nerve recovery time. It is widely accepted that peripheral neuropathy in patients treated with statins may have gone unnoticed by health care professionals [3]. This finding shows that despite normal electrophysiological findings, alterations of sciatic nerve morphology may be encountered, and this finding can clarify the symptoms of neuropathy noticed in patients under statin therapy.

The change in the sciatic nerve cMAP values of rats given simvastatin showed that this drug does not have a delaying affect on the peripheral nerve recovery time although we proposed that the drug would profusely interfere with sciatic nerves' electrical activity. Sciatic nerve light and electron microscopy studies of the whole rats given simvastatin showed more prominent degeneration in the center and prominent vacuolization of the myelin sheath. This finding may point to a structural dysfunction of sciatic nerve and myelin degeneration in the experimental animal resembling peripheral neuropathy although hard to differentiate these findings from artifacts related to preparation of sections [12].

Conclusions

In this study simvastatin was shown to delay regeneration of the nerve fibers in microscopic studies. However we could not prove this finding on electrophysiological measurements.

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