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Original Article

Evaluation of the effect of pitavastatin on motor deficit and functional recovery in sciatic nerve injury: A CatWalk study

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ABSTRACT

Objectives: This study aims to investigate the electrophysiological, scintigraphic, and histopathological effects of pitavastatin and its impact on functional status in rats with sciatic nerve injury.

Materials and methods: A total of 30 Wistar albino rats were divided into three equal groups including 10 rats in each group: sham group (no injury), control group (nerve injury induced), and pitavastatin group (nerve injury induced and 2 mg/kg of pitavastatin administered orally once a day for 21 days). Before and at the end of intervention, quantitative gait analysis with the CatWalk system and sciatic nerve conduction studies were performed. After the intervention, the gastrocnemius muscle was scintigraphically evaluated, and the sciatic nerve was histopathologically examined.

Results: There was no significant difference in the sciatic nerve conduction before the intervention and Day 21 among the groups ($p>0.05$). According to the quantitative gait analysis, there were significant differences in the control group in terms of the individual, static, dynamic, and coordination parameters ($p<0.05$). The histopathological examination revealed a significant difference in the total myelinated axon count and mean axon diameter among the groups ($p<0.001$).

Conclusion: Pitavastatin is effective in nerve regeneration and motor function recovery in rats with sciatic nerve injury.

Keywords: CatWalk, gait analysis, pitavastatin, pleiotropic effect of statin, sciatic nerve injury.

Peripheral nerve injury is a common disorder which may lead to disability. In addition to primary injury, the affected perineural environment, triggered inflammatory and immunological response, and oxidative stress can result in further damage.[1] An agent that can be effective particularly in the secondary injury process can improve recovery.

Besides their cholesterol-lowering effects, statins also exhibit antioxidant, anti-inflammatory, immunomodulatory, and neuroprotective properties and a pleiotropic activity.[2] There are many studies evaluating the effects of statins on the central nervous system, while only few have investigated their effects on peripheral nerves. In studies on sciatic

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nerve crush injury, simvastatin, atorvastatin, and lovastatin have been shown to exert neuroprotective effects.[3-5] Similar to other statins, pitavastatin has anti-inflammatory, immunomodulatory, and anti-oxidant properties. Some pleiotropic properties of pitavastatin (e.g., suppression of vascular inflammation and oxidative stress by endothelial protection) have been found to be highly potent.[6] In addition, pitavastatin has better pharmacokinetic and pharmacodynamic efficacy and minimal drug-drug interactions compared to other statins.[6] Owing to these advantages of pitavastatin over other statins, in the current study, we aimed to evaluate its effect on peripheral nerve injury, considering the lack of research in this area, and to examine the curative effect of pitavastatin using electrophysiological, scintigraphic, and histopathological methods and quantitative gait analysis in experimental models of sciatic nerve injury.

MATERIALS AND METHODS

Animals

To the best of our knowledge, there were no previous similar studies using pitavastatin and the CatWalk was not used in statin studies. Therefore, the sample size was unable to be calculated, since detailed data were not given in previous studies using statin. We arranged our study as 10 rats per group, as in the study in which the duration and practices of the study were most similar.[4] A total of 30 female Wistar albino rats weighing between 230 and 260 g were used in the study. The rats were kept in a 12-h light-dark cycle in standard rooms with a controlled airflow and humidity at a temperature maintained at 22 to 24°C. They were fed *ad libitum* with standard rat chow and tap water. After the surgical procedure, the animals were moved to separate cages.

Drugs administration

Thirty rats were randomized one after another into three groups: sham group (n=10), control group (n=10), and pitavastatin group (n=10). In the sham group, only a skin incision was made and repaired. Nerve injury was not induced, and no medication was given. In the control group, nerve injury was induced, but no medication was given. In the pitavastatin group, after the induction of nerve injury, 2 mg/kg of pitavastatin (Alipza®,Recordati SaRL, Italy) was orally administered. Oral gavage prepared by dissolving in 0.5% methyl cellulose was given to the rats using a 20-gauge blunt feeding needle.

Surgical procedures

After the induction of anesthesia with the administration of intramuscular ketamine hydrochloride (Ketalar®, Pfizer Pharmaceuticals, Istanbul, Türkiye) (87.5 mg/kg) and xylazine hydrochloride (Rompun®, Bayer Pharmaceuticals, Istanbul, Türkiye) (12.5 mg/kg), sterile conditions were achieved and the rats were placed in the prone position. With an incision made between the knee joint and the ischial tubercle, the skin and subcutaneous muscle tissue were passed to reach the sciatic nerve. Using a microsurgical needle holder, axonal injury (axonotmesis) was induced in a 1-mm segment on the nerve, immediately proximal to the left sciatic nerve where it trifurcated. Clamping was performed four times for 15 sec, until the mouth of the needle holder was fully closed. Totally, 5 sec were waited between each clamping. After the procedure, a transparent image was obtained in the axon segment (Figure 1). The muscles were approximated with two pieces of 5/0 poly (glycolide-co-lactide) (Pegelak®, Doğsan, Trabzon, Türkiye), and the skin was repaired with 4/0 polypropylene (Propilen®, Doğsan, Trabzon, Türkiye).

Electrophysiological evaluations

All the rats were electrophysiologically evaluated under anesthesia before surgery and on Day 21 after the intervention. Sciatic nerve conduction studies were performed using the Neuropack M1 (Nihon-Kohden Corp., Tokyo, Japan) device with the following technical settings: stimulation rate, 1 Hz; sampling time, 100 μs; and filter frequency, 5 kHz for high-cut and 10 kHz for low-cut. The room temperature was set at 25°C, and the extremity temperature, measured with a digital needle thermometer, was set at 34 to 36°C. The operation site was shaved. A bipolar stimulator needle electrode was placed on the left sciatic nerve, 10 mm proximal to the crush area, with the anode tip positioned distally. The monopolar recording needle electrode was positioned so that the anode electrode was in the middle of the gastrocnemius muscle and the cathode electrode was in the tendon. The ground electrode was placed on the back of the rats (Figure 2). Stimulation intensity was gradually increased, until a supramaximal response was obtained from the sciatic nerve. The compound muscle action potential amplitude, distal latency, and nerve conduction velocity were recorded for the sciatic motor nerve.

Functional evaluations

Functional evaluation was undertaken with the CatWalk XT (Noldus Information Technology,

Wageningen, the Netherlands), a quantitative gait analysis system used to automatically record the paw prints of mice and rats while walking. To teach the procedure to the rats, they were allowed to walk on CatWalk daily for two weeks before the operation. The animals were placed at the head of a track made of a standard 6-mm thick glass surface and black plastic walls. They were motivated to walk along the track by placing rewards at the end. After two weeks, all animals were able to complete the track without interruption, and their functional parameters were recorded before surgery.[7] On Day 21 of the intervention, walking and recording procedures were repeated. There were a high number of parameters measured by the CatWalk system, and only those that are frequently recommended for sciatic nerve injury in the literature were evaluated due to the limited data on reliability and validity. The print length (cm), print width (cm), and print area (cm^2) were used as individual paw parameters. Using these parameters, the length, width, and area values were calculated throughout the entire stance phase, as if the paws were inked. The following dynamic paw parameters were evaluated: stance duration (sec), swing duration (sec), swing speed (cm/sec), and duty cycle (%) [stance duration/(stance duration+swing duration)]. From the static paw parameters, the maximum contact area (cm^2), stride length (cm), and base of support (BOS) (cm) were used. The maximum contact area refers to the total floor area contacted by the paw during the stance phase. The BOS is obtained by taking the average width of the track made by the front paws and hind paws. Among the coordination parameters, average run speed (cm/sec) and regularity index (%) were selected. Regularity index refers to the exclusive use of regular step patterns during uninterrupted locomotion.[7,8]

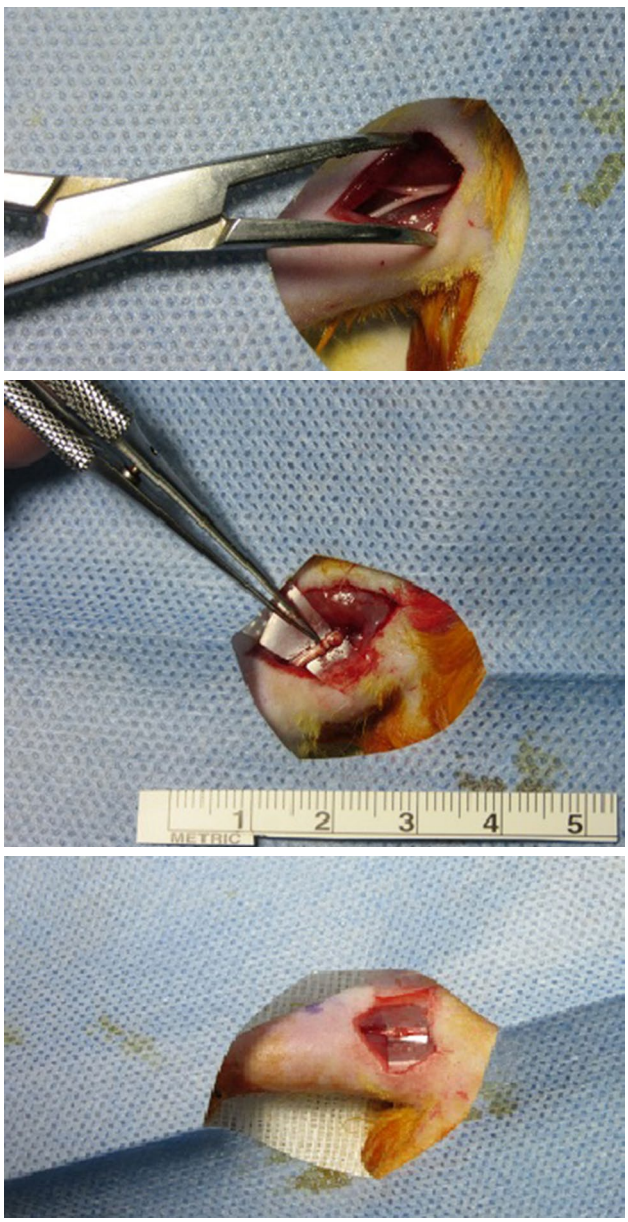

Figure 1. Surgical procedure.

Scintigraphic evaluations

At three weeks after the intervention, muscle perfusion scintigraphy with Tc99m-methoxy isobutyl isonitrile (MIBI) was applied to all the rats

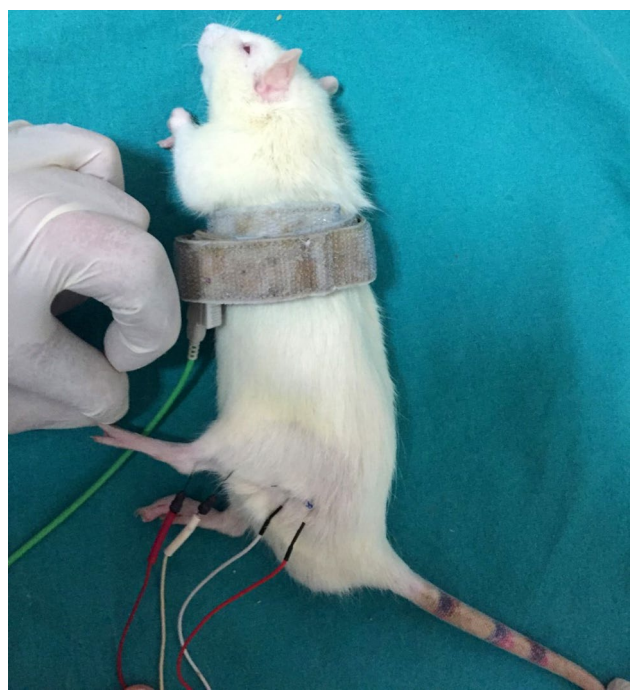

Figure 2. Location of electrodes in electrophysiological study.

under anesthesia to evaluate the perfusion of the gastrocnemius muscle. During the evaluation, a high inter-extremity index and a high calf retention index were considered as good perfusion findings.[9]

Scintigraphy was performed with the rats in the supine position using the MG dual-head SPECT gamma camera (General Electric Healthcare, WI, USA) for anterior-posterior imaging. A low-energy general purpose collimator was utilized in all scintigraphic imaging procedures. Dynamic and static imaging was performed based on a 256×256 matrix. Static images were acquired in the first 10 min during the early blood pool phase, starting simultaneously with the injection. Static blood pool images were acquired between 0 and 5 min in the early phase, with a 5-min late-phase static follow-up image also being obtained between 30 and 35 min. Qualitative and quantitative assessments were applied to the animals. For simultaneous imaging, scintigraphic imaging was initiated by an intravenous bolus injection of 5 mCi MIBI into the tail veins of the rats. In the quantitative evaluation, regions of interest (ROI) were drawn, and the results were obtained numerically. Images were taken bilaterally, and the healthy side was used as a control group. For the evaluation, the count value in each calf region was calculated with symmetrical angular ROI drawn on the acquired images. The count values of the ROI obtained from the static images for the right/left inter-extremity index calculation were determined separately for the right and left sides. The following formulas were used:

1. Inter-extremity index=early ROI left/early ROI right
2. Calf retention index=[(early ROI left-late ROI left)/early ROI left]×100

Histopathological evaluations

On Day 21 of the study, after all the evaluations were completed, the animals were euthanized under deep anesthesia. A sample of the left sciatic nerve was taken from the distal of the crush area. Nerve samples were fixed with formaldehyde and washed in running water overnight to remove formaldehyde. Then, the samples were subjected to routine pathological tissue procedures and passed through graded alcohol (50%, 75%, 96%, and 100%) xylol series and embedded in paraffin blocks. From the prepared blocks, 5 µ-thick sections, (the first three sections and, then, every 10th section), were taken using the Leica RM 2125 RT (Leica Microsystems, Wetzlar, Germany) and placed on

TABLE 1
Results of nerve conduction studies

	Sham		Control		Pitavastatin		
	Median	Min-Max	Median	Min-Max	Median	Min-Max	p^a (post-hoc)
Latency							
Before intervention	1.13	0.86-1.62	0.94	0.74-1.44	0.89	0.70-1.00	0.020 (S>P= C)
Day 21	0.94	0.82-1.18	1.84	1.18-6.08	0.92	0.74-1.08	<0.001 (S=P>C)
p^b	**0.033**		**0.005**		0.091		
Amplitude							
Before intervention	29.80	12.49-39.07	25.70	17.44-42.44	32.44	21.16-43.67	0.153
Day 21	47.65	32.19-75.20	8.53	0.87-19.01	35.01	20.12-40.22	<0.001 (S>P>C)
p^b	**0.005**		**0.005**		0.951		
Velocity							
Before intervention	56.25	50.00-62.50	62.50	50.00-62.50	62.50	50.00-83.30	0.100
Day 21	62.50	50.00-83.50	31.30	15.60-35.70	62.50	50.00-83.30	<0.001 (S=P>C)
p^b	**0.032**		**0.005**		0.999		

p^a: Kruskal-Wallis test; p^b: Wilcoxon test; S: Sham; C: Control; P: Pitavastatin.

TABLE 2
The CatWalk data at different time points

	Sham		Control		Pitavastatin		
	Median	Min-Max	Median	Min-Max	Median	Min-Max	p^a post-hoc
Individual paw parameters of Cat-Walk							
Print length							
Before intervention	1.51	0.58-1.77	1.71	1.10-1.90	1.66	0.98-1.78	0.220
Day 21	1.62	1.09-1.85	1.48	1.00-1.66	1.69	1.40-1.78	**0.005 S=P>C**
p^b		0.593		**0.005**		0.643	
Print width							
Before intervention	1.46	0.48-2.04	1.76	1.08-2.26	1.63	0.84-2.03	0.544
Day 21	2.01	1.15-2.44	1.70	0.96-2.41	2.03	1.53-2.41	**0.022 S=P>C**
p^b		**0.040**		0.721		**0.005**	
Print area							
Before intervention	1.16	0.17-1.57	1.39	0.78-1.77	1.23	0.47-1.41	0.346
Day 21	1.29	0.97-1.72	1.07	0.59-1.72	1.43	0.87-1.83	**0.001 S=P>C**
p^b		**0.007**		0.062		**0.022**	
Dynamic paw parameters of Cat-Walk							
Stance duration							
Before intervention	0.14	0.07-0.17	0.15	0.09-0.18	0.13	0.06-0.15	0.219
Day 21	0.14	0.10-0.16	0.11	0.11-0.19	0.13	0.08-0.16	**0.009 S=P>C**
p^b		0.799		**0.007**		0.501	
Swing duration							
Before intervention	0.11	0.09-0.14	0.11	0.09-0.13	0.11	0.09-0.13	0.301
Day 21	0.12	0.09-0.15	0.15	0.13-0.17	0.13	0.12-0.14	**<0.001 S=P>C**
p^b		0.112		**0.005**		**0.009**	
Swing speed							
Before intervention	94.7	82.6-138.1	104.2	86.2-116.3	106.7	91.9-145.1	0.368
Day 21	111.5	85.5-132.0	85.9	77.1-102.7	115.4	91.3-158.7	**<0.001 S=P>C**
p^b		**0.021**		**0.007**		**0.024**	
Duty cycle							
Before intervention	54.8	36.0-61.1	57.3	41.3-62.9	52.9	35.0-61.7	0.117
Day 21	53.8	49.9-53.3	42.8	29.1-54.2	49.0	38.1-54.4	**0.001 S=P>C**
p^b		0.799		**0.005**		0.096	
Static paw parameters of Cat-Walk							
Maximum contact area							
Before intervention	0.97	0.14-1.33	1.33	0.80-1.67	1.04	0.41-1.27	0.178
Day 21	1.14	0.28-1.47	0.89	0.65-1.27	1.14	0.61-1.46	**0.002 S=P>C**
p^b		0.799		**0.007**		0.877	
Stride length							
Before intervention	10.47	5.84-13.62	11.33	10.51-12.42	11.41	10.13-13.48	0.148
Day 21	12.11	9.46-14.60	12.33	9.66-14.11	12.99	11.32-14.92	0.166
p^b		0.005		0.064		0.073	

S: Sham; C: Control; P: Pitavastatin.

TABLE 2
Continued

	Sham		Control		Pitavastatin		
	Median	Min-Max	Median	Min-Max	Median	Min-Max	p^a post-hoc
BOS hind paws							
Before intervention	3.99	3.55-4.68	3.73	2.92-4.76	3.72	2.41-4.60	0.107
Day 21	4.21	3.69-4.95	3.12	2.41-4.22	3.70	2.68-4.68	**0.004 S=P>C**
p^b	0.071		0.021		0.152		
Coordination parameters of Cat-Walk							
Run speed							
Before intervention	39.2	33.7-50.8	40.6	34.1-53.8	45.8	35.5-55.9	
Day 21	47.6	37.2-52.9	32.0	23.3-37.1	47.7	41.3-62.5	**0.001 S=P>C**
p^b	**0.024**		**0.005**		0.501		
Regularity index							
Before intervention	96.1	57.1-100.0	100.0	94.1-100.0	100.0	80.0-100.0	0.103
Day 21	97.2	88.8-100.0	100.0	94.1-100.0	100.0	80.0-100.0	0.526
p^b	0.342		0.999		0.468		

p^a: Kruskal-Wallis test; p^b: Wilcoxon test; S: Sham; C: Control; P: Pitavastatin.

slides. The preparations were passed through alcohol and xylol series and stained with hematoxylin-eosin. All samples were analyzed under a high-resolution light microscope (Olympus DP-73 camera, Olympus BX53-DIC microscope; Tokyo, Japan) and processed with the CellSens Entry Imaging Software version 4.1 (Olympus, Tokyo, Japan). Photographs were taken from five random areas of each sample. The total myelinated axon count, mean axon diameter and axon-to-fiber diameter ratio (G-ratio) were calculated using XV Image Processing Software version 1.12 (Olympus, Tokyo, Japan).

TABLE 3
Scintigraphic and histopathological data

	Sham		Control		Pitavastatin		
	Median	Min-Max	Median	Min-Max	Median	Min-Max	*p* post-hoc
Scintigraphic data							
Early phase	17420	15359-26322	20057	2055-21742	17273	15165-25397	**0.034 S=P>C**
Late phase	14929	12445-23974	8889	1891-20064	14264	12098-23254	0.065
Calf retention index	12.7	8.9-19.7	9.1	2.4-19.2	13.8	8.4-23.0	**0.026 S=P>C**
Inter-extremity index-early phase	1.02	0.93-1.12	0.80	0.37-0.96	1.05	0.84-1.14	**0.001 S=P>C**
Histopathological data							
Total myelinated axon	4.76	3.95-5.20	1.62	1.26-1.74	2.25	1.58-2.57	**<0.001 S>P>C**
Mean diameter	4.95	3.89-5.21	2.57	2.15-2.76	3.10	2.98-3.38	**<0.001 S>P>C**
G-ratio	0.60	0.58-0.63	0.74	0.53-0.89	0.69	0.64-0.71	**<0.001 S>P=C**

Kruskal-Wallis test; S: Sham; C: Control; P: Pitavastatin.

Statistical analysis

Statistical analysis was performed using the IBM SPSS for Windows version 21.0 software (IBM Corp., Armonk, NY, USA). Since the data set did not fit into a normal distribution pattern, non-parametric approaches were used to describe data and test statistical hypotheses. Descriptive data were expressed in median (min-max) values. To test differences between two repeated measures, the Wilcoxon signed-rank test was used. The Kruskal-Wallis test was performed to analyze differences between three groups for each measure. For the pairwise comparison of groups, the Dunn-Bonferroni test was used. A *p* value of <0.05 was considered statistically significant.

RESULTS

There was no significant difference among the groups with respect to the body weights of the rats before the intervention and on Day 21 (Table 1).

Electrophysiological data

There was a significant difference in the sciatic nerve latency, amplitude and velocity values measured before the intervention and Day 21 among the groups (p<0.001). The detailed electrophysiological data of the subjects are given in Table 1.

CatWalk data

Concerning the individual paw parameters evaluated with the Catwalk XT gait analysis, a significant difference was found among the three groups before the intervention and on Day 21. The individual paw parameters showed significant differences among the groups on Day 21 (p<0.05) (Table 2). There was also a statistically significant difference for all variables among the groups on Day 21 in the dynamic paw parameters (p<0.05). The maximum contact area and BOS, the part of static part parameters, were found to be statistically significantly different among the groups on Day 21 (p<0.05). Of coordination parameters, only run speed showed a statistically significant difference among the groups on Day 21 (p=0.001). The results of the CatWalk gait analysis data are shown in Table 2.

Scintigraphic data

A significant difference was observed in the early phase, calf retention index, and inter-extremity index values in scintigraphic measurements among the groups (p<0.05) (Table 3, Figure 3).

Histopathological data

Significant differences were observed among the three groups in terms of all the three parameters of

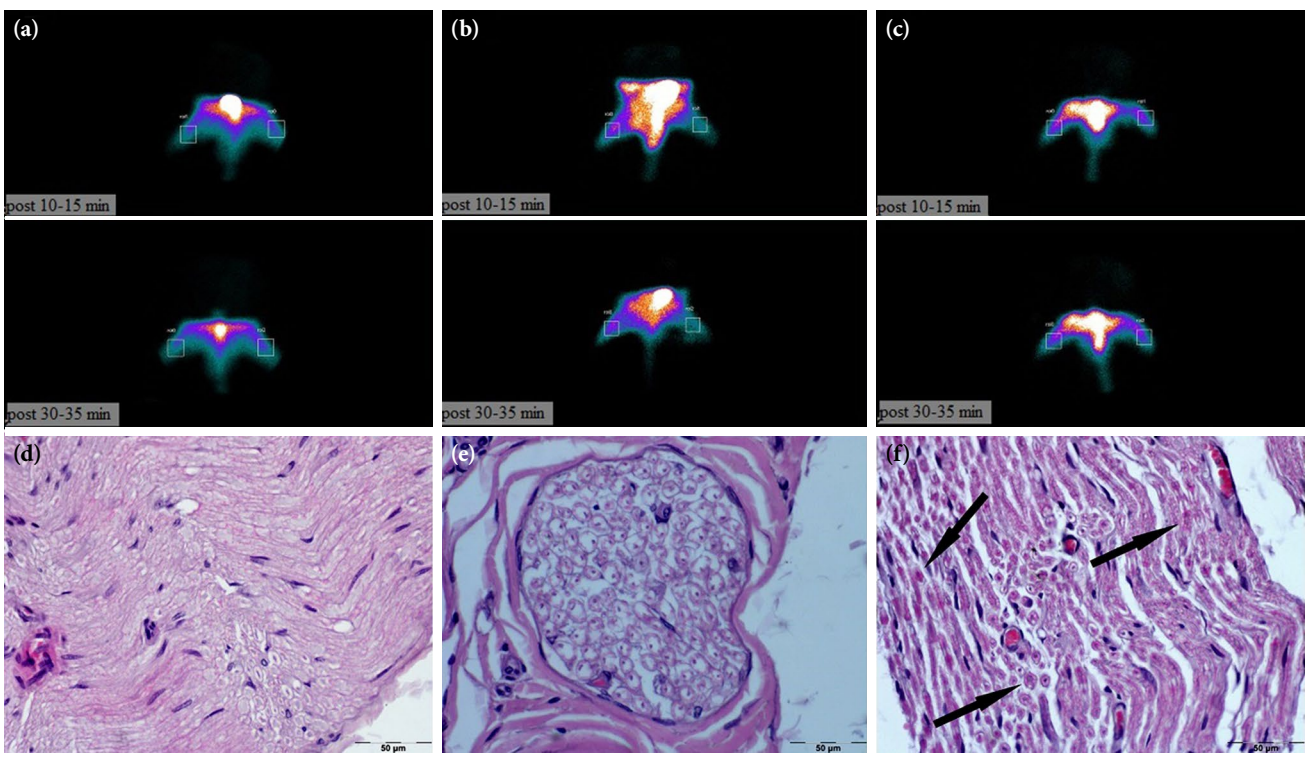

Figure 3. The perfusion of the gastrocnemius muscle. **(a)** Sham group. **(b)** Control group. **(c)** Pitavastatin group. Histopathological evaluations. **(d)** Sham group (H&E, ×50). **(e)** Control group (H&E, ×50). **(f)** Pitavastatin group (H&E, ×50).

the histopathological evaluation ($p<0.001$). The total myelinated axon count and the mean axon diameter had the highest value in the sham group and the lowest value in the control group. Concerning G-ratio, the value of the sham group was significantly higher than those of the remaining two groups (Table 3, Figure 3).

DISCUSSION

In this study, after sciatic nerve crush, pitavastatin resulted in electrophysiological, functional, scintigraphic, and histopathological improvements similar to the values observed in the sham group. This study is valuable, since it is the first to evaluate the effect of pitavastatin on sciatic nerve crush in a rat model.

Although there are no studies investigating the use of pitavastatin in peripheral nerve injury, research has been conducted with other statins. In a previous study, sciatic nerve injury was induced in rats, which were then administered either atorvastatin or saline, and electrophysiological and histopathological values were found to be significantly higher in the atorvastatin group at four weeks.[7] In another study evaluating the effects of lovastatin on sciatic nerve injury in rats, Ghayour et al.[3] reported lower latency and higher amplitude values and also higher mean axon count and myelin thickness in the lovastatin group. In this study, the post-intervention amplitude and latency values obtained by electrophysiological evaluation in the pitavastatin group were similar to the baseline values, and a significantly higher amplitude value and a lower latency. In addition to previous studies, sciatic nerve velocity was also calculated in our study, and the most decrease on Day 21 was observed in the control group. The increase in nerve conduction velocity is associated with an increase in the thickness of the myelin sheath, as well as axonal healing.[10] Moreover, the myelinated axon count and mean axon diameter values were found to be higher in the pitavastatin group than in the control group. The improvement in these values indicates both axonal healing and increased thickness of the myelin sheath. These results support the idea that pitavastatin contributes to neuroregeneration in sciatic nerve injury. In the literature, there are multiple sclerosis studies showing that statins have a positive effect on myelination.[11,12] Statins may exert their myelination effect by reducing edema with their anti-inflammatory properties.[13] Chang et al.[14] reported that pitavastatin reduced cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-alpha (TNF-α), and Chen et al.[15] similarly concluded that this statin showed anti-inflammatory properties by reducing IL-2, IL-6, interferon-gamma (IFN-γ), and TNF-α. In the current study, the anti-inflammatory properties of pitavastatin may have played a role in the increased myelination. Future studies evaluating the effect of pitavastatin on nerve injury can clarify this finding by investigating the anti-inflammatory properties of this statin.

In addition to electrophysiological studies, the sciatic function index (SFI) and CatWalk gait analysis system can be used to evaluate regeneration.[7,16,17] To the best of our knowledge, there is no previous study evaluating the effects of statins on functional recovery in sciatic nerve injury with CatWalk, a quantitative gait analysis method. In a study on atorvastatin, the improvement in SFI was found to be higher compared to the control group.[7] In another study, the group which was administered lovastatin had superior functional improvements at three, five, and seven weeks.[3] In a study evaluating the effects of simvastatin on sciatic nerve crush-induced rats, the simvastatin-administered group had superior SFI values to the controls on Days 7, 14, and 21.[4] Dynamic paw parameters and coordination parameters cannot be evaluated with the SFI. In this study, the differences in the print length, maximum contact area and BOS hind paw values were not found significantly in the pitavastatin group. Therefore, we can speculate that pitavastatin is effective in regeneration and functionality in rats subjected to sciatic nerve crush, consistent with previous statin studies. In our study, the post-crush change in the dynamic and coordination parameters in the control group are similar to the literature.[7,18] Moreover, we observed that the dynamic and coordination parameters of the pitavastatin group were similar to the preoperative values and were significantly superior to the control group and similar to the sham group. Based on these findings, we suggest that pitavastatin is effective in regeneration and improvement of motor function in the presence of static nerve injury.

In this study, scintigraphy measurements, in which the perfusion of the gastrocnemius muscle was evaluated, revealed that the early phase, calf retention index and inter-extremity index values were higher the pitavastatin group. High inter-extremity index and calf retention index values indicate better perfusion.[9] The literature contains positron emission

tomography studies evaluating denervated nerves after nerve injury;[19,20] however, we were unable to find any research involving such an evaluation based on the scintigraphy of the gastrocnemius muscle. Since it is expected that the innervated vascular structure would be affected as a result of the interruption or reduction of innervation, it is reasonable to observe a decrease in these values at three weeks. However, the fact that scintigraphy data in the pitavastatin group showed values closer to the sham group can be interpreted as the effectiveness of pitavastatin in accelerating nerve healing.

This study has certain strengths, such as being the first to evaluate the effects of pitavastatin on sciatic nerve injury, and also being the first statin study to perform the functional assessment of sciatic nerve injury using a quantitative method, CatWalk. On the other hand, the fact that biochemical markers were unable to be included in the evaluation and different doses of pitavastatin were unable to be tested is the main limitation to the study. Future studies may shed light on these issues more accurately.

In conclusion, in rats with sciatic nerve crush injury, the oral administration of 2 mg/kg of pitavastatin once daily for 21 days clinically improved the electrophysiological, scintigraphic, and histopathological markers and the individual, static, dynamic and coordination parameters of gait analysis. In the light of these findings, disability that may develop due to nerve injury can be reduced by pitavastatin through its effects on nerve regeneration and motor function. However, further experimental and clinical studies are needed to draw more reliable conclusions.

Ethics Committee Approval: The study protocol was approved by the University of Health Sciences, Ankara Training and Research Hospital, Animal Experiments Ethics Committee (date: 08.10.2018, no: 180049). All the experimental protocols applied in this study were carried out in accordance with international standards and declarations on animal experiments.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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