OBJECTIVE: This study planned to evaluate the effects of vitamin A (VA) and steroid treatment on nerve healing. Study design: we investigated electrophysiological, electron microscopic (EM), immunohistochemical, and biochemical findings in rat sciatic nerve model.

MATERIALS and METHODS: In total, 112 female rats were divided into 16 groups, each consisting of 7 animals. Subjects were evaluated according to compound muscle action potential (CMAP) findings, ultrastructural staging of myelinated axons with EM, nitric oxide (NO) and malondialdehyde (MDA) findings in serum and tissue, and immunohistochemical findings in the first week and first month.

RESULTS: NO and MDA levels in serum and tissue were found to be statistically lower after high-dose methylprednisolone (MP), normal-dose MP, high-dose MP+VA, normal-dose, and MP+VA treatment modalities compared to controls (p<0.05). The most similar EM findings compared to control animals were seen in the normal-dose MP and high-dose MP+VA treatment groups. The best amplitude values were seen in the high-dose MP and continuous normal-dose MP therapy groups in the first month. In the normal-dose MP and high-dose MP+VA treatment groups, intense staining was found compared to the control and sham groups according to the sciatic nerve immunohistochemical examination.

CONCLUSION: This study revealed noticeable VA effects on the inflammatory process during the healing of traumatic nerve paralysis by electron microscopy and immunohistochemistry. However, similar findings were not established by electrophysiology. Therefore, more experimental studies are needed to further investigate the efficacy and the mechanism of action of VA on steroid administration and the inflammatory process.

KEY WORDS: Facial nerve, peripheral nerve, vitamin A, steroids, traumatic nerve paralysis

INTRODUCTION
Arguments still exist about the appropriate treatment method used in peripheral facial nerve paralysis (PFNP). About 70% of patients recover without any treatment. However, incomplete recovery and/or healing with sequelae is also assumed in this ratio. The management of patients who do not respond to treatment still continues as a controversial issue [1]. Steroids, the most commonly used drugs in the treatment of idiopathic facial nerve paralysis (IFNP), have become the classic treatment method due to the anti-inflammatory and anti-edematous effects. In IFNP, it has been proven that using steroids is more effective than placebo [2]. Although steroid therapy, which will be given at an early stage in IFNP, slows the spontaneous progress in paralysis and accelerates recovery [3], some studies reported that steroids do not provide beneficial effects [4]. Steroids are also used in the treatment of PFNP. In traumatic PFNP, using steroids during the healing period is beneficial for reducing edema in the condition that nerve integrity still persists. After the injury, the inflammatory process is to be established in a balanced manner for the healing of the nerve tissue. The formation of excessive inflammatory reactions causes the occurrence of dense fibrous connective tissue, leading to scar formation. Similarly, intraneural inflammatory reactions are recognized in idiopathic facial nerve paralysis, as reported in some studies [5, 6]. Therefore, a traumatic facial nerve model is a suitable method for the investigation of the effectiveness of various treatment methods for facial nerve paralysis. It is reported that vitamin A (VA) helps to prevent the possible adverse effects of steroids [7]. Although the exact mechanism of retinol antagonism against the effects of corticosteroids in the wound healing cascade is unclear, the increase in the...
percentages of macrophages and monocytes in the inflammatory phase of healing and the counteraction of the effects of corticosteroids on the lysosome are thought to be responsible for the reversal of impaired healing. Retinoids regulate the growth and the differentiation of many cell types, and their actions are mediated through two families of nuclear receptors belonging to the steroid hormone receptor superfamily. These receptors—namely, retinoic acid and retinoid-x receptors—affect the differentiation and proliferation of epithelial tissues by binding and trans-activating the related genes [8]. Retinoids control the growth and differentiation in some cells. Steroids and retinoids show antagonistic effects on these growth factors. Retinoids also stimulate angiogenesis and contribute to the healing process by affecting collagen metabolism [9,10].

Based on available information in this work, the activity of combinations of high-dose methylprednisolone (MP), normal-dose MP, and VA treatments is evaluated in a rat sciatic nerve model with electrophysiological, electron microscopic, biochemical, immunohistochemical, and pathological examinations.

MATERIALS and METHODS
This experimental study was performed in the biophysics laboratory of Mersin University Medical Faculty on 112 female Wistar albino rats, each weighing 250-300 grams.

The experimental protocol was approved by the Animal Care and Use Committee of the University of Mersin (01. 02. 2010/09). The groups were assigned as follows: Group A (1, 2), only high-dose MP application (A1: 1. week, A2: 1. month); Group B (1, 2), only normal-dose MP application (B1: 1. week, B2: 1. month); Group C (1, 2), high-dose MP and vitamin A application (C1: 1. week, C2: 1. month); Group D (1, 2), normal-dose MP and vitamin A application (D1: 1. week, D2: 1. month); Group E (1, 2), high-dose MP and then continuous normal-dose MP application (E1: 1. week, E2: 1. month); Group F (1, 2), high-dose MP and then continuous normal-dose MP and vitamin A application (F1: 1. week, F2: 1. month); Group G (1, 2), only vitamin A application (G1: 1. week, G2: 1. month); Group (Control); and Group (Sham). In normal-dose treatment, MP was administered as 1 mg/kg/day, and high-dose MP was administered as 30 mg/kg/day intraoperatively. Animals in the VA groups received 10,000 IU/kg/day of vitamin A orally.

Rats were anesthetized with 8 mg/100 gr ketamine and 1 mg/100 gr xylazine intraperitoneally. The sciatic nerve longevity and thickness permitted use to harvest enough tissue for multiple evaluations in the same investigation, consisting of biochemical, electron microscopic, and histopathological assessments. The nerve lies relatively deep and stays under the muscles. This situation is advantageous during the re-exploration of the surgical field. The facial nerve is more prone to surgical trauma during re-exploration due to the greater incidence of adhesions to the overlying skin. Therefore, we prefer primarily to work on the sciatic nerve. The right sciatic nerve was exposed at the right gluteal region under sterile conditions. After exposure of the sciatic nerve under an operating microscope, the initial electrophysiological assessment was performed as described in the electrophysiological assessment part of the "Materials and Methods" section. Then, jeweler's forceps application was performed 1 cm proximal to the trifurcation point of the sciatic nerve during 20 seconds by the same surgeon under sterile operative conditions [11,12].

Electrophysiological Assessment
Data were collected by means of a BIOPAC MP 100 acquisition system (BIOPAC, Santa Barbara, USA). Bipolar surface electrodes (Medelec small bipolar nerve electrodes, 6894 T, Medelec, 6894 T, Oxford, UK) were used for recordings from the gastrocnemius muscle. The ground electrode was placed on the thigh on the side of stimulation. The sciatic nerve was stimulated proximal to the lesion site by bipolar electrodes. The supramaximal stimulus consisted of a single square pulse (intensity 0.5 V, duration 0.2 ms). CMAP records were raised on the amplifiers and then transferred to the computer, translated into numerical signals by a 16-bit A/D converter for off-line analysis. The sampling rate was chosen as 20,000 samples/s. BIOPAC Acknowledge Analysis software (ACK 100 W) was used to measure the amplitude of CMAP.

Pathological Assessment
Sciatic nerve specimens obtained from 36 test subjects were fixed in 10% formalin and embedded in paraffin; 4-μm thick sections were obtained from the formalin-fixed, paraffin-embedded tissues. Hematoxylin and eosin (H-E) staining and immunohistochemical staining were used for NGFR (ABBIOTEC, P75NTR) and VEGF (DAKO, CLONE VG1, CODE NO. M7273). Briefly, immunohistochemical staining of the slides was performed with a standard avidin-biotin-immunoperoxidase technique. The external controls for immunohistochemistry were Schwannoma and colon carcinoma for NGFR and VEGF, respectively. The H-E-, NGFR-, and VEGF-stained sections were evaluated using an Olympus BX50 microscope. Edema and the number of mast cells in the epineurium were evaluated using the H-E-stained sections and scored as follows: score 1: 0-2 mast cells, score 2: 2-4 mast cells, and score 3:>4 mast cells. The parameters evaluated for NGFR and VEGF staining were the staining intensity of the Schwann cells in the endoneurium, staining intensity of the vascular structures in the Schwann cells in the epineurium and endoneurium, staining intensity of the fibroblasts, and staining intensity of the perineurium. The staining intensity for both markers was scored as follows: score 0: no staining, score 1: weak staining, score 2: moderate staining, and score 3: strong staining.

Biochemical Assessment
Nitrite-nitrate (NN) Assay
The final products of NO in vivo are nitrite (NO2-) and nitrate (NO3-). The relative proportion of NO2- and NO3- is variable and can not be predicted with certainty. Thus, the best index of total NO production is the sum of both NO2- and NO3-. The oxidized endproducts of NO (NO2- and NO3-) were measured in serum samples by the following assay. Measurements of NO2- and NO3- were made using a procedure based on the Griess reaction [10]. The samples were obtained via indwelling catheters and immediately centrifuged at 4000 rpm for 10 minutes. The serum samples were ultrafiltered and used in the test. Nitrites were quantitatively converted to nitrates for analysis. Enzymatic reduction of nitrate to nitrite was carried out using enzyme cofactors in the presence of nitrate reductase. Enzyme and cofactors were added to each serum sample and standard. After a 1-hour incubation period at room temperature, Griess reagent was added, and all of these mixtures were incubated for 10 min at room temperature in dim light. Then, the absorbance of the samples and standards was measured at 540 nm using a plate reader. In the measurement of to-
tal NO products, only the nitrate standard was required for the standard curve preparation (Nitrate/Nitrite Colorimetric Assay Kit, 780001, Cayman Chemical Company, Ann Arbor, MI, USA). Total nitrite-nitrate levels were expressed as μM of serum.

**Lipid Peroxide Assay**
The levels of MDA, an index of lipid peroxidation, were determined by TBA reaction. The MDA levels in serum were determined by thio-barbituric acid (TBA) reaction according to Yagi. The principle of the method depends on the colorimetric measurement of the intensity of the pink color produced by the interaction of barbituric acid with MDA. The colored reaction 1,1,3,3 tetraethoxypropane was used as the primary standard. MDA levels were expressed as nmol/mL of serum.

**Tissue Assessment**
- **Nitrite-nitrate (NN) assay:** NO levels were studied according to the “Vanadium-3-chloride-Gries Reaction.”
- **Lipid Peroxide Assay:** MDA concentration was studied as described by Yagi et al.

**The Electron Microscopic Assessment**
For the transmission electron microscopic evaluation, the samples were fixed with 2.5% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated in graded alcohol series, cleared with propylene oxide, and embedded in Epon. Thin sections (50-70 nm) were cut by a microtome (Leica UCT-125) and contrasted with uranyl acetate and lead citrate. Sections were examined and photographed by an electron microscope (JEOL JEM-1011). All micrographs were collected at a standard magnification of ×6000. For evaluating myelin damage of the nerve fibers, myelin sheath grading was performed as described before by Tun and coworkers. The grading system is designed to grade the most severe histopathological findings and named according to the worst degree of damage seen at that view. Grade 0 represents normal morphology. Grade 1 consists of just separation in the myelin configuration, grade 2 represents a disruption in myelin configuration, grade 3 consists of a honeycomb view, and grade 4 indicates collapsed myelin-forming ovoids, in addition to all of the pathologies explained in grades 1 through 3.

**Statistical Analysis**
To assess whether the distribution of the continuous (MDA and NO) values was normal among the categorical groups (rat groups), Shapiro-Wilk test was used. One-way ANOVA test was used to control whether there was a statistically significant difference between at least two categorical groups.

For multiple comparisons of the rat groups according to NO and MDA levels, Tukey HSD test was performed. The statistical significance level was considered 0.05 for all types of statistical analysis, and SPSS 11.5.1 for Windows was used as the statistical software.

**RESULTS**
According to the electrophysiological assessment, the CMAP amplitude of group E2 (high-dose MP and then continuous normal-dose MP application/1 month) was found to be normal in range when compared to the control and sham groups (Figure 1).

The most similar EM findings compared to the control group were seen in the normal-dose MP and high-dose MP+VA treatment groups (Figure 2, 3). Intense staining of the normal-dose MP and high-dose MP+VA groups with NGF and VEGF compared to the control and sham groups was also in accordance with the similarity in EM assessment.
The levels of NO and MDA in serum and tissue were found to be statistically low after the high-dose MP, normal-dose MP, high-dose MP+VA, and normal-dose MP+VA treatment modalities (p<0.05) (Figure 4, 5).

In the normal-dose MP and high-dose MP+VA treatment groups, intense staining was found compared to the control and sham groups according to the sciatic nerve immunohistochemical examination (Figure 6, 7).

### DISCUSSION

Many drugs are used in FNP treatment. Steroids are also used in the treatment of traumatic PFNP under the condition that nerve integrity still persists. It is suggested that corticosteroids reduce edema and compression on the nerve by reducing capillary permeability, leading to reduced axonal degeneration, increased axonal regeneration, prevention of lipid peroxidation, and inhibition of fibrosis. Migration and phagocytosis of macrophages after nerve injury are accepted as normal functional progress. In addition to reducing inflammatory reactions, reducing scar tissue is also one of the important aims in nerve healing. To achieve this goal, usage of steroids and nonsteroid anti-inflammatory drugs is beneficial.

Steroids are known to have deleterious effects on wound healing, despite their benefits on nerve tissue. It is thought that the anti-inflammatory effects of VA help to prevent possible adverse effects of corticosteroids. Although the exact mechanism of retinol antagonism against the effect of corticosteroids in the wound healing cascade is unclear, the increase in the percentages of macrophages and monocytes in the inflammatory phase of healing and the counteraction of the effects of corticosteroids on the lysosome are thought to be responsible for the reversal of impaired healing. Retinoids regulate the growth and the differentiation of many cell types, and their actions are mediated through two families of nuclear receptors belonging to the steroid hormone receptor superfamily. These receptors-namely, retinoic acid and retinoid-x receptors-affect the differentiation and proliferation of epithelial tissues by binding and trans-activating the related genes.

The levels of MDA, an important indicator of lipid peroxidation, in serum and tissue were found to be statistically low after high-dose MP, normal-dose MP, high-dose MP+VA, and normal-dose MP+VA treatments. The most noticeable decrease in tissue and serum lev-
levels of MDA in VA groups may indicate the preventive effects of this substance on lipid peroxidation. NO, occurring in oxidative stress conditions, (surgery, trauma, hypoxia, etc.), is a pro-oxidative and antioxidant molecule [7]. NO levels in tissue injury are characterized by an instantaneous increase in the first 5 minutes. It returns to the initial level in 30 minutes and continues to fall in the subsequent days [18]. This information supports the finding that NO blood values of the control group were higher than other groups.

One of the objective assessments of nerve regeneration developing after peripheral nerve injury is the evaluation of the numerical distribution and spatial orientation of myelinated and unmyelinated nerve fibers [19]. According to the EM assessment, the greatest similarity to controls was found in the first month in the high-dose MP+VA and normal-dose MP treatment groups. So it was thought that steroid treatment increased nerve healing and VA used with steroids affected healing positively.

Although the beneficial effect of VA has been recognized in the high-dose steroid administered group by electron microscopy (p: 0.005), VA addition to normal-dose steroid therapy was not so (p: 0.0013). This can be attributed to the competition of steroids and VA through their mechanisms of action.

There are many studies on starting treatment in the early stage and determining the prognosis correctly. The most accurate results are obtained by electrophysiological tests in these studies [20]. EnoG is the most confidential electrophysiological test and is used to choose the treatment in the early stage and commonly determine the prognosis [21].

The decrease in CMAP amplitude shows axonal neuropathy and loss. When the amplitude is normal, the significant decrease in transmission speed (long latency) is in favor of demyelination. When the amplitude of proximal stimulation falls to half of the amplitude of the distal stimulation and dispersion occurs, transmission block and focal demyelination are seen. The occurrence of CMAP becomes late, and distal latency extends in demyelination [22].

According to first-week amplitude values, axonal degeneration could not be evaluated thoroughly. When first-month latency values were investigated, high-dose MP+VA had the lowest demyelination scores and was supported by EM findings. Therefore, VA addition to high-dose steroid treatment may be suggested to have a beneficial effect on myelination.

NGF promotes the vitality of sensitive nerve cells and nerve fiber growth. It was found that Schwann cells secrete NGF after nerve injuries, and this synthesis increases in distal Schwann cells after axotomy [23]. The effectiveness of treatment in the healing of nerves, examined by light microscope, was investigated by means of NGF and VEGF staining intensity immunohistochemically. The intense staining with NGF and VEGF of the normal-dose MP and high-dose MP+VA groups compared to control and sham animals was also in accordance with the similarity in the EM assessments. Only application of VA also resulted in more intense VEGF staining compared to sham and controls. Various studies showed that retinoic acid increases VEGF [24, 25]. If the contributions of the number of mast cells to nerve healing are considered, it is observed that addition of VA to MP increases the number of mast cells, creating a positive effect.

When all findings are evaluated, it was observed that the high-dose MP+VA treatment group was positively noticeable in terms of EM, first-month latency values, and NGF-VEGF. Moreover, the NO and MDA values of this group indicated better healing immunohistochemically.

In conclusion, the electrophysiological and electron microscopy findings revealed that MP administration was effective in a traumatic nerve paralysis model and reduced lipid peroxidation compared to the control group. Steroid treatment after the nerve injury probably decreases inflammation and prevents tissue injury. It was observed that continuous-dose steroid given after high-dose steroid decreases nerve injury. Also, addition of VA to steroid treatment may be suggested to have beneficial effects on the healing process. Additional experimental research and clinical studies are needed in terms of convenience and further support of this particular experiment. In terms of electrophysiology, experienced people are fundamental for the most appropriate and confident measurements of the repeated examinations; the same laboratory conditions and technical equipment are essential.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Mersin University (No: 01. 02. 2010/09)

Conflict of Interest: No conflict of interest was declared by the authors.

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