Current Problems in Surgery 63 (2025) 101706

Contents lists available at ScienceDirect

Current Problems in Surgery

journal homepage: www.elsevier.com/locate/cpsurg

Evaluation of the effects of low-level laser and steroid therapy, separately or in combination for the treatment of facial nerve injury: An experimental study in rats ☆

Zeynep Gümrükçü, PhD, DDS[a,*], Emre Balaban, DDS[a], Tolga Mercantepe, PhD[b], Kerimali Akyildiz, PhD[c], Andaç Doğan, DDS[a], Mert Karabağ, DDS[a], Muhammed Raşid Göksu, DDS[d], Levent Tümkaya, PhD[e], Fatih Mehmet Gökçe, MD[f], Adnan Yilmaz, MD, PhD[g]

Introduction

Facial palsy, complete or partial paralysis of the facial nerve, is a condition that deeply impairs the quality of life.[1] The most common cause of peripheral facial nerve palsy is Bell's palsy followed by Ramsay Hunt syndrome. Also, facial nerve palsy may see as a result of trauma or iatrogenic causes too. Post-traumatic facial nerve palsy is a rare condition occurring in approximately 1.5 % of people who sustain blunt craniofacial injuries. Furthermore, factors such as acoustic neuroma, head and neck cancers, chickenpox, benign tumors are also included in the etiology of facial palsy.[2] Clinically, facial palsy presents with aesthetic problems, asymmetry,

From the [a]Department of Oral and Maxillofacial Surgery, Faculty of Dentistry Recep Tayyip Erdoğan University, Rize, Turkey; [b]Department of Histology Embryology, Faculty of Medicine, Recep Tayyip Erdoğan University, Rize, Turkey; [c]School of Vocational Healh Care Services, Department of Medical Services and Techniques Recep Tayyip Erdoğan University, Rize, Turkey; [d]Private Practice, S Dental Polyclinic Osmaniye, Turkey; [e]Department of Histology Embryology, Faculty of Medicine, Ondokuz Mayıs University, Samsun, Turkey; [f]Department of Physiology, Faculty of Medicine, Recep Tayyip Erdoğan University, Rize, Turkey; and [g]Department of Biochemistry, Faculty of Medicine, Recep Tayyip Erdoğan University, Rize, Turkey

☆ Funding: This research is supported by the Scientific Research Project Fund of Recep Tayyip Erdoğan University, Rize Turkey (Grant number: TSA-2020-1045- Date:16.01.2020).

* Address reprint requests to Zeynep Gümrükçü, PhD, Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Recep Tayyip Erdoğan University, Menderes Street. No:612, Rize, 53020, Turkey.

E-mail address: zeynep_dt@hotmail.com (Z. Gümrükçü).

https://doi.org/10.1016/j.cpsurg.2024.101706

loss of function, changes in salivary flow and dysarthria.[3] Since it restricts the control of facial expressions, it poses a serious problem that directly concerns the social communication of individuals.[1] It often causes psychological trauma because of the facial asymmetry as well as serious loss of function, therefore, its treatment is of great importance.[4]

Peripheral nerve motor fibers are capable of regeneration, and there are many techniques currently available for the treatment of nerve injuries.[5] Treatments for nerve damage include both noninvasive and invasive methods, such as pharmacological approaches, surgery, and alternative treatments. Although the management of facial nerve palsy is still controversial, various surgical repair techniques and the use of adhesive agents have been reported in the literature for acute onset or complete palsy treatment. It has been reported that the success rate in recovery is quite variable from case to case, and the level of recovery can be supported by physiotherapy.[6] Apart from surgical treatments, hyperbaric oxygen (HBO), transcutaneous electrical nerve stimulation (TENS), percutaneous electrical nerve stimulation (PENS), cryotherapy, acupuncture, ozone therapy (OT), systemic steroid use and low-level laser therapy (LLLT) are among the preferred nonsurgical alternative treatment methods.[7] Low-level laser therapy (LLLT), and hyperbaric oxygen have provided remarkable results for partial palsy or delayed onset cases, as mentioned in literature reports.[8-11]

In the literature, corticosteroids such as methylprednisolone and dexamethasone have been applied systemically, locally and topically in the treatment of facial nerve damage.[12] Steroids are the conventional treatment agent for nerve paralysis due to their anti-inflammatory and immunosuppressive effects.[11] The steroid acts by reducing edema and protecting the cell membrane against peroxidation.[13] In traumatic nerve injuries, it is recommended to start the steroid within 24 h and use it at a dose of 1-2 mg/kg for approximately 10 to 14 days in the literature reports.[8,13]

It is known that LLLT application suppresses inflammation, stimulates fibroblasts to remodel scar tissue, increases myofibroblast formation by stimulating mitotic activity, accelerates the proregenerative process at the cellular level by increasing ATP synthesis (adenosine triphosphate), suppresses the inhibition of remyelination after nerve damage and even attenuates Wallerian degeneration.[14] Low-Level Laser Therapy helps the regeneration of traumatic nerves by preventing scar formation in the injured nerve tissue and by contributing to axonal outgrowth/myelination. Besides, LLLT increases the regeneration rate thanks to its effects on reducing edema, mononuclear cell migration, analgesia, and anti-inflammatory properties.[15,16] LLLT is applied with varying wavelengths, light beam energy, energy density and application times for different purposes along the path followed by the affected nerve fiber.[17]

The purpose of this study was to examine the effects LLLT and ST use (alone or in combination) on traumatic facial nerve injury. In the current study, the investigators hypothesize that the use of laser and steroids alone or in combination would provide healing in traumatic facial injuries and different healing results could be seen in different treatments groups. The specific aim of the study was to evaluate the effects of steroid tand laser treatment alone or in combination, in histopatholgical, electrophysiological and biochemical aspects.

Materials and methods

Study design/sample

To address the research purpose, the researchers designed and implemented an experimental animal study on rats. The study population was composed of rats, randomly assigned to 5 groups for evaluation of the effects of LLLT and ST in the treatment on facial nerve injury.

Thirty-five male Wistar rats weighing 200-300 g were housed under a 12-h light/dark cycle in 55%-60 % humidity and 22 $\pm$ 3 C° room temperature and received a standard rodent diet and water ad libitum. All surgical procedures were completed following the National Institutes of Health guidelines and approved by the Republic of Turkey Recep Tayyip Erdogan University Local Ethics Committee for Animal Experiments with decision no 2019/05. This research is supported

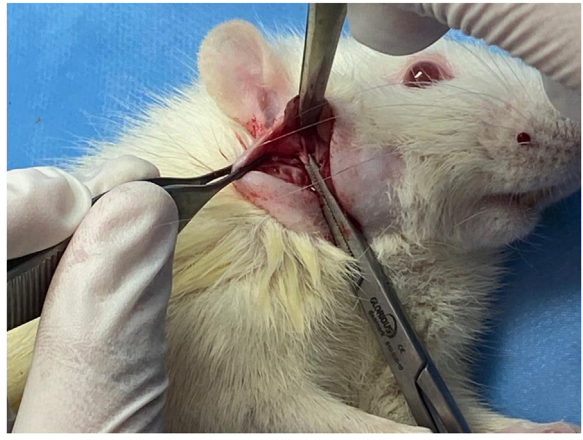

Fig. 1. Exposure and clamping of the external trunk of the facial nerve after the post-auricular incision.

by the Scientific Research Project Fund of Recep Tayyip Erdoğan University, Rize Turkey (Grant number: TSA-2020-1045- Date:16.01.2020).

The animals were randomly divided into 2 control groups; a healthy control group (HCG) and a damaged control group (DCG) and 3 experimental groups; Laser Group (LG), Steroid Group (SG) and Laser+ Steroid Group (LSG) Group before surgery;

1. Healthy Control Group (HCG): 7 rats were included randomly in this group. No damage was done on facial nerve. No surgical/medical approach was applied. EMG measurements were taken over the right facial nerve and recorded digitally.
2. Damaged Control Group (DCG): Facial nerve injury was created on the right facial nerve with the help of a mosquito clamp for 30 s (with 150 N force). No surgical/medical approach was applied. EMG measurements were taken over the right facial nerve after the injury and 4 weeks after from the treatment.
3. Laser Group (LG): Facial nerve injury was created on the right facial nerve. LLLT was applied 3 times a week for 4 weeks postoperatively, at 830 nm with an optical power output of 30 mW, an energy density of 4 J/cm2, an irradiation area of 0.116 cm2 and an exposure time of 16 s per spot. EMG measurements were taken over the right facial nerve after the injury and 4 weeks after from the treatment.
4. Steroid group (SG): Facial nerve injury was created on the right facial nerve. Intraperitoneal methylprednisolone (1mg/kg/day) was administered every day for 1 week postoperatively. EMG measurements were taken over the right facial nerve after the injury and 4 weeks after from the treatment.
5. Laser + Steroid Group (LSG): Facial nerve injury was created on the right facial nerve. Laser and steroid treatment were applied in combination. EMG measurements were taken over the right facial nerve after the injury and 4 weeks after from the treatment.

All rats were anesthetized with intraperitoneal (i.p.) ketamine hydrochloride at a dose of 50 mg/kg (Ketalar®, Eczacıbaşı Parke-Davis, İstanbul, Türkiye) and Xylazine HCl (Alfazyne®, Alfasan International B.V. Woerden, Hollanda) at a dose of 10 mg/kg (i.p.). A right postauricular incision was made under general anesthesia, and an external trunk of the facial nerve was found and dissected. In all experimental groups 30-second compression was applied to the nerve with a mosquito clamp with an average force of 150 N, a technique used for nerve damage, based on previous studies[18,19] (Fig. 1). Afterward, the surgical area was sutured in all experimental groups.

LLLT (Cheese Dental Diode Laser® DEN4A Class IV, Lotus Global Co., Ltd., London, UK) was applied 3 times a week for 4 weeks postoperatively, at 830 nm with an optical power output of

Fig. 2. LLLT application on facial nerve.

30 mW, an energy density of 4 J/cm2, an irradiation area of 0.116 cm2 and an exposure time of 16 s per spot (Fig. 2) Intraperitoneal methylprednisolone (1mg/kg/day) was administered every day for 1 week postoperatively.

At the end of 4 weeks, animals in all groups were sacrificed and samples were taken from the damaged facial nerve trunk and sent for histopathological and biochemical examination. Histopathological, biochemical and electrophysiological analyzes were performed by researchers who were not involved in the study between the surgery-scarification stage. Also the researchers, involved during the histopathological, biochemical and electrophysiological analyzes analysis, were blinded to the surgery-treatment stages.

Study variables

In this study, we believe that the predictor variables were the healthy control group (HCG); without trauma/treatment to the facial nerve, the damaged control group (DCG); with trauma and no treatment, the laser group (LG); with trauma and laser treatment, the steroid group (SG); with trauma and steroid treatment, and the laser-steroid group (LSG) with trauma and laser + steroid treatment together.

Primary outcome variables were histopathological parameters; Axonal Loss, Myelin Degeneration, Wallerian Degeneration, Histopathological Damage Score (HDS). Secondary outcome variables are immunohistochemistry positivity score; Anti-pan-AKT and Anti-Tau positivity grade score, electromyography (EMG) variables; Latency (LP) and amplitude (AMP) value changes to record any subtle weakness in nerve function and biochemical parameters; MDA- GSH level. The facial nerve function is recorded after facial nerve damage and before scarification at 4th week.

Data collection methods

At the end of the 4 weeks of study, tissue samples were taken from the facial nerve trunk with a second surgical procedure, preserved in formalin solution and sent for histopathological

and biochemical examination. Samples taken from the facial nerves of the study subjects were examined histopathologically, immunohistochemichally and biochemically. Electrophysiological (EMG) data were digitally measured and the data was digitally stored.

Histological examination

After the facial nerve tissue samples were fixed with Bouin's fixator, they were blocked by dehydration in alcohol, clearing in xylene, and buried in paraffin. Tissue cut at 3-4 µm thickness from the blocked facial nerve tissue with a microtome (Leica, Rm2525, Germany) were stained with hematoxylin-eosin and Goldner's Masson Trichrome dyes, and their photographs were taken with the help of a camera (Olympus, DP71, Japan) under a light microscope (Olympus, BX51, Japan) with digital attachment.

Immunohistochemical examination

Samples of facial nerve tissue cut with a thickness of 1-3 µm with a microtome (Leica, Rm2525, Germany) were taken on positively charged slides (PatoLab, Turkey) and stained manually with immunohistochemistry Anti-pan-AKT (phospho T308) antibody and Anti-Tau primary antibodies (ab38449, Abcam, England) following the manufacturer's instructions. After the necessary laboratory stages, it was evaluated immunohistologically and histopathologically.

Electrophysiological analysis

PowerLab 26T (ADInstruments Pty Ltd, Australia) device at the Department of Physiology Laboratory of Recep Tayyip Erdoğan University Training and Research Hospital was used to take EMG records. After shaving the right facial area of all animals under the depth of anesthesia, a stimulation electrode was placed just below the external auditory canal corresponding to the facial trunk projection, a recording electrode was placed inside the whisker pad, and a grounding electrode was placed on the auricle in order to obtain EMG recordings after the injury and after the 4 weeks from the treatment (Fig. 3). Stimulation was determined as 15 mA for 0.05 milliseconds at a frequency of 1 Hz. Stimulation. Stimulation was terminated after 15 consecutive responses of sufficient consistency were received for each recording. Amplitude and distal latency values were recorded.

Biochemical analysis

Determination of total glutathione was found by analyzing the amounts of reduced and oxidized glutathione. Reduced glutathione (GSH) was calculated by reading the yellow complex formed due to the reduction of 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) by sulfhydryl groups at 412 nm. Oxidized glutathione (GSSG) was made according to the Beutler method.

MDA, formed due to lipid peroxidation, is determined by the concentration of the pink complex (HPLC-DAD) formed from heating with Thiobarbituric Acid (TBA) solution. After the incubation, 900 µL was taken from the working tubes, and 600 µL of Trichloroacetic Acid (TCA) solution was added and centrifuged at 3000 rpm for 10 min. After centrifugation, 900 µL of supernatant was taken, and 300 µL of TBA solution, prepared fresh daily, was added and incubated in a boiling water bath for 15 min. The resulting-colored solution was applied to the High-Performance Liquid Chromatography (HPLC) column.[12]

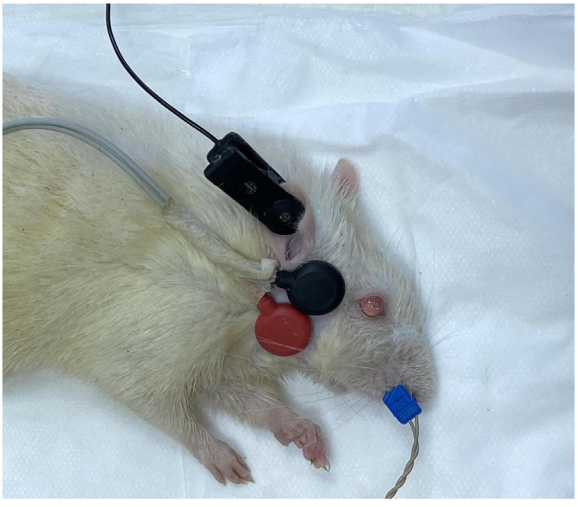

Fig. 3. Placement of stimulation and recording electrodes during EMG measurement.

Data analysis

The data of the groups were analyzed by using the SPSS 18.00 (IBM, NY, United States) statistical program. Parametric data were calculated as arithmetic mean ± standard deviation, and nonparametric data were calculated as median ± standard deviation. The conformity of the data obtained from the analysis to the normal distribution was evaluated with the Shapiro-Wilkinson test. Mann Whitney U test was used to evaluate the mean difference between independent groups and to determine the difference or equality between groups. Intergroup parametric data were analyzed with one-way ANOVA followed by the Tukey test. The results were considered statistically significant if the P was <0.05.

Results

Fig. 4 shows representative transmission electron microscopic section images of facial nerve tissue. Axon damage (asterix) is accompanied by myelin (arrowhead) loss were seen when the sections of the DCG group are examined under a light microscope.

Figs. 5 and 6 shows the representative light microscopic screenshot of $H+E$-stained facial nerve sections and facial nerve sections stained with Masson Trichrome respectively. In the sections of HCG, axons with normal axoplasm and myelin structure were seen in Fig. 5A and B for $H+E$ stained sections, and in Fig. 6A-B for Massom Thrichrome stained sections. When the facial nerve sections of the DCG were examined under a light microscope, Wallerian-type degenerative axons, widespread fibrotic areas and edematous areas were seen, accompanied by losses in the structure of the axoplasma and myelin in Fig. 5C-D for $H+E$ stained sections and Fig. 6C-D for Masson Thrichrome sections. In contrast, in the sections of the LG, we observed that axons and myelin losses and fibrotic areas were decreased in Fig. 5E-F for $H+E$ stained sections, in Fig. 6E-F for Masson Thrichrome stained sections. Similarly, when the sections of the SG group were examined, although a significant decrease was observed in degenerative axons showing Wallerian degeneration, axons with typical structure were observed too in Fig. 5G-H for $H+E$ stained sections. It was observed that fibrotic areas have decreased along with degenerative axons showing Wallerian degeneration in Fig. 6G-H for Masson Thrichrome stained sections. We the sections of LSG group were examined, degenerative axons, which commonly show Wallerian

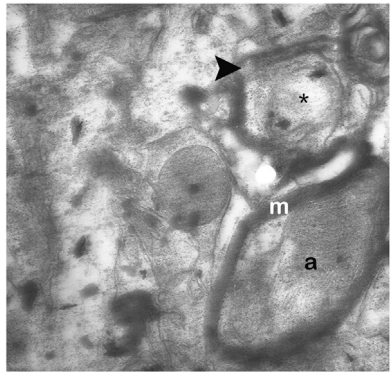

Fig. 4. Representative transmission electron microscopic images (x5000) of sections of facial nerve tissue. Axon (a), Myelin (m). When the sections of the facial nerve injury application group are examined under a light microscope, it is observed that axon damage (asterix) is accompanied by myelin (arrowhead) loss.

Table 1
Histopathologic Damage Score (HDS) (Median- [25 %–75 % Interquartile Range]).

Group	Axonal Loss	Myelin Degeneration	Wallerian Degeneration	HDS
HCG	0 (0-1)	0 (0-0)	0 (0-0)	0 (0-0)
DCG	2 (2-3)*	2 (2-2.5)*	2 (2-2)*	6 (6-7)*
LG	1 (0.5-2)*,‡	1 (0.5-1)§	1 (0-1)§	3 (2-3.5)*,§
SG	1 (1-2)†,§	1 (1-1)‖,¶	1 (0-1)§	3 (2-4)*,¶
LSG	1 (1-1)§	1 (0-1)§	0 (0-1)§	1.5 (0.5-2)§

* $P = 0.001$; vs. healthy Control Group.
† $P = 0.003$; vs. healthy Control Group.
‡ $P = 0.009$; vs. Damaged Control Group.
§ $P = 0.001$; vs. Damaged Control Group.
‖ $P = 0.006$; vs. healthy Control Group.
¶ $P = 0.002$; vs. Damaged Control Group.
Mann Whitney U test with Benferroni Corrections.

degeneration accompanied by axon and myelin losses, decrease and typical axon structures were observed in Fig. 5I-J for $H+E$ stained sections. and nerve fascicles with typical axon and myelin structures were seen. It was observed that degenerative axons, which commonly show Wallerian degeneration accompanied by axon and myelin loss, decreased, and fibrotic areas also decreased Fig. 6I-J for Masson Thrichrome stained sections.

Table 1 shows the difference in axonal loss, myelin degeneration, Wallerian degeneration and HDS score between groups, with the results of Mann Whitney U test with Bonferroni Corrections. There was a statistically significant decrease seen in axonal loss in the LG group ($P = 0.009$), SG group ($P = 0.001$) and LSG group ($P = 0.001$) compared to the DCG group. Statistically significant decrease were seen in myelin degeneration in the LG ($P = 0.001$), SG ($P = 0.002$) and LSG groups ($P = 0.001$) compared to the DCG group. Statistically significant decrease were seen in Wallerian degeneration in the LG ($P = 0.001$), SG ($P = 0.001$) and LSG groups ($P = 0.001$) compared to the DCG group. According to the HDS score a statistically significant decrease was seen in LG ($P = 0.001$), SG ($P = 0.002$) and LSG group ($P = 0.001$), according to DCG Group.

Fig. 7 represents a representative light microscopic screenshot of facial nerve sections incubated with Anti-pan-AKT primary antibody. In Fig. 7A immune-negative nerve fascicles containing normal axons and myelin structures belonging to the HCG group were seen. When the sections of the DCG group were examined Schwann cells (arrow with tail) showing intense Anti-pan-AKT positivity were observed among the degenerative axons (Fig. 7b). In the LG group a decrease in cells showing anti-pan-AKT positivity were seen (Fig. 7C). Similarly, in the SG

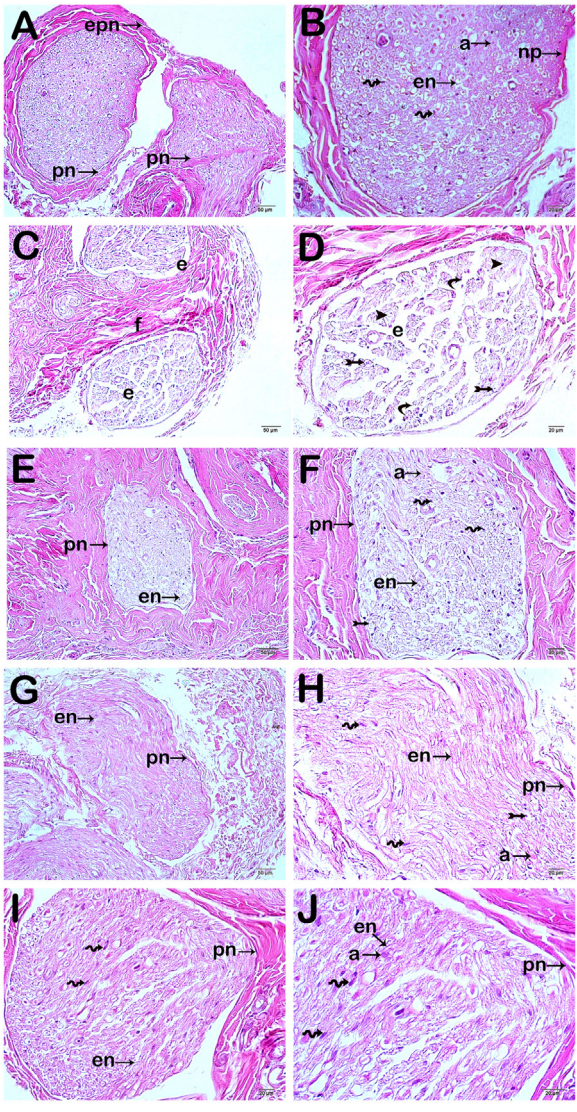

Fig. 5. Representative light microscopic screenshot of $H+E$-stained facial nerve sections. Axon (a). Endoneurium (en), Perineurium (pn), Epineurium (en), Schwann Cell (spiral arrow). Healthy Control Group (A, x10) - (B, x40): Nerve fascicles containing normal axons (a) and myelin structures are observed in the facial nerve. Normally structured Schwann cells (spiral arrow) are observed (HDS 0(0-0)). Damage Control Group (C, x20) - (D, x40): Widespread edematous (e) areas are observed among degenerative axons (spiral) accompanied by myelin sheath losses (arrowhead). In addition, degenerative ascones commonly showing Wallerian degeneration accompanied by axon and myelin losses are observed (HDS 6 [6-7]). Laser Treatment Group (E, x20) - (F, x40): It is observed that myelin and axon losses are reduced (HDS 3 [2-3.5]). Steroid Treatment Group (G, x20) - (H, x40): Although a significant decrease is observed in degenerative axons showing Wallerian degeneration, axons with typical structure are observed (HDS 3 [2-4]). Laser + Steroid Group (I, x20) - (J, x40): Degenerative axons, which commonly show Wallerian degeneration accompanied by axon and myelin losses, decrease, and typical axon structures are observed (HDS 1.5 [0.5-2]).

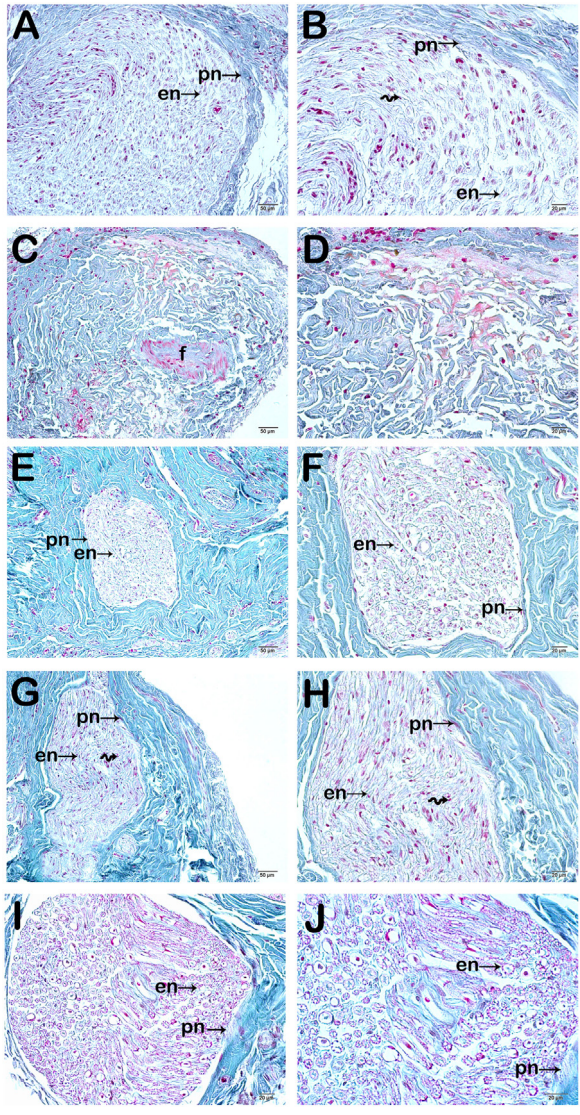

Fig. 6. Representative light microscopic screenshot of facial nerve sections stained with Masson Trichrome. Axon (A). Fascicle (f), Perimysium (p). Schwann Cell (arrow). Healthy Control Group (A, x10) - (B, x40): Nerve fascicles containing normal axons (a) and myelin structures are observed in the facial nerve. Normally structured Schwann cells (spiral arrow) are observed (Fibrosis score 0 [0-0]). Damage Control Group (C, x20) - (D, x40): Widespread fibrotic (f) and edematous (e) areas are observed among degenerative axons (spiral) accompanied by myelin sheath losses (arrowhead). (Fibrosis score 2 [2-3]). Laser Treatment Group (E, x20) - (F, x40): In addition to myelin and axon losses, fibrotic areas are observed to decrease (HHS 1(0.5-1)). Steroid Treatment Group (G, x20) - (H, x40): It is observed that fibrotic areas have decreased along with degenerative ascones showing Wallerian degeneration (Fibrosis score 1 [1-1.5]). Laser + Steroid Group (I, x20) - (J, x40): It is observed that degenerative axons, which commonly show Wallerian degeneration accompanied by axon and myelin losses, have decreased, and fibrotic areas have also decreased (Fibrosis score 0.5 [0-1]).

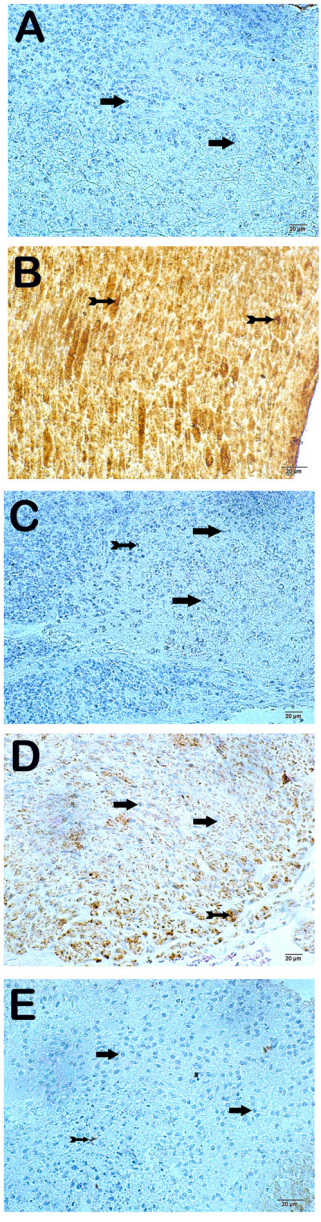

Fig. 7. Representative light microscopic screenshot of facial nerve sections incubated with anti-pan-AKT primary antibody. Axon (A). Fascicle (f), Perimysium (p). Schwann Cell (arrow). Healthy Control Group (A, x40): When facial nerve sections are examined under a light microscope, immune-negative nerve fascicles containing normal axons and myelin structures are observed. (Anti-pan-AKT positivity score 0 [0-0.5]). Damage Control Group (B, x40): Schwann cells (tailed arrow) showing intense Anti-pan-AKT positivity are observed among the degenerative axons. (Anti-pan-AKT positivity score 2 [2-3]). Laser Treatment Group (C, x40): It is observed that the cells showing anti-pan-AKT positivity are reduced (HHS 0.5 [0-1]). Steroid Treatment Group (D, x40): It is observed that there is a decrease in Schwann cells showing Anti-pan-AKT positivity along with degenerative axons showing Wallerian degeneration (Anti-pan-AKT positivity score 1 [0-1]). Laser + Steroid Group (E, x40): Although it is observed that there is a decrease in Schwann cells showing anti-pan-AKT positivity, Schwann cells with typical structure are observed (Anti-pan-AKT positivity score 0.5 [0-1]).

Table 2
Immunohistochemistry Positivity Scores Results (Median-25 %–75 % Interquartile Range).

Group	Anti-pan-AKT positivity grade score	Anti-Tau positivity grade score
HCG	0.00 (0-0)	0.00 (0-0.5)
DCG	2 (2-3)*	2 (2-2)*
LG	0.5 (0-1)†	0 (0-1)†
SG	1 (0-1)†	1 (0-1)†
LSG	0.5 (0-1)†	0 (0-0.5)†

* $P = 0.001$ compared to the healthy Control Group.
† $P = 0.001$ compared to the Damage Control Group.
Mann Whitney U test with Benferroni Corrections.

Table 3
Quantitive Analysis Results (Mean ± Standard Deviation).

Group	Axon Area (AA; μm²)	Myelin Thickness (FA; μm²)
HCG	84.47 ± 7.05	45.43 ± 5.23
DCG	37.062 ± 5.65†	21.10 ± 5.23†
LG	68.21 ± 7.12‡	34.24 ± 5.14‡
SG	64.47 ± 7 5.23‡	35.16 ± 3.20‡
LSG	75.01 ± 8.45‡,§,‖	42.45 ± 0.21‡,§,‖

FA: Fasicle area.
† $P = 0.001$; vs. healthy Control Group.
‡ $P = 0.019$; vs. Damage Control Group.
§ $P = 0.004$; vs. Laser Group.
‖ $P = 0.003$; vs. Stereoid Group.

group, it was seen that Schwann cells and axons showing Anti-pan-AKT positivity were decreased (Fig. 7D). Schwann cells and axons showing Antipan AKT positivity in the LSG group were decreased, commonly immun-negative typical Schwann cells and axon structures were seen (Fig. 7E).

Fig. 8 represents a representative light microscopic screenshot of facial nerve sections incubated with Anti-Tau primary antibody. In Fig. 8A immune-negative normal axons, myelin structures and Schwann cells are observed. When the sections of the DCG group were examined, it was seen that the Schwan cells showing intense Anti-Tau positivity between degenerative axons were increased (Fig. 8B). In the LG group, it was seen that cells showing Anti-Tau positivity were decreased (Fig. 8C). It is observed that there was a decrease in Schwann cells showing Anti-Tau positivity along with degenerative axons showing Wallerian Degeneration (Fig. 8D). Although Schwann cells showing Anti-Tau positivity in the LSG group decreased, typical Schwann cells were seen (Fig. 8E).

Table 2 shows immunohistochemistry positivity scores results with Mann Whitney U test with Bonferroni Correction. According to the Anti-Pan-AKT score LG, SG and LSG groups showed a statistically significant decrease according to DCG group ($P = 0.001$). Similarly, according to the Anti-Tau positivity score LG, SG and LSG groups showed a statistically significant decrease according to DCG group ($P = 0.001$).

Table 3 shows quantitive analysis results in terms of the change of axon area and myelin thickness between groups. It was seen that Axon area showed a statistically significant increase in LG ($P = 0.019$), SG ($P = 0.019$) and LSG group ($P = 0.019$) according to the DCG group. Statistically significant higher values were detected in the LSG group in terms of axon area compared to the LG group ($P = 0.004$), and SG group ($P = 0.003$). In terms of axon area, the statistically highest value among the experimental groups was found in the LSG group $P < 0.05$. Similarly, the Myelin thickness showed a statistically significant increase in LG ($P = 0.019$), SG ($P = 0.019$) and LSG ($P = 0.019$) group according to the DCG group. Statistically significant higher values were de-

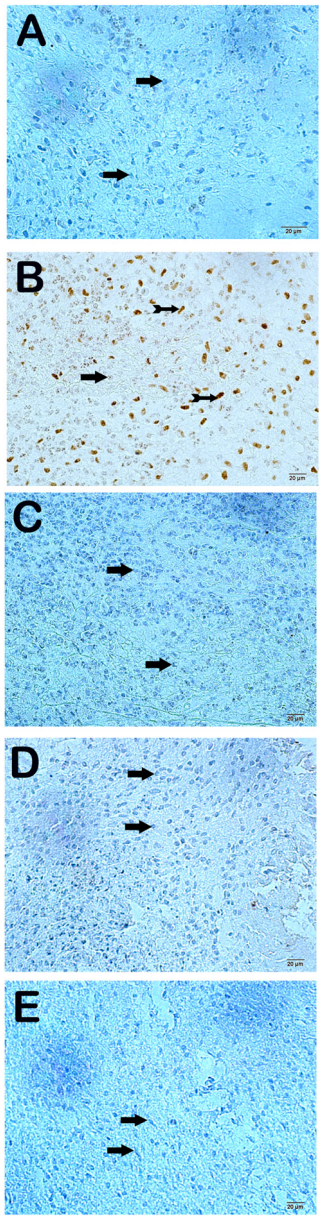

Fig. 8. Representative light microscopic screenshot of facial nerve sections incubated with anti-Tau primary antibody. Axon (A). Fascicle (f), Perimysium (p). Schwann Cell (arrow). Healthy Control Group (A, x40): When facial nerve sections are examined under a light microscope, immune-negative normal axons, myelin structures and Schwann cells are observed. (Anti-Tau positivity score 0 [0-0.5]). Damage Control Group (B, x40): Schwann cells (arrow with tail) showing intense Anti-Tau positivity are observed among the degenerative axons. (Anti-Tau positivity score 2 [2-3]). Laser Treatment Group (C, x40): It is observed that the cells showing anti-Tau positivity are reduced (HHS 0.5 [0-1]). Steroid Treatment Group (D, x40): It is observed that there is a decrease in Schwann cells showing Anti-Tau positivity along with degenerative axons showing Wallerian degeneration (Anti-Tau positivity score 1 [0-1]). Laser+Steroid Group (E, x40): Although it is observed that there is a decrease in Schwann cells showing anti-Tau positivity, Schwann cells with typical structure are observed (Anti-Tau positivity score 0.5 [0-1]).

Table 4
Latency (LP) and Amplitude (AMP) Value Changes (Mean ± Arithmetic Mean).

Group	LP	AMP
DCG Pre-Op.	17.32 ± 6.85	27.24 ± 12.24
DCG Post-Op.	17 ± 6.05	97.54 ± 6.91[*,‡]
LG Pre-Op.	15.34 ± 4.91	33.64 ± 31.16[‡]
LG Post-Op.	16.71 ± 5.88	74.85 ± 36.19[†]
SG Pre-Op.	17.71 ± 5	38.83 ± 22.49[‡]
SG Post-Op.	11.85 ± 2.99	91.52 ± 12.05[*,#]
LSG Pre-Op.	17.64 ± 5.2	46.47 ± 9.9[§,¶,**]
LSG Post-Op.	13.71 ± 6.33	64.43 ± 31.23

[*] $P = 0.001$: vs. DCG Pre-Op.
[†] $P = 0.007$: vs. LG Post-Op.
[‡] $P = 0.001$: vs. DCG Post-Op.
[§] $P = 0.003$: vs. DCG Post-Op.
[¶] $P = 0.03$: vs. LG Pre-Op.
[#] $P = 0.02$: vs. SG Pre-Op.
[**] $P = 0.013$: vs. SG Post-Op.
One-Way ANOVA-Tukey HSD test.

Table 5
Biochemical Analysis Results (Mean ± Standard Deviation).

Group	MDA (nmol/g tissue)	GSH (nmol/g tissue)
HCG	1.30 ± 0.33	0.91 ± 0.019
DCG	1.50 ± 0.26[*]	0.76 ± 0.39[¶]
LG	1.43 ± 0.21[*,†,‡]	0.94 ± 0.049[†]
SG	1.38 ± 0.20[*,†]	0.97 ± 0.057[†]
LSG	1.34 ± 0.01[†,§,‖]	1.01 ± 0.128[†]

[*] $P = 0.001$; vs. HCG.
[†] $P = 0.001$; vs. DCG.
[‡] $P = 0.01$; vs. SG.
[§] $P = 0.027$; vs. SG.
[‖] $P = 0.001$; vs. LG.
[¶] $P = 0.011$; vs. HCG,
One-Way ANOVA-Bonferroni.

tected in the LSG group in terms of myelin thickness compared to the LG group ($P = 0.004$), and SG group ($P = 0.003$). The statistically highest myelin thickness among the experimental groups was found in the LSG group ($P < 0.05$).

Table 4 shows the Latency (LP) and amplitude (AMP) value changes between the groups as a result of One-way Anova Tukey HSD Test. Postoperatively, statistically significantly prolonged amplitude values were detected in all study groups ($P < 0.05$). A decrease was observed in the latency value after treatment in the LG, SG, and SLG groups, but these differences were not found statistically significant ($P > 0.05$).

Table 5 shows the changes in MDA and GSH level between all groups as a result of One-Way Anova test with Bonferroni Corrections. It was seen that the MDA level in SG, LG and LSG groups were significantly decreased compared to the DCG group. ($P = 0.001$) It was observed that the MDA level in the LG group was showed a significant decreased compared to the HCG ($P = 0.001$), compared to DCG ($P = 0.001$) and compared to SG groups $P = 0.001$). It was seen that the MDA level in the SG group was significantly decreased compared to the both HCG and DCG group ($p = 0.001$). The MDA level in the LSG group decreased significantly compared to the DCG Group ($P = 0.001$), compared to SG Group ($P = 0.027$) and compared to LG Group ($P = 0.001$).

In Table 5, GSH level in the DCG group was significantly decreased compared to the HCG Group ($p = 0.011$). GSH level in the LG group showed a statistically significant increased compared to the DCG Group ($p = 0.001$) Similarly, it was observed that the GSH level in the SG group showed a statistically significant increased compared to the DCG Group ($P = 0.001$) and

the GSH level in the LSG Group showed a statistically significant increase compared to the DCG Group (P-Value = 0.001).

Discussion

The purpose of this project was to assess the regenerative effects of single or combined use of LLLT and steroid treatment in traumatic facial nerve injury. The study team hypothesized that different treatment protocols may show different healing results in facial nerve injury cases.

Procedures for the treatment of nerve damage include surgical treatment, pharmacological agents (Steroid hormones Erythropoietin, 4-Aminopyridine, Ozone, Hyperbaric oxygen etc.), use of physical agents (Physical therapy, Electrical stimulation, Low-level laser therapy, Therapeutic ultrasound), cellular therapies and epigenetic applications. Among these methods, steroid use was preferred in our study due to its predictable results and easy applicability, while LLLT was preferred due to its minimally invasive nature and its accepted effectiveness in peripheral nerve damage.[20]

In order to benefit from its anti-inflammatory and antiedema effects, 1 mg/kg/day systemic methylprednisolone was administered to rats every day for 1 week in SG and LSG groups. As a result, a statistically significant increase were detected in axon area and myelin thickness in the SG group compared to the DCG group. A significant decrease in axonal loss, myelin degeneration, and Wallerian degeneration was detected in the LG group compared to the DCG group. Anti Pan-AKT and Anti Tau values were showed a statistically significantly decrease in the SG group compared to the DCG group. A statistically significant increase in GSH level was observed in the SG group compared to DCG group. These findings are evidence of the therapeutic effect of laser and steroid therapy in traumatic facial nerve injury.

A statistically significant increase in axon area and myelin thickness, and a decrease in Myelin and Wallerian degeneration were detected in the LG group compared to the DCG group. A statistically significant decrease was detected in Anti-Pan AKT and Anti-Tau immunohistochemical positivity scores in the LG group compared to the DCG group. When EMG values were examined, a statistically significant decrease in amplitude was detected in the LG group compared to the DCG group. As a result of biochemical analysis, the decrease in MDA levels and increase in GSH levels in the LG group proves the treatment-supportive effects of LLLT on nerve damage.

In the present study, as a result of our histopathological examination, statistically significantly greater axon area and myelin thickness were detected in the LS group than in the DCG. In addition, the numerical values, closest to the healthy control group, regarding axon area and myelin thickness were seen in the LSG group. These data support that combined treatment of steroids and laser, rather than using them separately, has a significant positive contribution to nerve regeneration. When the damage scores were evaluated, compared to the DCG group, it was observed that axonal loss decreased in the S-LS group, and myelin and Wallerian degeneration decreased in the L-S-LS group. According to this finding, it can be concluded that although laser treatment provides benefits in terms of axonal loss, it may not be sufficient alone for treatment, and the use of laser treatment together with steroids will produce better results.

It is known that one of the treatment drugs used in the literature for facial nerve injuries is methylprednisolone. In a previous study, Gao and his colleagues examined the effects of pyrrolidine dithiocarbamate and methylprednisolone in the treatment of nerve injury.[21] As a result of the immunofluorescence examination, they stated that the nerve fibers in the methylprednisolone group were arranged regularly and with a few vacuoles similar to the normal inferior alveolar nerve. They emphasized that steroids significantly improved the regeneration of crushed inferior alveolar nerves by inhibiting Nuclear factor kappa NF-κB activation in adult rats.[21] Feng and colleagues studied damaged sciatic nerves in rats and provided strong evidence that dexamethasone increases sciatic nerve regeneration and function recovery through immunosuppressive and potential neurotrophic effects.[22]

Low-Level Laser Therapy (LLLT) is a treatment method that was accepted many years ago as a treatment method that reduces pain, inflammation, and edema and can help heal wounds and

prevent tissue injury. LLLT is a complementary, safe, noninvasive, easy-to-apply method with very few side effects in the treatment of facial paralysis. It is a special type of laser that affects biological systems by nonthermal means.[23] LLLT contributes to functional recovery thanks to its positive effect on mitochondrial ATP synthesis and photobiostimulant effect.[24] During the regeneration of nerves, Schwann cells transform themselves into a regenerative phenotype and contribute to regeneration by supporting the production of the basal lamina. LLLT contributes to nerve regeneration by increasing Schwann cells, reducing inflammatory cytokines, and contributing to neurofilament repair and axonal remodeling.[25,26]

There are many studies in the literature investigating the effectiveness of low-level laser treatment in facial nerve injuries. Gigo-Benato et al.[6] evaluated the effectiveness of continuous, pulsed, and combined laser use in their animal study. They concluded that laser biostimulation significantly accelerates the healing of the lesion, provides a statistically faster recovery in muscle mass, and statistically significant myelin fiber regeneration in nerve fibers.[6] Mashhoudi Barez and colleagues investigated the effects of low-level laser therapy on nerve regeneration in their study of 12 rats. As a result of their study, they reported a significant acceleration in revascularization and angiogenesis of the injury site, a decrease in bleeding and increased blood supply, a decrease in Wallerian degeneration, and a higher axonal density in the experimental group.[27]

Similarly, in terms of myelin and Wallerian degeneration, while each of the L and S treatments provides statistically significant better results according to the DCG group, it is seen that the best result will be obtained in the LSG group. Regarding immunohistochemical positivity scores, it was determined that using laser and steroids separately or in combination created a significant difference compared to the DCG group. In contrast, no significant difference was observed between L-S and LS groups.

In the literature reports, although electrodiagnostic testing (EDX) is considered the ideal method for determining nerve function, EDX is a diagnostic method that is more directed at the distal segment of nerve fibers. In addition, EDX may not provide accurate results in the early stages of nerve injury. Therefore, EMG was used in our study to determine nerve function in early stage of facial nerve injury.[28] Electromyography (EMG) is a type of measurement of nerve function. EMG is an objective test in which motor unit action potentials occurring with muscle contraction are recorded by electrodes placed inside the muscle. EMG recordings can be taken at rest and during voluntary contraction. It is not useful in the early stages of paralysis. However, detecting motor unit potentials (MUPs) during voluntary muscle movement efforts in early tests shows no full-thickness cut in the nerve.[29] The electrophysiological test that can provide the most useful information after the third week following paralysis is EMG. Fibrillation potentials can be detected in denervated muscle fibers by EMG measurement 2-3 weeks after paralysis. Fibrillation potentials are MUP with low amplitude, an indicator of Wallerian degeneration. Potentials occurring in regeneration are another indicator that determines the prognosis of polyphasic MUP. It indicates that the healing period begins with regeneration.[29,30] Among the parameters used in EMG measurements, the latent period measures myelination, and the amplitude measures the depolarization waves of active muscle fibers. In our study, amplitude and latency parameters were recorded with EMG after facial nerve damage in rats and secondly after 4 weeks of treatment before the scarification. A significantly prolonged amplitude was detected in post-operative in all study groups. Latency is considered an important indicator of myelination, but in our study, any significant increase/decrease in latency values was not detected after treatment in all study groups.[31,32] Some studies have reported that the latency value increases in the early phase after trauma and decreases after treatment. In the current study, while a decrease was observed in the latency value after treatment in the LG, SG, and LSG groups, this difference was not found significant. The peak-to-peak amplitude value is the sum of the depolarization waves of active muscle fibers, so a decrease in amplitude values is expected after injury.[33] In light of this information, the increase in amplitude value after treatment of the damaged nerve may indicate recovery. Considering the post-treatment amplitude values, it was observed that prolonged amplitude values were obtained at the end of treatment in all our groups.

MDA is the end product of the enzymatic or nonenzymatic decomposition of arachidonic acid and larger polyunsaturated fatty acids (PUFA). It is also known that the increase in MDA levels

increases the production of free radicals and reduces antioxidant activity, resulting in the disruption of the balance between antioxidants and free radicals.[34] According to our study results, the MDA value in the L, S, and LS groups showed a statistically significantly decreased according to the DCG group. This data supports that the treatments applied separately in each group in our study have a positive effect on healing nerve damage. Although no statistically significant difference were found in MDA levels between L, S, and LS groups, the fact that the lowest MDA level was seen in the LSG group may suggest that using laser and steroids together supports the therapeutic effect more strongly. GSH is an antioxidant that protects cells from the toxic effects of reactive oxygen species such as free radicals, peroxides, and heavy metals. Our study results show that while GSH is higher in the HCG group, it decreases in the DCG group, and there is a statistically significant increase in the LG, SG, and LSG groups after laser/steroid treatments. Similar to MDA levels, although the LG, SG, and LSG groups do not show a statistically significant difference within themselves, higher GSH levels were seen in the LSG group. These results support the positive effect of laser and steroid use, separately or together, on GSH levels.

Although there are many studies in the literature evaluating the effect of diode laser and steroid on traumatic nerve injuries, the current study is a valuable study in which the separately and combined use of diode laser and steroid is evaluated in a multidisciplinary manner in terms of histopathological, electrophysiological and biochemical aspects. In light of the results of this study, it can be concluded that the effects of laser and steroids on the tissue-damaged facial nerve are more evident when applied together. These data may contribute to the literature by shedding light on the clinical treatment protocol of patients with traumatic facial nerve injuries.

The main limitation of this study is that no comparison was made regarding different growth factors that would contribute to traumatic facial nerve healing. In this study, the biochemical methods used in the study are routinely used in our laboratory. In GSH and MDA methods, control solutions were prepared from pure GSH and MDA. Tissue sample concentrations were determined by spectroscopic measurement using control solutions. These methods have been used in many studies we have conducted and reliable results have been obtained. These methods were used instead of immunoblot methods due to project planning, laboratory facilities and budget. The fact that the immunoblot method was not used in the current study can be considered a limitation, but there are reports in the literature that obtained reliable results with the current method used.[35] It would be beneficial to conduct more comprehensive animal studies in the future where these limitations are eliminated.

Conclusions

When the study results are evaluated, the following key findings can be reported;

1. Regarding axon area and myelin thickness, using steroids and laser together instead of separately supports nerve regeneration more evidently.
2. In terms of damage scores, although laser treatment provides a positive effect in terms of axonal loss, it may not be sufficient alone in the treatment; application together with steroids and laser provides better results in terms of axonal loss. While each of the L and S treatments provides a positive effect regarding Myelin and Wallerian degeneration, the most effective treatment will be obtained when laser and steroid are used together.
3. The use of laser and steroids separately or together provides a significant difference compared to the DCG group in terms of immunohistochemical positivity scores, However, no difference was detected between the treatment groups.
4. Both laser and steroid treatment groups had a significant difference compared to the control group In terms of MDA and GSH levels. No significant differences were seen within treatment groups.

According to our research results in traumatic facial injuries, we see that the combined use of laser and steroid in the postoperative period produces more effective results in healing. Although

our current results provide very beneficial results for the treatment of facial nerve injury, they should be supported by more comprehensive animal studies and clinical studies.

Declaration of competing interest

None.

Author contributions

Conceptualization, Zeynep Gümrükçü-Emre Balaban.
Methodology, Emre Balaban, Andaç Doğan, Mert Karabağ, Muhammed Raşid Göksu.
Software, Mert Karabağ-Fatih Mehmet Gökçe.
Validation, Zeynep Gümrükçü.
Formal analysis, Tolga Mercantepe, Kerimali Akyıldız, Adnan Yılmaz, Fatih Mehmet Gökçe.
Investigation, Zeynep Gümrükçü.
Resources, Tolga Mercantepe, Kerimali Akyıldız, Adnan Yılmaz, Fatih Mehmet Gökçe.
Data Curation, Tolga Mercantepe.
Writing—Original Draft, Zeynep Gümrükçü.
Writing—Review and Editing, Zeynep Gümrükçü.
Visualization, Zeynep Gümrükçü.
Supervision, Zeynep Gümrükçü.
Project administration, Zeynep Gümrükçü.
Funding acquisition, Zeynep Gümrükçü- Scientific Research Project Fund of Recep Tayyip Erdoğan University, Rize Turkey (Grant number: TSA-2020-1045- Date:16.01.2020).

Acknowledgments

This research is supported by the Scientific Research Project Fund of Recep Tayyip Erdoğan University, Rize Turkey (Grant number: TSA-2020-1045- Date:16.01.2020).

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