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Original article

Histopathological, immunohistochemical and biochemical evaluation of electroacupuncture treatment of nervus radialis and nervus ulnaris injuries in rabbits

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Abstract

The aim of this study was to evaluate the efficacy of electroacupuncture in acute and chronic phases of radial and ulnar nerve injuries in histopathological, immunohistochemical and biochemical aspects. In the study, the rabbits were divided into four groups namely acute nerve injury (ANI) group, chronic nerve injury (CNI) group, positive control (PC) group and negative control (NC) group. In the ANI, CNI and PC groups, damage was created on the nervus radialis and nervus ulnaris by applying pressure for 60 seconds using a hemostatic forceps under anesthesia. No damage was created in the NC group. Fifteen sessions of electroacupuncture were applied to the rabbits in the ANI, CNI, and NC groups every other day using LI-4 (Large Intestine Meridian-4, He Gu), LI-10 (Large Intestine Meridian-10, Shou San Li), LR-3 (Liver Meridian-3, Tai Chong), and ST-36 (Stomach Meridian-36, Zusanli) electroacupuncture points. Electroacupuncture was not applied to the rabbits in the PC group. Decapitation was performed under general anesthesia at the end of electroacupuncture applications. After the euthanasia procedure, the samples obtained were evaluated for histopathological, immunohistochemical and biochemical parameters. In conclusion, degenerative foci in the treatment groups were found to be fewer than in the PC group whereas NGF and S-100 immunoreactivity were higher in the treatment groups than in the PC group. Whereas no statistically significant difference was observed between the treatment groups and the NC group in terms of oxidative stress factors, there was a statistically significant difference between the treatment groups and the PC group. In light of all these data, we have concluded that electroacupuncture is an effective treatment method for peripheral nerve injuries.

Key words: electroacupuncture, histopathological, immunohistochemical, nerve injury, oxidative stress

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Introduction

With a history of 4700 years, acupuncture is known as one of the oldest treatment modalities in medical history. The name originates from Latin and is terminologically defined as the treatment of pain and dysfunctions caused by diseases via inserting needles at specific points on the body (Nur 1995, Günday 1997, Loken 2001, Cabıoglu and Ergene 2003, Kalyon 2007, Horasanlı et al. 2008, Zhao 2008, Horasanlı et al. 2009, Cheng 2011, Atalar et al. 2013).

Although acupuncture treatment is mainly based on inserting needles to the special points on the body, acupuncture points have been started to be stimulated by using laser beams, electric current, heat, and sterile fluids apart from needles in the course of time, leading to the emergence of several acupuncture techniques such as laser acupuncture, electroacupuncture, moxibustion and aqua-acupuncture. Acupuncture treatment has a wide area of use in medicine including respiratory diseases, gastrointestinal diseases, behavioral disorders, neurological diseases, and musculoskeletal diseases (Captug and Bilgili 2005, Gulanber 2008, Chang 2013).

Peripheral nerve injury is one of the most common neurological disorders in veterinary medicine as in human medicine. Peripheral nerve damages or injuries can be classified into two ways. In the Seddon method, classification is made according to the severity of injury in the damaged nerve whereas classification is made according to structural, histological and functional losses in the damaged nerve in the Sunderland method (Kavlak 2012, Tanyeri 2013).

According to Seddon method, nerve injuries are divided into three classes: neuropraxia, axonotmesis, and neurotmesis. Neurotmesis is damage in which all structures of the neurons, including the axon and endoneurium, are damaged and which do not have a good prognosis. Such nerve injuries often result in neuromas. Axonotmezis is the disruption of the anatomical structure of the damaged nerve by undergoing Wallerian degeneration in the distal part of injury. The recovery of this type of nerve injury, in which the structures of the epineurium, perineurium, and Schwann cells are preserved, varies according to the age and condition of the patient and localization of the lesion and positive results are achieved in most of the cases. Neuropraxia is mostly due to compression and neither axonal, i.e. nerve integrity, nor Wallerian degeneration is observed. There might be motor dysfunction, paresthesias, decreases in the sense of deep pain and loss of strength. Complete or partial recovery can be seen in neuropraxia- and axonotmesis-type nerve injuries without requiring surgery whereas there is a need for surgical intervention in neurotmesis-type nerve injuries (Ademoglu et al. 2003, Kahn and Line 2007, Kavlak 2012, Tanyeri 2013).

According to the Sunderland method, nerve injuries are divided into six classes. In first-degree nerve injuries, demyelination occurs while axon and other structures maintain their integrity. There is a block of nerve conduction in the area of damage. In second-degree nerve injuries, degeneration is observed in the axonal structure of the nerve and Wallerian degeneration is seen in the distal part of the damage. The overall axonal continuity is preserved. In the third-degree nerve injuries, there is an axonal and Wallerian degeneration in the damaged area of nerve and the integrity of the endoneurial tubes is impaired. In fourth-degree nerve injuries, the internal structure is disrupted and fibrous reproduction occurs in the damaged area although the nerve body maintains its integrity. In the fifth-degree nerve injuries, the integrity of the nerve tissue is impaired and the nerve is separated from the damaged area. Sixth-degree nerve injury is the combination of all these injuries (Kavlak 2012, Tanyeri 2013).

Regeneration is defined as the restoration of degenerating nerves and regaining their normal functions. Nerve regeneration occurs in two stages. Firstly, the nerve fibers move from the part that remains connected to the nerve cell by acting as a pseudopod. Secondly, the nerve fibers formed are wrapped by a myelin sheath and gain the ability to be stimulated (Yaman 2009, Reece 2012).

Endorphin and dynorphin, which are among the endogenous opioids released in the body during acupuncture applications, play an important role in pain management in spinal cord injuries. Furthermore, the release of neurotrophic factors that initiate the regeneration process of axons of damaged nerves is increased with electroacupuncture and needle acupuncture applications. Neurotrophic factors are sometimes released by the damaged tissue and guide the neurons to find new axons correctly (Bragin et al. 1989, Al Majed et al. 2000, Inoue et al. 2003, Alrashdan 2010, Kenney 2010, Manni et al. 2011, Tangjitjaroen 2011, Zhang et al. 2013).

In the treatment of peripheral nerve damage, B1 and B6 vitamin complexes (to compensate for the loss of myelin), local strychnine applications (to stimulate nerve cells) and massage applications with alcoholic solutions are performed. Again, nonsteroidal anti-inflammatory drugs and glucocorticoids are used for symptomatic treatment (Kahn and Line 2007). However, safety problems and drug resistance caused by long-term drug use are among the most important problems. Electroacupuncture is widely used in peripheral nerve injuries because it increases cellular proliferation by stimulating nerve cells, acti-

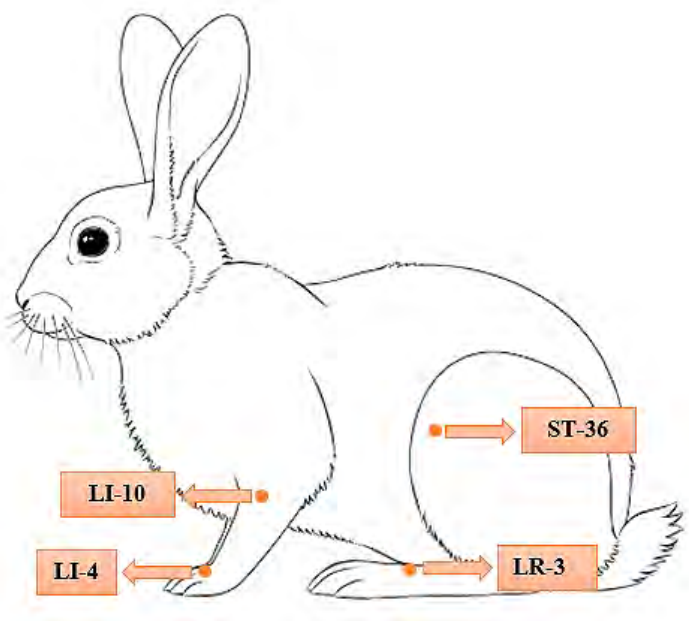

Fig.1. Locations of LI-4, LI-10, LR-3 and ST-36 acupuncture points.

vates atrophied muscles, is easy to apply and has few side effects.

In this study, it was decided to use LI-4 and LI-10 acupuncture points because of their effect on paralysis, pain and atrophy conditions in the forelegs. Since ST-36 and LR-3 acupuncture points are the main influence points, it was decided to use them (Fig. 1). In this study, electroacupuncture technique was used in order to have the same intensities, frequencies and amplitudes of stimulation applied to acupuncture points. In this study, experimental nerve injury created on the nervus radialis and nervus ulnaris by applying compression was treated with electroacupuncture and the results were evaluated in the light of histopathological, immunohistochemical and biochemical findings obtained as a result of the therapy.

Materials and Methods

Experimental groups and creating experimental nerve injury

A total of 28 adult New Zealand rabbits (14 female and 14 male), aged 8 to 12 months, which were obtained from the Firat University Experimental Research Center, were used in the study. Official approval of the Firat University Animal Experiments and Ethics Committee (2016/43-Decision no: 65) was obtained prior to the study. The rabbits were divided into four equal groups namely acute nerve injury (ANI) group, chronic nerve injury (CNI) group, positive control (PC) group and negative control (NC) group, each containing 7 animals. Experimental damage was created on the radial and ulnar nerves of rabbits in the ANI and CNI groups, and then electroacupuncture therapy was applied. The treatment was started in the rabbits in the ANI group immediately after the injury, while the treatment was started on the thirtieth day after the injury in the rabbits in the CNI group. The radial and ulnar nerves of rabbits in the PC group were damaged, but electroacupuncture was not performed. No radial and ulnar nerve damages were created in rabbits in the NC group, but electroacupuncture was performed.

While creating experimental nerve injury, the rabbits in ANI, CNI, and PC groups were given 5 mg/kg xylazine hydrochloride (Rompun® 23.32 mg/mL, Bayer, Istanbul, Turkey) and 35 mg/kg ketamine hydrochloride (Ketalar® 50 mg/mL, Eczacıbaşı, Istanbul, Turkey) intramuscularly for anesthesia. Necessary asepsis procedures were applied to the right anterior legs of the rabbits in the groups where experimental damage would be created and they were, then, placed on the operation table in the lateral position. A skin incision was made from the distal part of the articulatio humeri to the distal part of the articulatio cubiti by approaching from the medial part of the leg. The fascia was excised and anterior and medius muscles of the musculus scalenus were excluded to reveal the root of the plexus brachialis. The nerves were exposed to pressure for 60 seconds using a hemostatic forceps 1 cm below the area where the plexus brachialis was branching as the nervus radialis and nervus ulnaris (Al-Majed et al. 2000, Inoue et al. 2003, Sencar 2007, Alrashdan et al. 2010, Manni et al. 2011, Zhang et al. 2013, Li et al. 2015). Then, hemostatic forceps were removed and the area was closed via an appropriate surgical method in accordance with the asepsis and antisepsis rules. Parenteral cephalexin (Bavet Cepha-

lexin; 150 mg/mL; Bavet, Istanbul, Turkey) therapy (15 mg/kg) was applied for five days and dressing was performed for the surgical wound for a week to prevent the risk of postoperative infection.

Electroacupuncture application

Following the experimental damages, electroacupuncture applications were started on the same day for rabbits in the ANI group and after 30 days for rabbits in the CNI group. For this purpose, ITO-ES 160 electroacupuncture device *(ITO Physiotherapy & Rehabilitation, Japan, Human Device)* was used. The animals in the ANI, CNI, and NC groups received electroacupuncture therapy for 15 sessions (30 days) on alternate days. For the application, LI-4, LI-10, LR-3, and ST-36 acupuncture points were used. Electroacupuncture was performed at 50 Hz and 400 µs constant current for 30 minutes. Rabbits were given 5 mg/kg xylazine hydrochloride intramuscularly for each session (Unsaldi 2011). Following the electroacupuncture applications, rabbits were decapitated under general anesthesia and tissue samples required for laboratory tests were taken.

Histopathological examination

After the decapitation process, the samples taken from the radial and ulnar nerves were placed in separate tissue monitoring cassettes (Isolab GmbH, Wertheim, Germany) (56 samples from 28 animals) and fixed in a 10% neutral formalin solution for 48 hours. Tissues samples fixed in formaldehyde were washed with running tap water for about two hours and were, then, passed through a series of alcohol, xylol, and paraffin in an automated tissue processor (Leica TP 1020, Wetzlar, Germany) and blocked with paraffin in a tissue embedder (Leica EG 1150 H, Wetzlar, Germany). Numerous sections of 3-5 micron thickness were taken from paraffin blocks into positively charged slides (Thermo Fisher Scientific, Superfrost, Massachusetts, USA) using the rotary microtome (Leica RM2125, Wetzlar, Germany). The sections were subjected to hematoxylin-eosin staining in an automated tissue dyeing machine (Leica Autostainer XL, Wetzlar, Germany). Nerves were examined semiquantitatively for necrosis, inflammation, vascularization, congestion and fibrosis (0: None, 1: Mild, 2: Moderate, 3: Severe). The examination was performed under a trinocular light microscope (Olympus DP72, Tokyo, Japan) with a camera imaging analysis system (CellSens Standard) and fluorescence attachment (Olympus BX43, Tokyo, Japan).

Immunohistochemical examination

Immunohistochemical analyses were performed by immunoperoxidase (IP) and indirect immunofluorescence (IF) techniques. Immunohistochemical evaluation was performed semiquantitatively considering the number of cells, cytoplasm or nucleus of which was stained red (IP) with chromogen or bright green with fluorescent (IF), and the staining intensity (0: None, 1: Mild, 2: Moderate, 3: Severe).

Immunoperoxidase technique

Nerve growth factor (NGF) and S-100 immunoreactivity were analyzed in the previously prepared preparations by streptavidin-biotin peroxidase complex (ABC) technique. The chemicals available in the ready-to-use immunohistochemistry kit (Ultra Vision Detection System, Anti-Polyvalent, HRP, Thermo Fischer Scientific, Massachusetts, USA) were used and the standard procedure recommended by the manufacturer was followed. Tissues in positively charged slides were boiled in a 600-watt microwave oven in a citrated buffer solution for 20 minutes to eliminate the masking effect of the fixing solution on antigenic elements. Sections were waited in hydrogen peroxide block solution for 10 minutes to inhibit the endogenous peroxidase activity. The excess liquid was removed from the sections without washing after they were waited in the protein block solution for five minutes to prevent nonspecific binding and then, they were left for incubation with primary antibodies (S100 and NGF) for an hour at room temperature in a humid medium. Then, they were incubated with a secondary antibody for 30 minutes. The sections were, then, incubated with streptavidin peroxidase for 30 minutes and the labeling of the desired parameter was completed. Sections were incubated in a controlled manner with 3-amino-9-ethylcarbazole (AEC) (Ultra Vision AEC Substrate System, Thermo Fischer Scientific, Massachusetts, USA). After incubating the sections for one to five minutes depending on the parameter to be analyzed, incubation was ended by washing the sections with distilled water. In contrast staining, the sections were incubated in Gill's hematoxylin solution for about five seconds. Sections covered with water-based adhesive (Lerner Laboratories, Chicago, USA) were examined under light microscopy (Hsu et al. 1981a,b).

Indirect immunofluorescence technique

Tissues in positively charged slides were boiled in a 600-watt microwave oven in a citrated buffer solution for 20 minutes to eliminate the masking effect of the fixing solution on antigenic elements. The excess

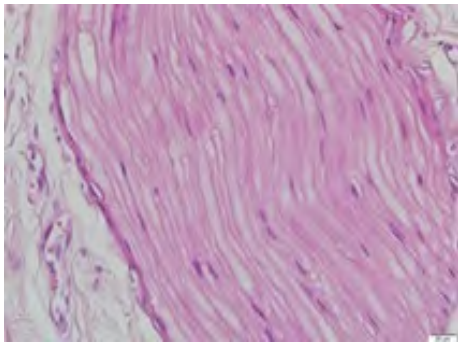

Fig. 2. Normal appearance of the nervus ulnaris in rabbits of the NC group. Hematoxylin-eosin staining (HE); ×40

liquid was removed from the sections without washing after they were incubated in the protein block (Ultra V Block, Thermo Fischer Scientific, Massachusetts, USA) for five minutes and then, they were left for incubation with primary antibodies (S100 and NGF) for an hour at room temperature in a humid medium. The sections were then incubated with the secondary antibody (Goat Anti-Mouse IgG Seconder Antibody, Alexa Fluor 488 Plus, Thermo Fischer Scientific, Massachusetts, USA) labeled with fluorescein isothiocyanate (FITC) for an hour at room temperature in a humid medium. At the end of the incubation, the sections were covered with a water-based adhesive and examined with a FITC filter under a fluorescent microscope (Lerner Laboratories, Chicago, USA).

Biochemical analysis

At the end of the applications, the animals were decapitated under anesthesia and nerve tissues were removed. Prior to the analyses, the collected nerve tissues were washed with physiological saline solution, diluted at a ratio of 1:10 with distilled water, and homogenised in a Potter-Elvehjem homogeniser (CAT R50D, Germany). The homogenate was centrifuged at +4°C at 3.000 g for 15 minute with cooled centrifuge (NUVE NF 800R). Malondialdehyde (MDA), reduced glutathione (GSH) levels, and catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) activities, and protein levels were determined by spectrophotometric methods in nerve tissue samples.

The MDA level was tested according to the method described by Placer et al. (1966). This method was based on the reaction of thiobarbituric acid (TBA) with MDA, one of the aldehyde products of lipid peroxidation. The GSH level was determined by the method of Ellman et al. (1961). This method was a spectrophotometric method based on the formation of highly stable yellow colour of sulphhydryl groups when 5,5'dithiobis-2-nitrobenzoic acid (DTNB) was added. The CAT activity was carried out by using Aebi's method (1981). It was determined by measuring the resolution of hydrogen peroxide (H_2O_2) at 240 nm. The GSH-Px activity was measured by the Beutler method (1984). GSH-Px catalyses the oxidation of GSH to oxide glutathione (GSSG) using H_2O_2. The rate of formation of GSSG was measured by the glutathione reductase reaction. The SOD activity was tested by quantifying superoxide anion (O_2.-) generated by xanthine and xanthine oxidases reacting with nitroblue tetrazolium. The determination of protein concentration was performed using the method described by Lowry et al. (1951).

Statistical analysis

All analyzes were made in SPSS®22 package program. Histopathological and immunohistochemistry scores were subjected to chi-square independence analysis. A pairwise comparison was made for determining the differences within the groups. Statistical significance was accepted when $p<0.05$. The results of the immunohistochemistry analysis were applied Bonferroni correction due to eliminating Type-II error and for these variables, significance was accepted when $p<0.0125$ (McHugh 2013, Armstrong 2014). In the evaluation of biochemical findings, differences between the groups were examined by one-way analysis of variance (ANOVA) and Tukey post hoc test was used for paired comparisons between groups. The data were expressed as mean ± standard error ($\bar{x} \pm SE$).

Results

Histopathological results

The structure of the radial and ulnar nerves (Fig. 2) of the rabbits in the NC group were completely normal and showed peripheral nerve histology (Schwann cells, endoneurium, perineurium, and epineurium). The remarkable microscopic lesions in the ANI and CNI groups were as follows: degeneration (Waller degeneration), capillary proliferation (vascularization)/ congestion (Fig. 3), necrosis (Fig. 4A), inflammatory

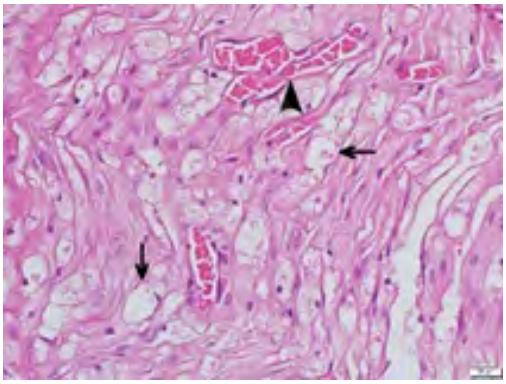

Fig. 3. Waller degeneration (arrows) and vascular proliferation (arrowhead) in the radial nerve of rabbits in the ANI group. HE; ×40

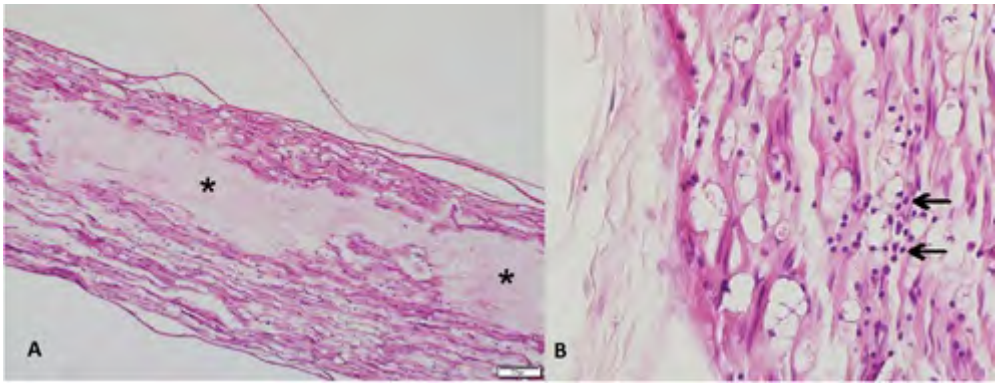

Fig. 4. (A) Diffuse necrosis of the ulnar nerve of rabbits in the PC group (stars). HE; ×20; (B) Focal mononuclear cell infiltration in the radial nerves of rabbits in the PC group (arrows). HE; ×40

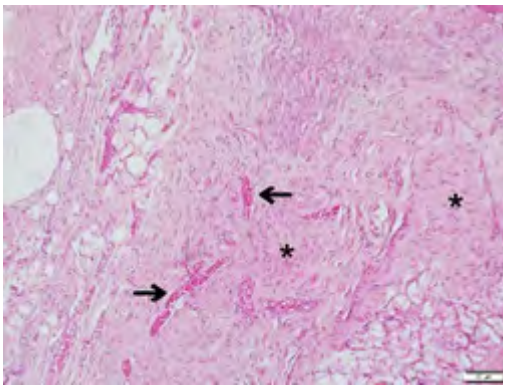

Fig. 5. Diffuse fibrosis (stars) and vascularization (arrows) in the radial nerves of rabbits in the CNI group. HE; ×20

mononuclear cell infiltration (Fig. 4B) and fibrosis (Fig. 5). The frequencies and statistical significance of the histopathological variables are presented in Table 1.

Immunohistochemical results

In the nerve tissues of the rabbits in the ANI, CNI, PC and NC groups, S-100 immunoreactivity showed strong cytoplasmic positivity in Schwan cells by both IP and IF techniques (Fig. 6A, 6B, 7A, 7B). Whereas NGF immunoreactivity showed moderate cytoplasmic positivity in ANI, CNI and NC groups in the IP technique, it was found to have mild cytoplasmic positivity in the IF technique. Similarly, NGF immunoreactivity had a mild cytoplasmic positivity in the PC group in the IP technique whereas no cytoplasmic positivity was observed in the IF technique (Fig. 8A, 8B, 9A, 9B). Frequencies and statistical significance of immunohistochemical variables are presented in Table 2.

Table 1. Number of rabbits (n), degrees of freedom (df), chi-square distribution (X2), significance status and total scoring (Σ) distribution according to severity of degeneration/necrosis, inflammation, vascularization, congestion and fibrosis.

	Degeneration- Necrosis								
	Histopathological score (frequency)						df	X^2	P value
None	Mild	Moderate	Severe	n	Σ				
PC[a]	-	-	2	5	7	3			
NC[bd]	7	-	-	-	7	19	20.33	<0.001	
ANI[ab]	-	4	3	-	7	0			
CNI[cd]	-	1	4	2	7	10			
	Inflammation					15			
	None	Mild	Moderate	Severe	n	Σ			
PC[a]	-	2	4	1	7	13			
NC[b]	7	-	-	-	7	0	3	43.44	<0.001
ANI[bc]	-	1	5	1	7	14			
CNI[d]	-	1	5	1	7	14			
	Vascularization								
	None	Mild	Moderate	Severe	n	Σ			
PC	1	5	1	-	7	7			
NC	7	-	-	-	7	0	3	5.44	0.142
ANI	-	-	3	4	7	18			
CNI	-	2	3	2	7	14			
	Congestion								
	None	Mild	Moderate	Severe	n	Σ			
PC[a]	-	3	4	-	7	11			
NC[ac]	7	-	-	-	7	0	3	35.78	<0.001
ANI[bc]	-	2	3	2	7	14			
CNI[d]	-	1	5	1	7	14			
	Fibrosis								
	None	Mild	Moderate	Severe	n	Σ			
PC[a]	-	1	5	1	7	14			
NC[b]	7	-	-	-	7	0	3	17.00	0.001
ANI[bc]	7	-	-	-	7	0			
CNI[d]	-	-	1	6	7	19			

Different superscripts in the same column indicate the differences between groups. n=number of subjects in a group.
(0: None, 1: Mild, 2: Moderate, 3: Severe)

Biochemical results

The effect of electroacupuncture application on the MDA and GSH concentrations and SOD, GSH-Px and CAT activities in nervus radialis tissue

The MDA levels, CAT and GSH-Px activities in the radial nerve tissue were found to increase ($p<0.001$, $p<0.05$, and $p<0.001$, respectively) whereas GSH level and SOD activities decreased ($p<0.05$ and $p<0.05$, respectively) in the PC group compared to the NC group. There was no statistically significant difference between the ANI group and NC group in terms of all parameters. No statistically significant difference was observed between CNI and NC groups in terms of all parameters other than GSH-Px activity and the decrease in GSH-Px activity was not statistically significant. When ANI and CNI groups compared with the PC group, MDA levels, and CAT and GSH-Px activities were found to significantly decrease ($p<0.001$, $p<0.05$, and $p<0.001$, respectively) whereas an increase was observed in the GSH levels. There was a statistically significant increase in SOD activity only in the ANI group ($p<0.05$) and the increase in the CNI group was not statistically significant (Table 3, Fig. 10).

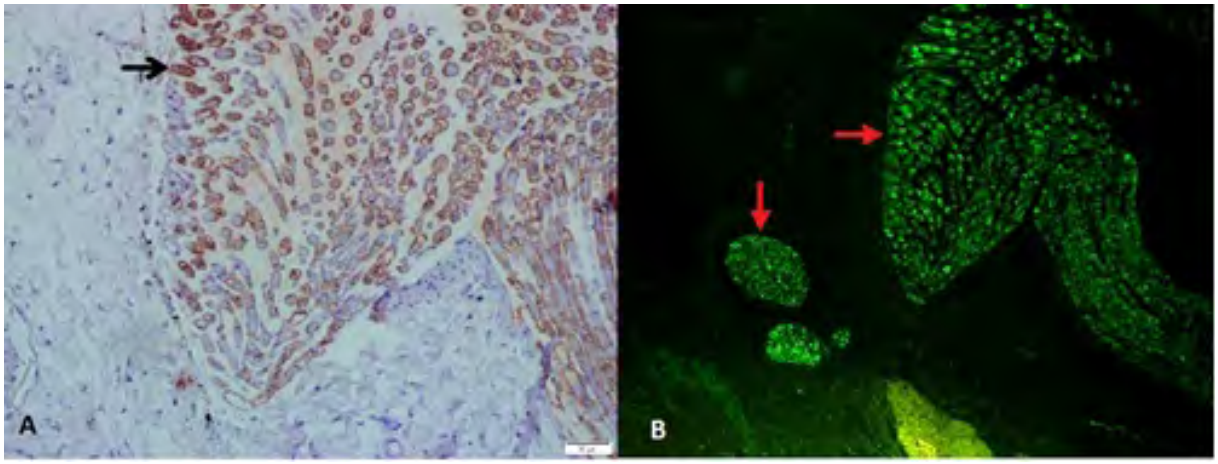

Fig. 6. (A) Cytoplasmic S100 immunoreactivity in rabbits of the CNI group (arrow). Immunoperoxidase staining (IP); ×20; (B) S-100 fluorescence reactivity (arrows) in the radial nerves of rabbits in the CNI group. Immunofluorescence staining (IF); ×20

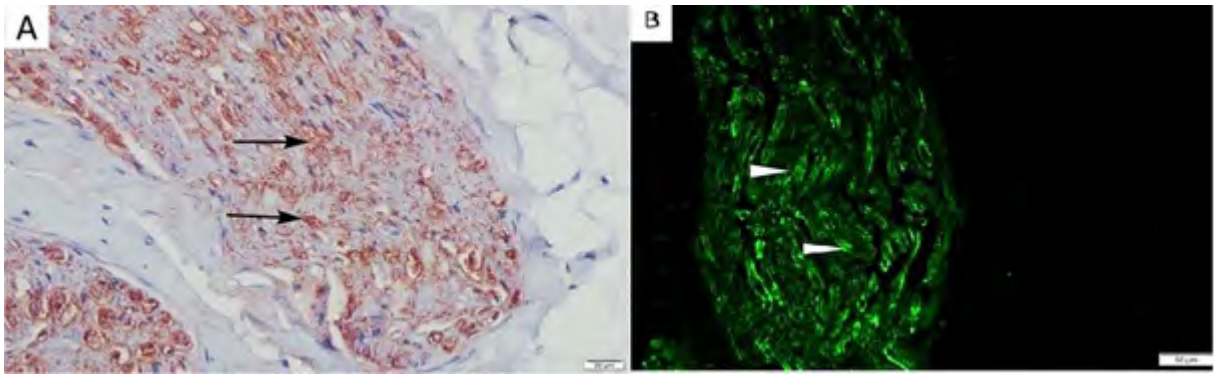

Fig. 7. (A) Cytoplasmic S100 immunoreactivity in rabbits in the ANI group (arrow). IP; ×20; (B) S-100 fluorescence reactivity (arrows) in the radial nerves of rabbits in the ANI group. IF; ×20

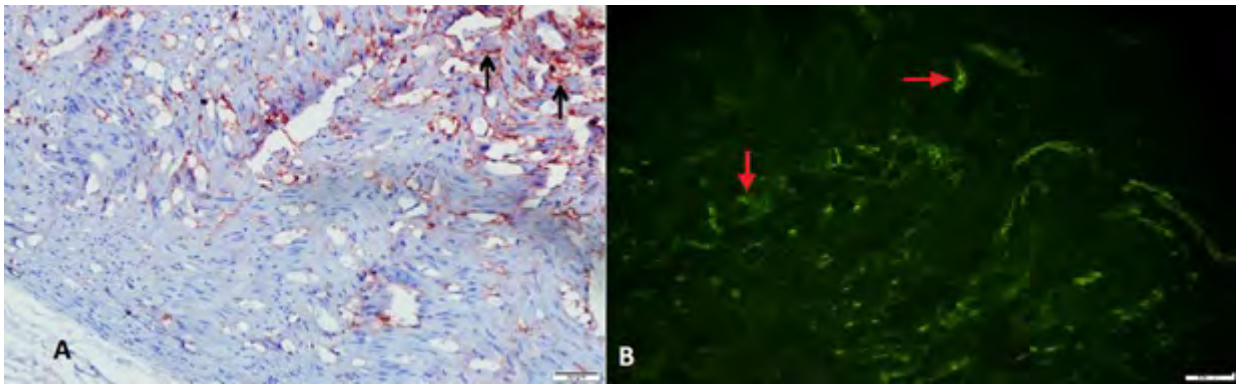

Fig. 8. (A) Nerve Growth Factor (NGF) immunoreactivity (arrows) in ulnar nerves of rabbits in the CNI group. IP; ×20; (B) NGF fluorescence positivity in the ulnar nerves of rabbits in the CNI group (arrows). IF; ×10

The effect of electroacupuncture application on the MDA and GSH concentrations and SOD, GSH-Px and CAT activities in nervus ulnaris tissue

The MDA levels, CAT and GSH-Px activities in the ulnar nerve tissue were found to increase ($p<0.001$, $p<0.001$, and $p<0.001$, respectively) whereas GSH level and SOD activities decreased ($p<0.001$ and $p<0.001$, respectively) in the PC group compared to the NC group. When the ANI and CNI groups were compared with the NC group, no statistically significant difference was observed between the groups in terms of all parameters. When ANI and CNI groups compared with the PC group, MDA levels, and CAT and GSH-Px activities were found to significantly decrease ($p<0.001$, $p<0.001$, and $p<0.001$, respectively) whereas an increase was observed in the GSH level and SOD activity ($p<0.001$ and $p<0.001$, respectively) (Table 4, Fig. 11).

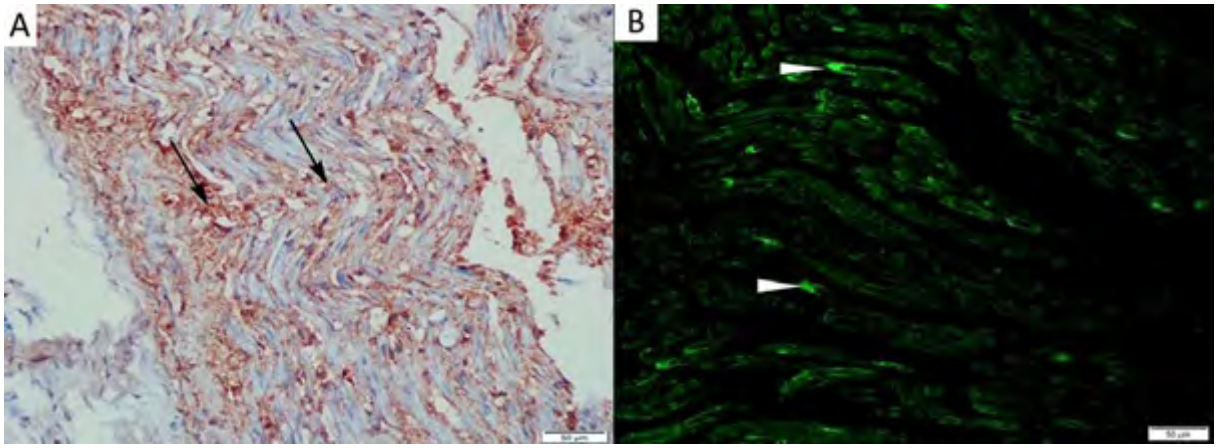

Fig. 9. (A) NGF immunoreactivity (arrows) in ulnar nerves of rabbits in the ANI group. IP; ×20; (B) NGF fluorescence positivity in the ulnar nerves of rabbits in the ANI group (arrows). IF; ×10

Table 2. Distribution of S100 and NGF immunoreactivity detected by IP and IF methods according to the number of rabbits (n), degrees of freedom (df), chi-square distribution (X^2), significance (p) and total score (Σ).

S100 (IP)									
None Mild	**Immunohistochemistry score (frequency)**						df	X^2	P value*
	Moderate	Severe	n	Σ					
PC	-	2	4	1	3	6.93	0.031		
NC	-	-	2	5	7	13			
ANI	-	-	2	5	7	19			
CNI	-	1	3	3	7	19			
					7	16			
NGF (IP)									
	None	Mild	Moderate	Severe	n	Σ			
PC	5	2	-	-	7	2	3	4.36	0.113
NC	-	5	2	-	7	9			
ANI	-	4	3	-	7	10			
CNI	-	3	4	-	7	11			
S100 (IF)									
	None	Mild	Moderate	Severe	n	Σ			
PC	-	2	4	1	7	13	3	6.50	0.039
NC	-	-	2	5	7	19			
ANI	-	-	3	4	7	18			
CNI	-	1	3	3	7	16			
NGF (IF)									
	None	Mild	Moderate	Severe	n	Σ			
PC	6	1	-	-	7	1	3	3.50	0.174
NC	1	5	1	-	7	7			
ANI	-	4	3	-	7	10			
CNI	-	4	3	-	7	10			

df – degree of freedom, X2 – Table chi-square value * – After Bonferroni correction, the new P value was calculated as 0.0125 and the comparison was made according to the new P value. n=Number of subjects in a group

(0: None, 1: Mild, 2: Moderate, 3: Severe)

Table 3. Statistical evaluation of the levels of oxidative stress factors in the rabbit radial nerve tissue ($\bar{x}$±SE).

N. radialis Dokusu	NC ($\bar{x}$±SE)	PC ($\bar{x}$±SE)	ANI ($\bar{x}$±SE)	CNI ($\bar{x}$±SE)	P
MDA (nmol/g tissue)	0.48±0.02[a]	1.32±0.08[b]	0.54±0.03[a]	0.46±0.01[a]	0.001
GSH (µmol/ml)	4.50±0.13[a]	3.85±0.12[b]	4.62±0.18[a]	4.36±0.18[a]	0.05
CAT (k/g protein)	18.72±0.96[a]	24.70±1.74[b]	18.98±1.95[a]	23.52±1.00[a]	0.05
GSH-Px (U/mg protein)	0.255±0.01[a]	0.413±0.01[c]	0.230±0.02[a]	0.300±0.01[b]	0.001
SOD (U/mg protein)	0.350±0.01[a]	0.309±0.01[b]	0.344±0.01[a]	0.319±0.01[ab]	0.05

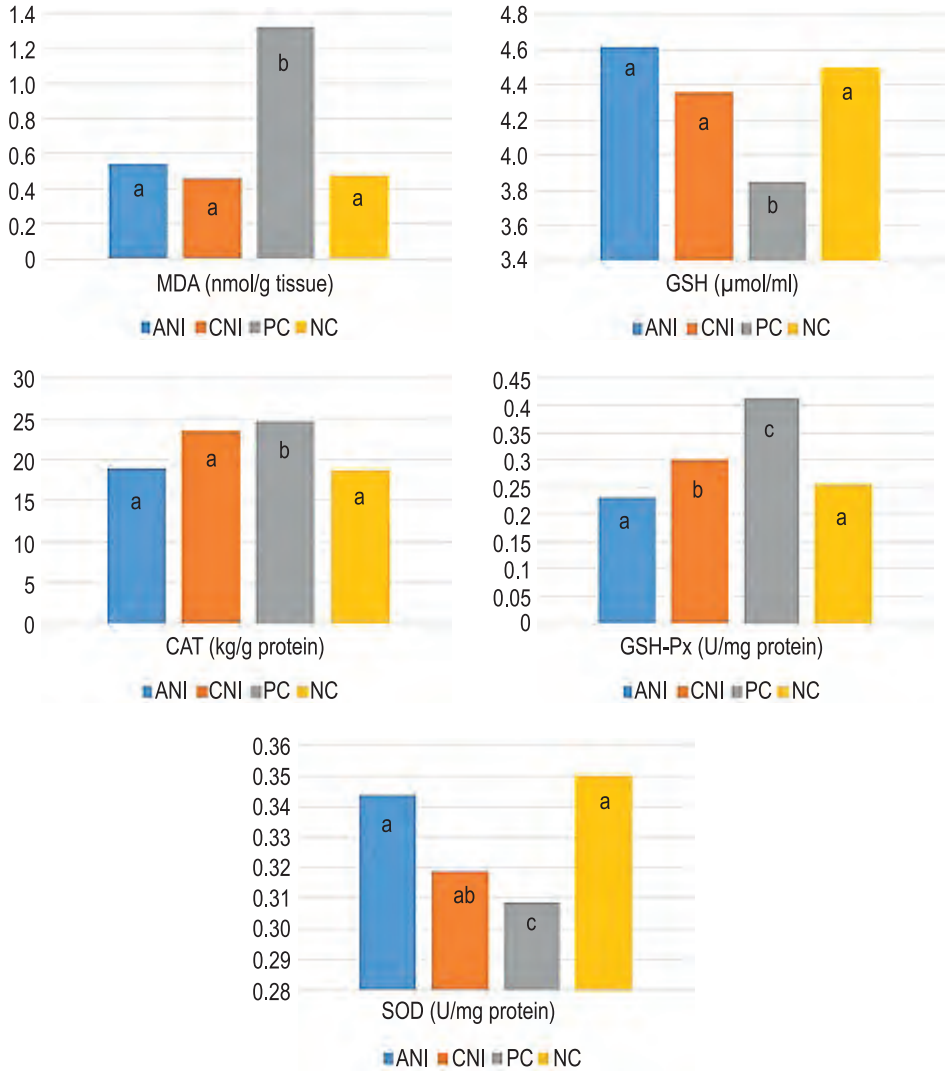

Fig. 10. Effects of electroacupuncture on oxidative stress parameters in radial nerve tissue (The differences between the means with different letters in the same graph are statistically significant).

Discussion

Histopathological and immunohistochemical findings are important indicators in experimental studies on peripheral nerve injuries. Histopathological findings such as degeneration/necrosis of peripheral nerves, inflammatory reaction, vascularization, congestion, and fibrosis are important data showing regeneration status. In a study by Sütçü (2010) investigating the effect of melatonin on nerve regeneration in peripheral nerve transections, fibrous connective tissue and blood vessel were observed in the epineural layer on the damaged nerve region. In a study investigating the efficacy of interferon beta in rats with sciatic nerve damage by Zengin (2014), it was reported that axon and myelin damage in the sciatic nerves of rats in the control groups

Table 4. Statistical evaluation of the levels of oxidative stress factors in the rabbit ulnar nerve tissue ($\bar{x}$±SE).

N. ulnaris tissue	NC ($\bar{x}$±SE)	PC ($\bar{x}$±SE)	ANI ($\bar{x}$±SE)	CNI ($\bar{x}$±SE)	P
MDA (nmol/g tissue)	0.47±0.02[a]	0.67±0.02[b]	0.51±0.01[a]	0.47±0.02[a]	0.001
GSH (µmol/ml)	4.05±0.11[a]	3.30±0.08[b]	4.07±0.09[a]	3.87±0.09[a]	0.001
CAT (k/g protein)	16.26±0.90[a]	24.31±0.83[b]	14.90±1.15[a]	14.39±0.66[a]	0.001
GSH-Px (U/g protein)	82.34±3.47[a]	110.49±3.74[b]	75.81±2.18[a]	72.88±1.68[a]	0.001
SOD (U/mg protein)	0.285±0.02[a]	0.221±0.01[b]	0.266±0.01[a]	0.278±0.01[a]	0.001

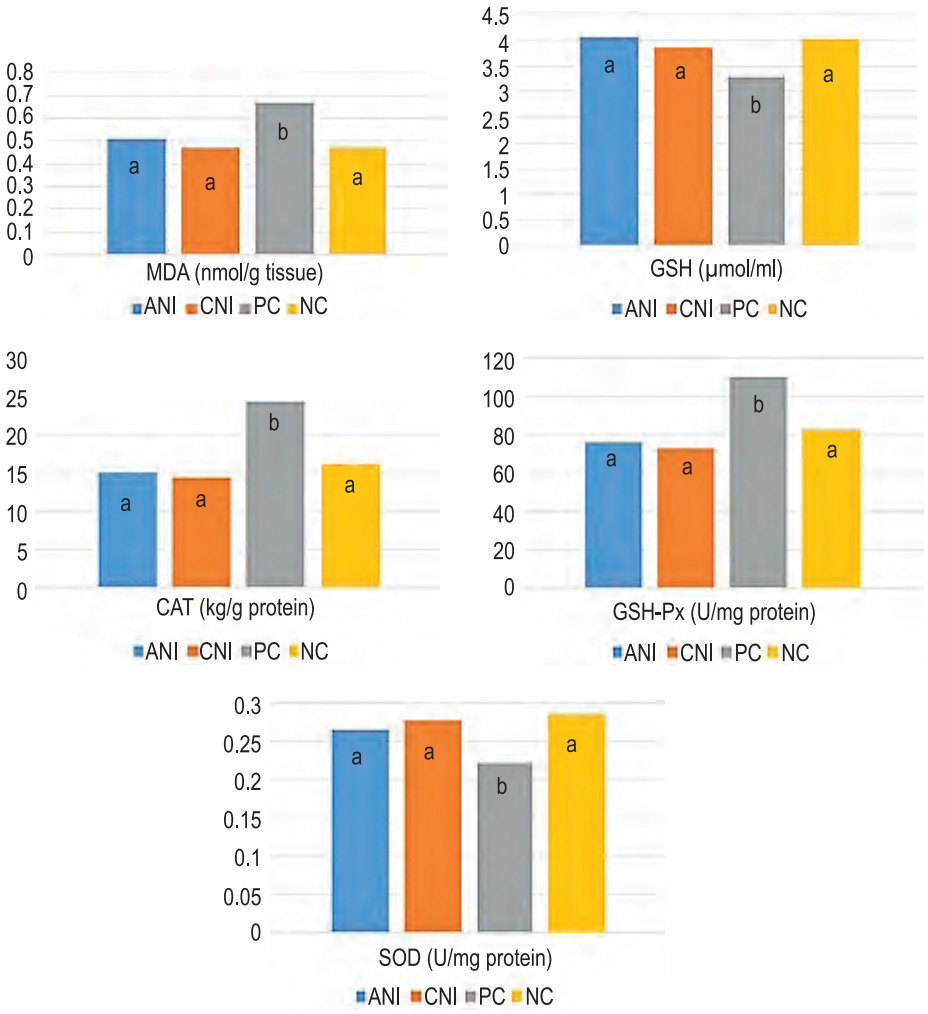

Fig. 11. Effects of electroacupuncture on oxidative stress parameters in ulnar nerve tissue (the differences between the means with different letters in the same graph are statistically significant).

was more severe than in the rats in the treatment groups (intraperitoneal interferon beta administered groups). It was reported that new axon formation was more prominent in rats in interferon beta administered groups. In the present study, there were more common and severe degenerative and necrotic areas in the damaged nerve region of rabbits in the PC group compared to the rabbits in the ANI and CNI groups. The degenerative changes in the damaged nerve region of rabbits in the CNI group were found to be more intense than the rabbits in the ANI group. This histopathologically indicates that electroacupuncture is a more effective treatment method in acute peripheral nerve injury compared to chronic peripheral nerve injury.

Scar tissue formation consisting of the connective tissue has been reported to be one of the greatest problems encountered during peripheral nerve injury treatment and after operative procedures. In a study by Huseyinoglu et al. (2012), in which neurorrhaphy was performed following the damage created on the

sciatic nerves of rats, silicone tubes were used to prevent the formation of scar tissue and silicone tubes containing hyaluronic acid were used to remove adhesions. Furthermore, there are also studies reporting that hyaluronic acid improves the regeneration of peripheral nerves (Huseyinoglu et al. 2012). In the present study, relatively less regeneration was observed in some of the rabbits in the CNI group despite the electroacupuncture therapy. We believe that this might be due to the formation of scar tissue called neuroma formed at the end of the injured nerve. Macroscopic examination of the injured nerves, which was performed prior to the pathological examination, revealed an excessive thickening in the injured areas of nerves in some subjects. Histopathological examinations showed that degeneration, necrosis, and fibrosis in the nerve tissues of rabbits in the PC and CNI group were more severe than the ANI group. In light of these findings, the use of silicon tubes filled with hyaluronic acid in combination with the electroacupuncture applications in the treatment of peripheral nerve injuries is important in terms of preventing scar tissue formation in the injured nerve region.

Levels of S-100 and NGF proteins are important indicators for the assessment of recovery status during regeneration of peripheral nerves. The NGF is a protein-structured neurotrophin particularly secreted from mammalian neuronal cells which can be also secreted from epithelial and endothelial cells. It is responsible for the proliferation and differentiation of neurons during the regeneration process of the injured nerves. The S-100 proteins are calcium-binding proteins found in vertebrates. They are located in the extracellular area and involved in neuronal differentiation and proliferation of astrocytes by stimulating the activity of inflammatory cells. Both NGF and S-100 proteins show a positive correlation with recovery during regeneration (Pagani et al. 2006, Sun et al. 2009, Yediel et al. 2017). Hu et al. (2018) determined the S-100 levels and serum NGF levels in the injured nerve regions of rats treated via electroacupuncture and moxibustion following the damage to sciatic nerves. A significant increase was reported to be observed in the S-100 levels and serum NGF levels in the injured nerve regions of the rats undergoing electroacupuncture and moxibustion compared to the control group. In the present study, S-100 levels in the damaged nerve regions of rabbits in the ANI group were found to be higher than in rabbits of the PC and CNI group whereas there was no difference between the CNI and PC groups in this regard. Moreover, the NGF levels in the damaged nerve region of rabbits in the ANI and CNI groups were found to be higher than in the PC group. Sun et al. (2009) investigated the efficacy of NGF in sciatic nerve regeneration and found that the significant regeneration difference between the control and treatment groups occurred in the eighth week. In the present study, there was a difference between the ANI and CNI groups and PC group in terms of S-100 and NGF levels, however, this difference was not statistically significant, which may be due to the short duration of treatment (4 weeks).

Fei et al. (2019) investigated the efficacy of electroacupuncture in rabbits with facial nerve damage. They reported that axonal demyelination was less and the rate of inflammatory cells was higher in rabbits in the electroacupuncture group. They also reported that the level of glial cell-derived neurotopic factor (GDNF), which protects neuronal structures, was higher in the electroacupuncture group compared to the control group. In this study, it was determined that the level of degeneration in the nerves of the rabbits in the treatment groups (ANI and CNI) was lower, but the level of inflammation was similar to the PC group. At the same time, it was determined that the vascularization level in the nerves of the rabbits in the ANI group was higher than the rabbits in the PC and CNI groups. Again in this study, it was determined that the NGF level, which is effective in the growth, protection and proliferation of neurons, was high in the treatment groups (ANI and CNI). This result is similar to the GDNF level, which is effective in the protection of neuronal structures in the study of Fei et al. (2019).

Excessively produced oxygen free radicals must be neutralized by GSH, glutathione peroxidase (GPO), glutathione reductase (GRx), glutathione s transferase (GST), SOD, CAT, and other antioxidant defense systems to maintain the integrity and normal functioning of tissues in living organisms. If antioxidants are failed to neutralize oxidants, i.e. if the balance which is in favor of antioxidants in normal physiological structure turns in favor of oxidants, tissue damage known as oxidative stress occurs (Suleyman et al. 2018). Lipids in cellular membranes undergo oxidation with the increasing concentrations of oxygen free radicals, leading to the formation of toxic products such as MDA (Suleyman et al. 2018). Antioxidants show their efficacy by preventing the formation of oxygen free radicals during the production phase and eliminating the harmful effects of oxygen free radicals formed. GSH, which is one of the most important antioxidants, renders hydrogen peroxide and organic oxides chemically harmless, SOD converts superoxides to hydrogen peroxide and molecular oxygen, and CAT catalyzes the conversion of hydrogen peroxide to molecular oxygen and water (Suleyman et al. 2018). In this study, GSH, MDA, GSH-Px, CAT and SOD concentrations in the tissue samples taken from the radial and ulnar nerves of the subjects which were divided into four groups

namely ANI and CNI groups (damage was created on radial and ulnar nerves and electroacupuncture treatment was performed), NC group (no damage was created), and PC group (damage was created but no treatment was applied), were measured and the results were evaluated biochemically.

In a study by Santos et al. (2013) including 28 female rats, in which the effects of needle acupuncture and electroacupuncture performed following the estradiol-induced inflammation on oxidative stress were investigated, a statistically significant increase was reported in both GSH and MDA plasma and ovary concentrations in both treatment groups compared to the control group. Similarly, Acioli et al. (2014) investigated the efficacy of needle acupuncture and electroacupuncture on oxidative stress and inflammation in 30 male rats with testicular torsion/detorsion and reported that there was a statistically significant increase in GSH concentration in the electroacupuncture group and in MDA concentration in the experimental groups compared to the control group. In the present study, the data obtained from rabbits in both the ANI and CNI groups were statistically different from those found in the rabbits of the PC group in terms of all criteria (GSH, MDA, GSH-Px, CAT, and SOD). No statistically significant difference was observed between the rabbits in the ANI group and the in rabbits of the NC group in terms of GSH, MDA, GSH-Px, CAT, and SOD concentrations in the radial and ulnar nerves. There was a statistically significant difference between the rabbits in the CNI group and the rabbits in the NC group only in terms of SOD and GSH-Px concentrations in the radial nerves. This difference was also present between NC and PC groups in terms of SOD and GSH-Px concentrations.

In this study, S-100 immunoreactivity was found to be lower in groups (PC and CNI) with severe degeneration and fibrosis compared to the other groups (ANI and NC). In addition, MDA, GSH-Px and CAT levels were higher in the PC group with severe degeneration compared to the other groups (ANI, CNI and NC). The increase in MDA, a marker of oxidative damage, is normal. However, the increase in GSH-Px and CAT levels is due to the fact that they are responsible for the removal of hydrogen peroxide catalyzed by SOD.

Conclusion

In conclusion, this study has proven that electroacupuncture is an effective method in the treatment of peripheral nerve injuries in the light of histopathological, immunohistochemical and biochemical data. Electroacupuncture has been further shown to be more effective in acute peripheral nerve injury than in chronic peripheral nerve injury.

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