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ORIGINAL ARTICLE

Effects of N-acetylcysteine on sciatic nerve healing: A histopathological, functional, and biochemical study of the rat sciatic nerve

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Peripheral nerve injuries can lead to serious problems. The uncertainties during the healing process have inspired studies aiming to improve healing and nerve regeneration. Preventing oxidative stress after peripheral nerve damage can accelerate the repair process and enhance functional healing following a nerve injury.[1]

N-acetylcysteine (NAC) is primarily used in the treatment of respiratory diseases and hepatotoxicity caused by high doses of paracetamol. It also

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ABSTRACT

Objectives: This study aims to evaluate the histopathological, biochemical, and functional effects of N-acetylcysteine (NAC), which has antioxidant, anti-inflammatory, and cytoprotective activity, on nerve regeneration in rats with sciatic nerve crush (axonotmesis) injury.

Materials and methods: This study used 16 male Wistar rats, which were divided into treatment and control groups. A standard axonotmesis-type surgical injury was induced in the left sciatic nerves of all rats. The treatment group was given 300 mg/kg of intraperitoneal NAC once a day, whereas the control group received an equal volume of saline solution. After conducting gait analyses, the sciatic functional index (SFI) was used for functional assessment. After gait analysis, all animals were euthanized. Blood samples were examined biochemically. The left sciatic nerves and left triceps surae muscles were examined histopathologically.

Results: Histopathologically, the thickness of the perineurium, axonal degeneration, axonolysis, edema, inflammation, muscle atrophy, and muscle degeneration were all significantly lower in the treatment group ($p<0.05$). Functionally, SFI-1, SFI-2, and SFI-3 were significantly higher in the treatment group ($p<0.05$). Biochemically, while the native thiol level and native thiol/total thiol ratio were significantly higher in the treatment group ($p<0.003$), the disulfide/total thiol ratio was significantly higher in the control group ($p<0.005$). Significant correlations were found between six of the seven gait parameters and the histopathological findings ($p<0.05$).

Conclusion: Our study results suggest that NAC may contribute positively to the histopathological and functional recovery of sciatic nerve injury in rats. Furthermore, NAC may have an antioxidant effect on thiol-disulfide homeostasis at a biochemical level. We believe that NAC has a stimulatory effect on healing following nerve injuries.

Keywords: Animal experiment, antioxidant, N-acetylcysteine, sciatic nerve, thiol-disulfide.

possesses strong antioxidant and anti-inflammatory properties.[2] The NAC can reduce neuronal death following peripheral nerve injury.[3,4] However, no experimental study has examined the therapeutic effects of NAC on sciatic nerve injuries so far. In the present study, we hypothesized that the antioxidant, anti-inflammatory, and cytoprotective effects of NAC might contribute to sciatic nerve healing. We, therefore, aimed to investigate the effects of NAC on this type of healing in rats.

MATERIALS AND METHODS

In this study, 16 male Wistar rats weighing between 350 and 500 g and aged between 16 and 18 weeks were used. The rats were given water and standard pellet feed ad libitum and kept in a room under a 12-h light/12-h dark cycle with 50 to 60% humidity and a temperature of 18 to 22°C. The rats were divided into NAC and placebo (control) treatment groups. No information was provided to the experts who evaluated the samples sent for analysis and coding.

Sciatic nerve injury model: Anesthesia and surgical method

All surgical procedures were performed on the rats with inhalation anesthesia in İstanbul Bağcılar Training and Research Hospital Experimental Research and Skills Development Center operating rooms. A standard 2-cm longitudinal skin incision was made along the hip crease line. The biceps femoris muscle was reached after passing through the skin and subcutaneous tissues and blunt dissected along the intermuscular septum. The sciatic nerve was identified directly below and released from soft tissue up to the bifurcation. The sciatic nerve was clamped 1 cm proximal to its bifurcation with a 54-Newton clamp for 30 sec, creating axonotmesis type nerve damage (Figure 1).[5,6] The skin incision was sutured, and a single dose of meloxicam (2 mg/kg) was administered subcutaneously for pain control (Figure 1).[7] These procedures were performed on

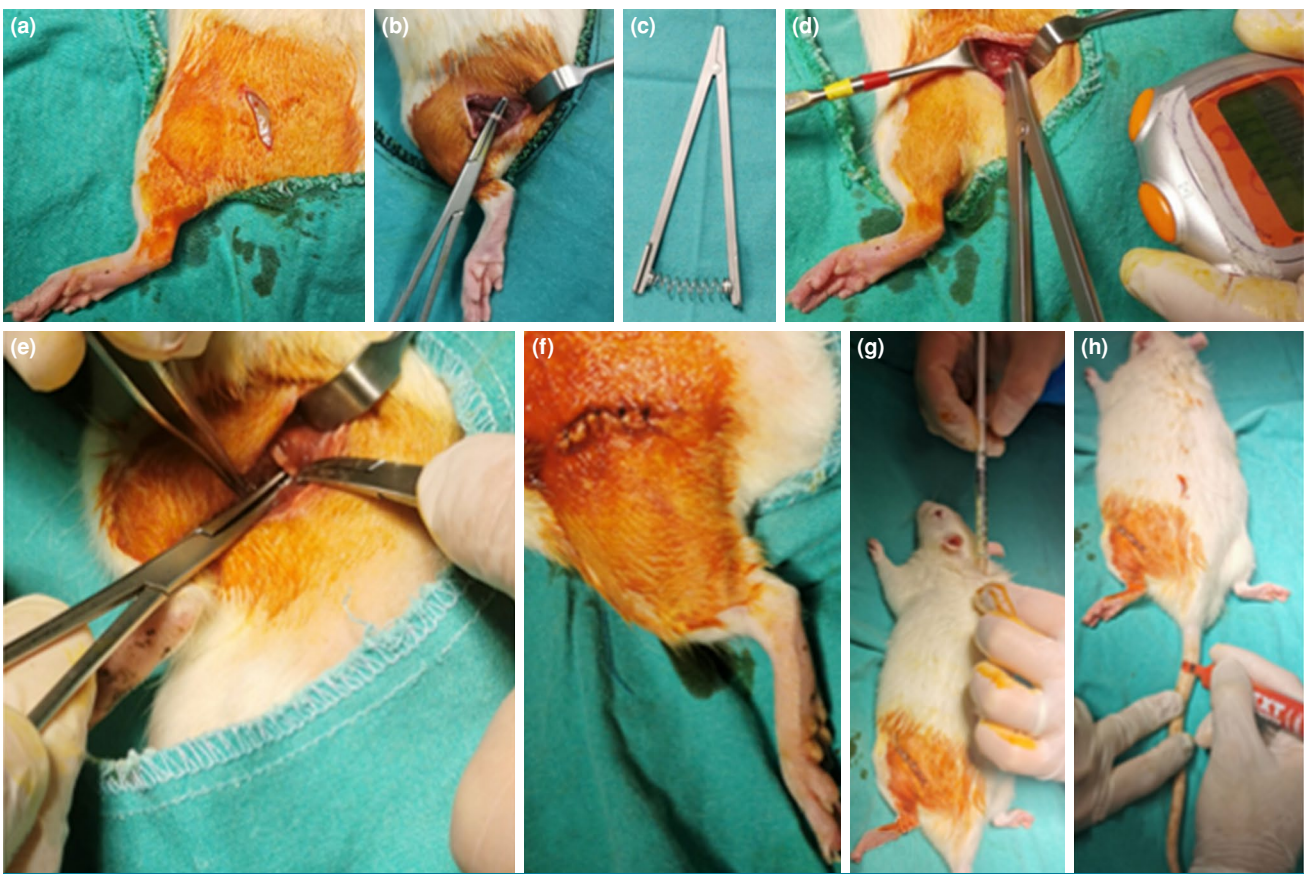


FIGURE 1. Creating damage to the sciatic nerve. **(a)** Skin incision. **(b)** Reaching the sciatic nerve. **(c)** A specialized clamp with a 3 mm wide tip, applying a force of 54 Newtons, and without serrations. **(d)** Compression of the sciatic nerve with a clamp for 30 seconds. **(e)** Damaged area in the nerve. **(f)** Suturation and dressing. **(g)** Subcutaneous administration of analgesics. **(h)** Painting the tails of rats according to the work scheme.

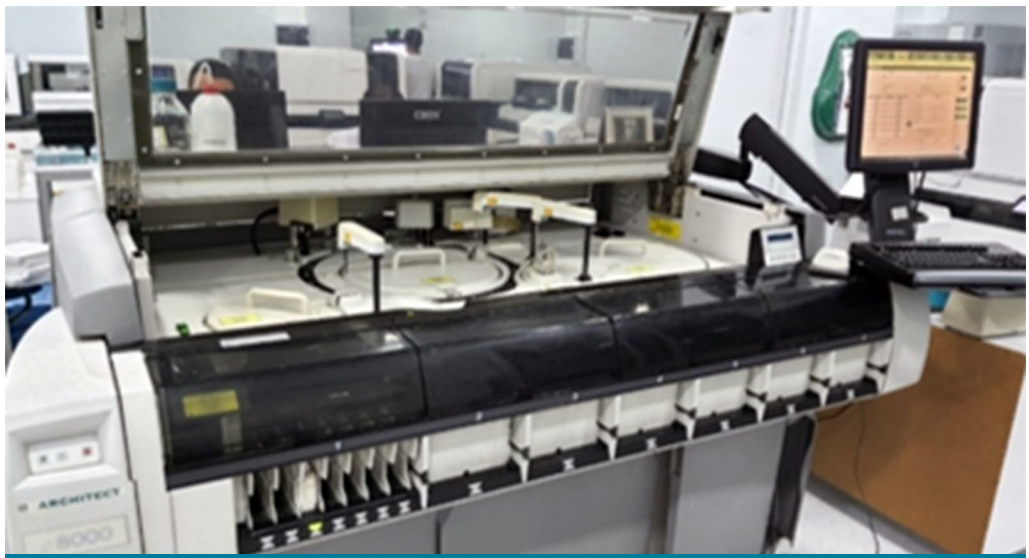


FIGURE 2. The device used for conducting biochemical tests is the "Architech c8000 - Abbott."

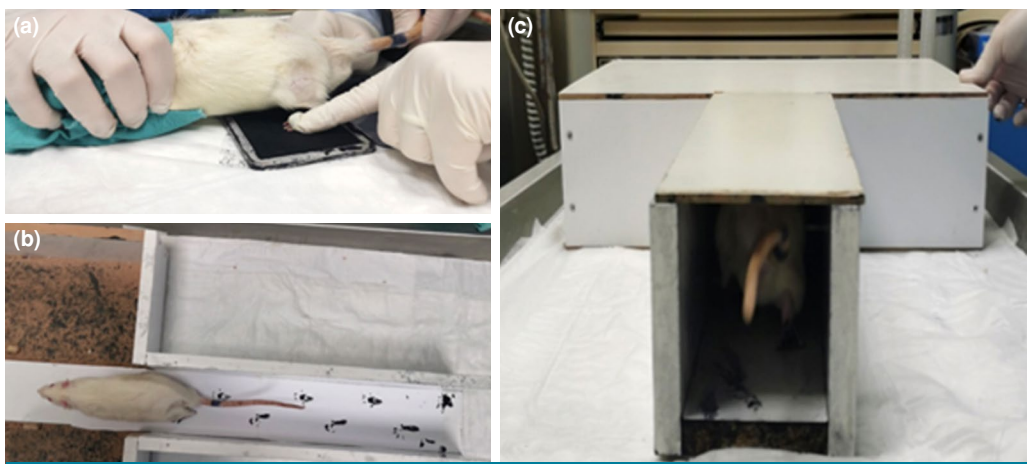


FIGURE 3. (a) Pressing the hind legs of the rat onto the ink pad. **(b)** A rat walking in a corridor (Sample shot). **(c)** A rat walking in a hallway (viewed from behind).

TABLE I
Histopathological evaluation findings

	Control (n=8)			Treatment (n=8)			
	Mean±SD	Median	Min-Max	Mean±SD	Median	Min-Max	*p*
Nerve histopathology							
The thickness of the perineurium (fibrosis) (μ)	37.13±6.41	36.2	29.4-45.4	27.71±7.42	29.5	10.2-33.4	0.012*
Axonal degeneration (%)	50±7.56	50	40-60	31.25±5.82	30	25-40	0.001*
Axonolysis (%)	25.63±8.21	25	15-40	12.5±4.63	10	10-20	0.003*
Edema	1.5±0.53	1.5	1-2	0.25±0.46	0	0-1	0.002*
Inflammation	1.38±0.74	1.5	0-2	0.13±0.35	0	0-1	0.002*
Muscle histopathology							
Muscle atrophy	1.5±0.53	1.5	1-2	0.5±0.53	0.5	0-1	0.006*
Muscle degeneration	1.5±0.76	2	0-2	0.63±0.52	1	0-1	0.021*

SD: Standard deviation; μ: micron; * p<0.05; Mann-Whitney U test.

the same day by the same surgeon. No complications were observed after the procedure in any rat.

N-acetylcysteine administration protocol

Starting from the day of surgery, rats in the treatment group were given 300 mg/kg/day NAC (Mucinac® 300 mg/3 mL ampoule, Vem İlaç, Tekirdağ, Türkiye) via intraperitoneal injection until euthanized.[8] The control rats were given an isosmotic solution of 0.9% NaCl in the same manner (Polifleks®; Polifarma İlaç, Tekirdağ, Türkiye). The rats were injected with appropriate amounts according to their weight, which was measured at intervals.

Euthanasia method, sample preparation, and preservation

Following gait analysis, all animals were euthanized at 30 days after surgery by anesthetic overdose followed by intracardiac blood sampling. The blood samples were centrifuged and transferred to Eppendorf™ tubes (Eppendorf SE: Barkausenweg, Hamburg Germany). The left sciatic nerves of the rats were removed post-euthanasia, from 1 cm distal to 2 cm proximal to the bifurcation. Then, the left triceps surae muscles were dissected to include their proximal and distal attachments. The samples were placed in labeled pathology containers containing a 10% formaldehyde solution.

Histopathological staining and outcome measures

Sciatic nerve and triceps surae muscle samples from each group were placed in 10% formaldehyde and sent to Department of Pathology, Istanbul Kanuni Sultan Süleyman Training and Research Hospital for histopathological examination. For the sciatic nerve samples, the thickness of the perineurium was measured digitally at 1,000× magnification to determine the severity of fibrosis, and the measurements were recorded in μm. The percentages of cells showing axonal degeneration and axonolysis were calculated. Edema was categorized as absent (0-5%), mild (6-30%), or marked (>30%) relative to the histopathology of the intact nerves. Based on the number of inflammatory cells observed, inflammation was categorized as absent (0–1 cell), mild (2-15 cells), or marked (>15 cells). Triceps surae muscle samples were compared based on the histopathology of the healthy side. The atrophy and degeneration of the triceps surae muscle was categorized as absent (0%), mild (1-10%), moderate (11-50%), or severe (>50%).

Biochemical tests and outcome measures

Plasma samples were stored at –80°C until the day of testing. The samples were thawed at room temperature without pretreatment and evaluated using an Architect™ c8000 Clinical Chemistry analyzer (Abbott Diagnostics, Chicago, IL, USA) based on spectrophotometric principles (Figure 2).

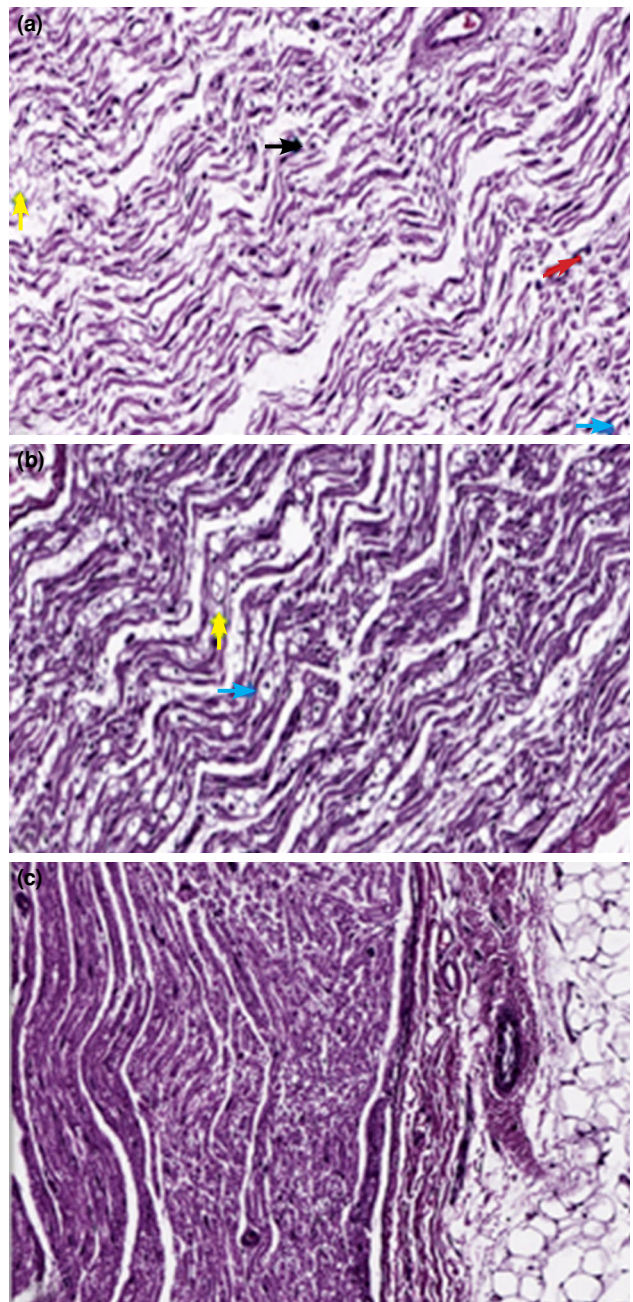


FIGURE 4. Samples of sciatic nerve specimens stained with Hematoxylin-Eosin (H-E). **(a)** Control group: a section with widespread axonal degeneration (blue arrow), axonolysis (yellow arrow), inflammation (black arrow histiocytes, red arrow mast cells), and significant edema. **(b)** Treatment group: a section showing axonal degeneration (blue arrow), axonolysis (yellow arrow), inflammation, and mild edema. **(c)** Typical histomorphology (H-E, ×400).

Total and native thiol and disulfide were measured. The direction of change in oxidative/antioxidative status was examined by determining the native thiol/total thiol and disulfide/total thiol ratios of each group.[9,10]

Functional test procedures and outcome measures

Gait was analyzed on postoperative Day 30 using the sciatic functional index (SFI) equations described by Medinaceli (SFI-1), Carlton and Goldberg (SFI-2), and Bain and Maccinon (SFI-3) (Figure 3).[11,12] The footprints of the rats were measured to determine the experimental and normal distances to the opposite foot (ETOF and NTOF, respectively), experimental and normal print lengths (EPL and NPL, respectively), experimental and normal toe spreads (ETS and NTS, respectively), and experimental and normal intermediate toe

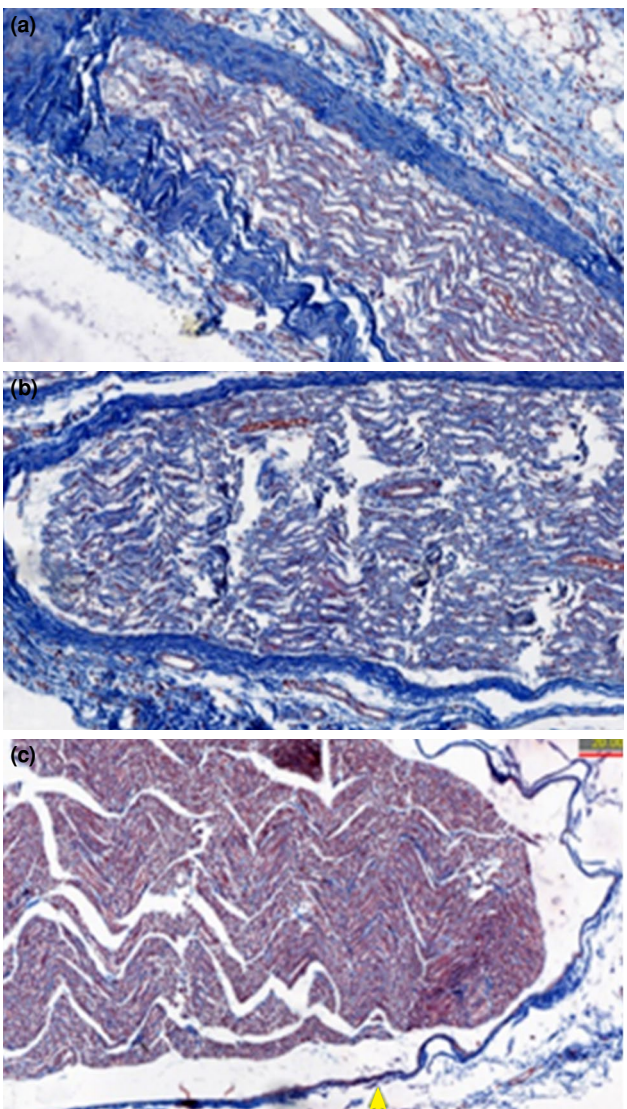


FIGURE 5. Sciatic nerve samples stained with MT. **(a)** Control group: a section with excessive fibrous thickening. **(b)** Treatment group: less fibrosis in the section compared to the control group. **(c)** Typical histomorphology (yellow arrow: perineurium thickness) (MT, ×200).
MT: Masson's trichrome.

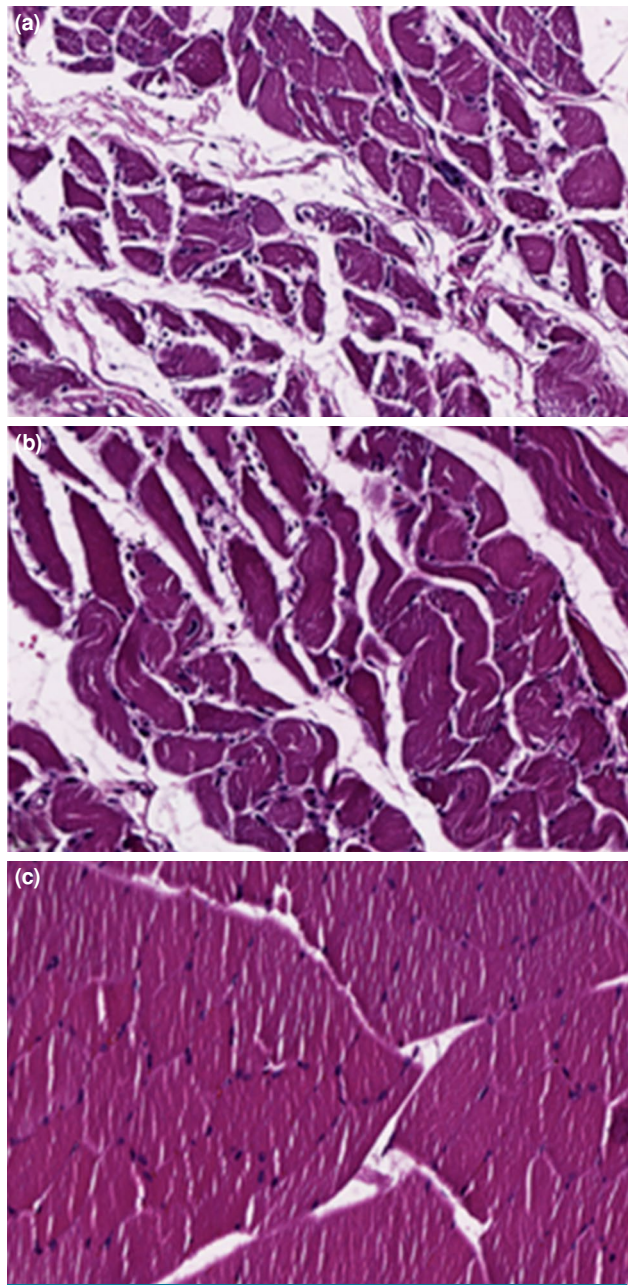


FIGURE 6. Triceps surae muscle examples with H-E staining. **(a)** Control group: A section showing moderate signs of atrophy and degeneration. **(b)** Treatment group: a section showing mild atrophy and degeneration signs. **(c)** Typical histomorphology (H-E, ×400).

spreads (EIT and NIT, respectively). Based on these values, the same individual calculated SFI-1, SFI-2, and SFI-3.

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). The normality of the distribution of the parameters was assessed using the Shapiro-Wilk test. Descriptive data were expressed in mean ± standard deviation (SD), median (min-max), or number and frequency, where applicable. Non-normally distributed data were compared using the Mann-Whitney U test. Spearman rho was used to examine relationships between parameters not following a normal distribution. A p value of <0.05 was considered statistically significant.

RESULTS

Histopathological findings

On examining the nerve and muscle histopathology, the treatment group showed significant improvements in all parameters compared to the control group ($p<0.05$) (Table I). Figures 4-6 depict examples of histopathological staining.

Functional assessment (gait analysis) findings

In the gait assessments, the treatment group had significantly higher scores for all SFIs compared to the controls ($p<0.05$) (Table II).

Biochemical findings

The treatment group had significantly higher native thiol levels and native thiol/total thiol ratios

TABLE II
Functional assessment (gait analysis) findings

	Control (n=8)			Treatment (n=8)			
	Mean±SD	Median	Min-Max	Mean±SD	Median	Min-Max	*p*
Gait analysis							
Experimental distance to opposite foot	15.46±1.39	15.2	13.5-17.6	15.88±1.37	16.2	13.7-17.4	0.462
Normal distance to opposite foot	15.99±0.98	15.8	14.9-17.5	15.7±1.18	15.4	14-17.2	0.528
Experimental print length	3.04±0.38	3.1	2.3-3.5	2.98±0.31	3	2.4-3.4	0.672
Normal print length	2.64±0.28	2.6	2.2-3	2.83±0.24	2.9	2.4-3.2	0.256
Experimental toe spread	2.03±0.16	2.1	1.8-2.2	2.25±0.18	2.3	1.9-2.5	0.014*
Normal toe spread	2.35±0.14	2.4	2.1-2.6	2.28±0.1	2.3	2.1-2.4	0.227
Experimental intermediate toe spread	1.09±0.11	1.1	0.9-1.2	1.18±0.1	1.2	1.1-1.4	0.202
Normal intermediate toe spread	1.26±0.07	1.3	1.2-1.4	1.18±0.15	1.2	1-1.4	0.212
Sciatic functional index-1	–7.8±5.39	–6.5	–17.2 - –0.4	0.27±3.73	–0.1	–4.3-5.5	0.002*
Sciatic functional index-2	–29.36±9.7	–30.7	–45 - –16.5	–3.89±8.18	–4.3	–13.4-8.3	0.001*
Sciatic functional index-3	–31.58±5.07	–32.9	–40.5 - –24.3	–11.87±7.68	–12.2	–25.2 - –3.2	0.001*

SD: Standard deviation; * p<0.05; Mann-Whitney U test.

TABLE III
Biochemical evaluation findings

	Control (n=8)			Treatment (n=8)			
	Mean±SD	Median	Min-Max	Mean±SD	Median	Min-Max	*p*
Biochemical analysis							
TTHIOL	394.9±70.15	398.7	292.8-487.8	426.39±66.84	415.5	348.6-554.8	0.600
NTHIOL	49.46±13.77	47.1	32.1-69.8	88.86±23.69	85.5	45-117.2	0.005*
Disulfide	345.44±63.32	350.1	252.4-439.6	337.53±54.81	334.2	275-449.2	0.674
NTHIOL/TTHIOL	0.13±0.03	0.1	0.1-0.2	0.21±0.05	0.2	0.1-0.3	0.003*
Disulfide /TTHIOL	0.87±0.03	0.9	0.8-0.9	0.79±0.05	0.8	0.7-0.9	0.003*

SD: Standard deviation; TTHIOL: Total thiol; NTHIOL: Native thiol; * p<0.05; Mann-Whitney U test.

TABLE IV
Assessment of the correlation between nerve and muscle histopathology parameters and walking analysis parameters

Gait analysis		Nerve histopathology					Muscle histopathology	
		The thickness of the perineurium (fibrosis) (μ)	Axonal degeneration (%)	Axonolysis (%)	Edema	Inflammation	Muscle atrophy	Muscle degeneration
ETOF	r	0.300	–0.122	0.086	–0.240	0.192	–0.086	–0.200
	p	0.259	0.652	0.751	0.371	0.477	0.751	0.458
NTOF	r	0.707	0.163	0.440	0.008	0.477	0.029	0.107
	p	0.002*	0.546	0.088	0.977	0.062	0.916	0.693
EPL	r	0.356	0.182	0.408	0.163	0.468	0.087	–0.169
	p	0.176	0.499	0.117	0.547	0.068	0.749	0.531
NPL	r	0.097	–0.151	–0.024	–0.278	0.132	–0.277	–0.538
	p	0.721	0.578	0.93	0.298	0.627	0.300	0.032*
ETS	r	–0.293	–0.538	–0.293	–0.419	–0.407	–0.527	–0.284
	p	0.270	0.031*	0.271	0.106	0.118	0.036*	0.286
NTS	r	0.464	0.082	0.327	0.262	0.354	0.060	0.259
	p	0.070	0.763	0.216	0.328	0.179	0.825	0.333
EIT	r	–0.390	–0.376	–0.253	–0.473	–0.388	–0.598	–0.266
	p	0.135	0.152	0.345	0.064	0.137	0.014*	0.319
NIT	r	0.122	0.006	0.076	0.055	0.172	–0.168	–0.106
	p	0.654	0.982	0.779	0.840	0.525	0.533	0.695
SFI–1	r	–0.600	–0.536	–0.531	–0.623	–0.614	–0.499	–0.384
	p	0.014*	0.032*	0.034*	0.010*	0.011*	0.049*	0.142
SFI–2	r	–0.621	–0.603	–0.596	–0.673	–0.697	–0.575	–0.439
	p	0.010*	0.013*	0.015*	0.004*	0.003*	0.020*	0.089
SFI–3	r	–0.618	–0.594	–0.494	–0.679	–0.671	–0.633	–0.489
	p	0.011*	0.015*	0.055	0.004*	0.004*	0.009*	0.054

ETOF: Experimental distance to opposite foot; NTOF: Normal distance to opposite foot; EPL: Experimental print length; NPL: Normal print length; ETS: Experimental toe spread; NTS: Normal toe spread; EIT: Experimental intermediate toe spread; NIT: Normal intermediate toe spread; SFI: Sciatic functional index; * $p<0.05$; Spearman Rho Correlation Analysis.

compared to the controls ($p<0.003$), while the controls had significantly higher disulfide/total thiol ratios than the treatment group ($p<0.005$). No significant differences were found for any other parameters (Table III).

Compatibility of the histopathological and gait analysis data

Correlation analysis revealed no significant relationship between muscle degeneration and the SFIs. However, significant relationships were found between the SFIs and all other histopathological data ($p<0.05$) (Table IV).

DISCUSSION

In this experimental study, we investigated the effects of NAC on this type of healing in rats. Our study has four important findings. First, the treatment group had significantly better results than the control group for all histopathological parameters examined. Second, all SFIs were significantly higher in the treatment group. Third, the treatment group had significantly better antioxidant activity than the control group based on the native thiol, native thiol/total thiol, and disulfide/total thiol values. Finally, a significant correlation was found between histopathological and gait analysis data.

Histopathologically, we observed that NAC contributes to sciatic nerve healing and reduces triceps surae muscle atrophy. Considering the prolonged healing process following peripheral nerve injury, histopathological examinations are crucial for early assessment. In their study

investigating sciatic nerve damage following intramuscular injection, Zeynal and Kadıoğlu[13] histopathologically examined fibrosis, Wallerian degeneration, edema, and inflammation parameters in a semi-quantitative manner, similar to Faroni et al.[14] and Sezer et al.[15] Quantitative or semi-quantitative methods are commonly used in such studies, although there is no universally accepted method. Therefore, we used quantitative and semi-quantitative histopathological analyses in our study. Somay et al.[16] observed that the ozone molecule, which has antioxidant and anti-inflammatory activity, enhanced rat sciatic nerve healing. Karlidag et al.[17] observed positive effects of NAC on rabbit facial nerve regeneration compared to methylprednisolone. Consistent with the literature, our findings suggest NAC enhances nerve regeneration.

Many studies have focused solely on nerve damage and its treatment, but the main goal of regenerative medicine is to protect muscles and prevent joint contractures. Examining the muscle groups stimulated by a nerve is of importance to assess the quality of end-organ innervation in the context of nerve healing. Mandelbaum-Livnat et al.[18] investigated the effects of laser phototherapy on the healing of rat sciatic nerves and the prevention of muscle atrophy and compared gastrocnemius muscle atrophy between the affected and healthy sides semi-quantitatively. Feng et al.[19] observed that dexamethasone, which has anti-inflammatory properties, had a healing effect on gastrocnemius muscle atrophy after sciatic nerve damage in rats by comparing muscle wet weights between the atrophic and healthy sides. To evaluate the microarchitecture specifically, we semi-quantitatively evaluated muscle atrophy and degeneration by comparing the affected and sides and observed that NAC could reduce the muscle atrophy and muscle degeneration that might develop as a result of nerve damage.

In the current study, we observed a contribution of NAC to functional healing of the sciatic nerve. The rat sciatic nerve model is commonly used for the simultaneous evaluation of motor and sensory nerve function. While the functions of the sciatic nerve in humans can be evaluated through careful motor, sensory, and reflex examinations, this is impossible in rats. Numerous methods have been used to conduct functional analysis of rats. Naik et al.[20] investigated the effect of NAC treatment on rat sciatic nerve recovery and performed behavioral analysis and hyperalgesia, mechanical, and cold allodynia tests, reporting a significant benefit. Walking analysis and SFI are used to evaluate the overall nerve function in rats, since walking involves complex innervation and is a reliable, repeatable, cost-effective, quantitative method for evaluating sciatic nerve healing and function. In SFI evaluations, significant results often emerge after three to four weeks.[19] Therefore, we performed gait analysis and SFI on postoperative Day 30. Our results suggest that NAC has a strong effect on sciatic nerve functional recovery.[21] Some studies have combined SFI with electromyography, and we believe that this enhances functional evaluation.

Biochemically, we observed that NAC had antioxidant effects on thiol-disulfide homeostasis (TDH). Of note, NAC exerts most of its antioxidant effects via intracellular pathways.[22,23] Other studies have investigated the antioxidant effects of NAC on intracellular pathways using tissue samples or cell cultures,[24] while TDH reflects cysteine metabolism and is a system that buffers free oxygen radicals.[9] Recent studies of TDH have revealed its association with several diseases.[25] Therefore, we evaluate TDH using blood samples. While NAC shows antioxidant activity, its correlation with TDH parameters should be investigated to explore its potential use as a biomarker. Future similar studies can be enhanced by using tissue samples and cell culture examinations.

In the present study, we identified a consistent relationship between the histopathological and gait data. Terzis et al.[26] showed that the results of nerve regeneration studies were highly correlated without actually performing a correlation analysis. Based on this, Munro et al.[27] compared functional, electrophysiological, histopathological and histomorphometric parameters and reported that researchers should choose tests according to their study aims. Based on our correlation analysis, we believe that we have confirmed the relationship between the evaluation methods used and the results.

Nonetheless, there are some limitations to our study. Due to ethical concerns, we studied a small number of animals and lacked a sham group. Moreover, our injury model was a standard axonotmesis injury; injuries in humans have different mechanisms. Furthermore, we did not use immunohistochemical methods or electromyography. While we concluded that NAC had a positive effect on nerve regeneration, there is still no evidence regarding the mechanisms involved. In addition, our experiment lasted 30 days, which was too short to provide information about the late stage of regeneration. Finally, while our methods are suitable in rats, they are unlikely to be used in human clinical studies.

In conclusion, we believe that NAC has a healing effect on peripheral nerve injuries. Experimental studies should investigate the potential mechanisms through which NAC affects peripheral nerve regeneration. If future clinical studies support our results, NAC may be considered for use in the treatment of nerve tissue pathologies.

Ethics Committee Approval: The study protocol was approved by the Istanbul Bağcılar Training and Research Hospital Animal Experiments Local Ethics Committee (date: 25.02.2021, no: 2021/41). The study was conducted in accordance with the principles of the Declaration of Helsinki.

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

Author Contributions: Idea/concept, design, writing the article: T.B.S., C.E.; Control/supervision, analysis and/or interpretation, critical review: C.E., N.G., Ş.S.; Data collection and/or processing, literature review: T.B.S., H.B., B.Y.; References and fundings: T.B.S., C.E., H.B.; Materials: T.B.S., B.Y., N.G., Ş.S.

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