Erciyes Med J 2022; 44(1): 56–62 • DOI: 10.14744/etd.2021.87405
ORIGINAL ARTICLE – OPEN ACCESS

Comparison of the Effectiveness of Rapamycin and Gabapentin Treatment in Rats with Induced Sciatic Nerve Injury

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ABSTRACT

Objective: The purpose of this study was to compare the therapeutic effects of gabapentin (GBP) and different doses of rapamycin (RAPA) in an induced sciatic nerve (SN)-injury rat model.

Materials and Methods: The study consisted of 7 groups: Control, Sham, High-dose rapamycin (RAPA-H), Low-dose rapamycin (RAPA-L), GBP, DMSO and DMSO+nerve injury (DMSO+NI). Medical treatment was administered intraperitoneally for 30 days after the induction of SN injury.

Results: Significant differences ($p<0.001$ for all) were found in comparisons between the groups in terms of axon diameter, axon number, and neurofilament (NF) and S100 immunointensity. Among the treatment groups, the highest mean axon diameter value, close to that of the Control group, was seen in the RAPA-L group. In terms of axon number, the value closest to that of the Control group was measured in the GBP group. The NF and S100 immunodensity in the RAPA-L group was similar to that of the GBP group. The S100 immunodensity in the RAPA-L group was closest to that of the Control group. The highest conduction velocity and distal latency values were recorded in the RAPA-L group.

Conclusion: The histological and electrophysiological findings observed in this study suggest that RAPA-L treatment is a promising alternative to GBP.

Keywords: Gabapentin, nerve damage, rapamycin, regeneration

Cite this article as:
Canpolat M, Çolpak HA, Günay Canpolat D, Önder GÖ, Yetkin MF, Yay AH, et al. Comparison of the Effectiveness of Rapamycin and Gabapentin Treatment in Rats with Induced Sciatic Nerve Injury. Erciyes Med J 2022; 44(1): 56-62.

This study was presented as an oral presentation at the 23rd National Pediatric Neurology Congress

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Submitted
05.03.2021

Accepted
10.06.2021

Available Online
26.11.2021

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INTRODUCTION

Traumatic peripheral nerve damage is an important clinical and public health issue, as it frequently leads to important functional losses and permanent disability (1). There is currently no standardized approach to the treatment of peripheral nerve injury (PNI) and neuropathic pain; however, numerous invasive and noninvasive medical techniques have been described in the literature (1, 2).

Gabapentin (GBP) is a structural analog of gamma-aminobutyric acid (3). GBP is widely employed in the treatment of neuropathic pain resulting from nerve damage. One of the most important problems of GBP therapy is a risk of misuse (3). The need for other agents that provide the nerve regeneration effectiveness of GBP for cases of nerve damage and neuropathic pain persists.

The axon-regenerating capacity of neurons in the peripheral nervous systems (PNS) and central nervous systems (CNS) differs (4). The mechanisms that control axon regenerative capacity are still not fully understood. Although nerve regeneration in the PNS is known to be associated with several intrinsic factors released by damaged neurons, recent studies have revealed that the control of proteins released by the mammalian target of rapamycin (mTOR) pathway for regeneration plays a critical role (4). mTOR essentially consists of 2 different multi-protein complexes, mTORC1 and mTORC2 (5). mTORC1 is susceptible to sirolimus, while mTORC2 is not (6). A few studies have evaluated the effects of rapamycin (RAPA) on nerve regeneration (7, 8).

The aim of this study was to analyze the effects of RAPA and GBP treatment on nerve regeneration in rats with induced sciatic nerve (SN) damage with context from the current literature.

MATERIALS and METHODS

This research was performed with the approval of the Erciyes University animal experiment ethics committee on December 13, 2017 (no: 17/136). This was Erciyes University Scientific Research Unit project no. TSA-2018-7853. This study was carried out at the Erciyes University Experimental Research and Application Center (DEKAM).

In all, 50 female Wistar albino rats (weighing 200-250 g) were used in the study: 36 in the experimental groups and 4 as a control group. In addition, in order to assess the toxicity of the dimethyl sulfoxide (DMSO) used to

prepare the rapamycin and GBP infusion solutions, 4 animals were assigned to each of the DMSO and DMSO + nerve injury (DMSO+NI) groups, with 1 animal also in reserve for each.

Sciatic Nerve Damage Model

Following an appropriate acclimatization, the study animals were fasted overnight, and anesthesia of 60 mg/kg ketamine (Ketalar 50 mg/mL, Pfizer Inc., New York, NY, USA) and 10 mg/kg xylazine (Rompun 23.32 mg/mL; Bayer AG, Leverkusen, Germany) was administered via the intramuscular route. The area of the left SN was shaved with a dermatome and was cleansed with povidone iodine for skin antisepsis. The SN region was accessed with an incision approximately 1 cm in length and the nerve was exposed. Next, one-third to one-half of the left SN was ligated with silk using the neuropathic nerve pain model described by Seltzer et al (9). All of the skin incisions were subsequently closed with 3-0 resorbable sutures.

Group I (Control group): Only healthy nerve tissue was taken before ligation of the SN (n=4).

Group II (Sham group): The SN was ligated, but no procedure was performed (n=9).

Group III (Low-dose rapamycin group; RAPA-L): The SN was ligated, and afterwards, 3 mg/kg RAPA (R-5000; LC Laboratories, Woburn, MA, USA) was administered for 30 days in an infusion solution via the intraperitoneal route (n=9). Two rats from this group died due to diarrhea during the study; the research was completed with 7 rats in this group.

Group IV (High-dose rapamycin group; RAPA-H): The SN was ligated, after which 6 mg/kg RAPA (R-5000; LC Laboratories, Woburn, MA, USA) was administered for 30 days in an infusion solution via the intraperitoneal route (n=9).

Group V (Gabapentin group; GBP): The SN was ligated, followed by administration of 90 mg/kg GBP (PHR1049-1G; MilliporeSigma, Burlington, MA, USA) for 30 days via the intraperitoneal route (n=9).

Group VI (Dimethyl sulfoxide group; DMSO): The SN was not ligated; DMSO was used as a solution for GBP and rapamycin was administered via the intraperitoneal route to evaluate toxicity (n=4).

Group VII (DMSO+nerve injury group; DMSO+NI): SN damage was induced with ligation and DMSO was administered intraperitoneally (n=4).

DMSO was used as a common solution for the intraperitoneal administration of both RAPA and GBP.

Each animal was placed in a separate plastic cage after the surgical procedure. The experimental animals were then maintained in the same room with the standard environmental and dietary conditions. The experiments and 30-day intraperitoneal treatments (RAPA and GBP) were conducted at the Erciyes University Experimental Research Practice and Research Center.

The rats were anesthetized on the 31st day via intramuscular administration of 60 mg/kg ketamine (Ketalar 50 mg/mL, Pfizer Inc., New York, NY, USA) and 10 mg/kg xylazine (Rompun 23.32 mg/mL; Bayer AG, Leverkusen, Germany) for electrophysiological tests and subsequently euthanized. A histologist participated in the collection of SN specimens, which were then stored under appropriate conditions for histomorphological analysis.

Electrophysiological Evaluation

A nerve conduction study was performed to assess nerve regeneration. A device to measure electromyography, nerve conduction velocity, and evoked potential (Sierra Summit Industries, Kennewick, WA, USA) was used to assess electrophysiological function in the test groups and the Control group on day 31 before the sacrifice. Motor nerve conduction velocity, compound muscle action potential (CMAP) amplitude, CMAP area, and distal latency (DL) were evaluated.

Histological Evaluation

Histomorphometric Examination

Transverse SN sections from the experimental groups were stained with Oil Red O and examined under a light microscope (BX51; Olympus, Tokyo, Japan) at 100x magnification, and photographs were taken of 5 randomly selected areas. The number of myelinated nerve sheaths in 40 areas of each group were calculated and recorded using Image J software (US National Institutes of Health, Bethesda, MD, USA). In addition, 1000-axon diameters from each experimental group were calculated using Image J software, and statistical comparisons were performed between the groups.

Immunohistochemical Application

Immunohistochemical staining was performed using the avidin-biotin-peroxidase method to determine S-100 and neurofilament (NF) expression in SN tissue in the study groups. An S-100 (Anti-S100; Invitrogen, Waltham, MA, USA) or NF primary antibody (ab8135; Abcam, Cambridge, UK) was used. Finally, they were examined using a fluorescence microscope (BX51; Olympus, Tokyo, Japan). Image J software was used to calculate the S-100 and NF immunoreactivity intensity in the immunochemical stained SN sections. Light microscopic photographs were taken at 40x magnification from 5 randomly selected regions of each tissue sample.

Statistical Analysis

The data distribution was assessed using a Q-Q plot and the Shapiro-Wilks test. The Kruskal- Wallis test and one-way analysis of variance were applied to compare more than 2 independent groups. Homogeneity of variance was evaluated using Levene's test. Post hoc analysis was applied for multiple group comparisons using the Tukey and Tamhane tests. The mean and SD, 25th-75th percentile values, and median values were calculated as descriptive statistics. A p value of <0.05 was regarded as statistically significant. The analyses were performed using TURCOSA statistical software (Turcosa Analytics, Kayseri, Turkey).

RESULTS

Electrophysiological Findings

A comparison of the DL, CMAP amplitude, CMAP area, and conduction velocity values between the groups is shown in Table 1. A significant difference was detected in the DL values ($p<0.001$) (Table 1). The mean value in the DMSO+NI group was higher than that of the DMSO, Control, RAPA-L, and Sham groups. The

Table 1. Distal latency, CMAP amplitude, CMAP area, and conduction velocity values of the study groups

	DL (ms)	CMAP amplitude (mV)	CMAP area (mvms)	Conduction velocity (m/s)
Control	1.96±0.23[a]	7.39±1.40[a]	7.34±0.56[a]	36.01±3.47[a]
Sham	2.48±0.28[ab]	2.77±2.63[b]	3.05±2.42[b]	28.57±3.21[b]
RAPA-L	2.50±2.29[ab]	5.16±3.28[ab]	4.04±2.88[ab]	28.35±3.53[b]
RAPA-H	2.84±0.62[bc]	5.42±6.08[ab]	4.68±3.17[ab]	25.60±5.27[bc]
GBP	2.81±0.30[bc]	4.25±2.17[ab]	4.53±3.00[ab]	25.17±2.88[bc]
DMSO	2.30±0.28[ab]	6.72±0.44[a]	8.16±2.25[ab]	30.75±3.49[ab]
DMSO+NI	3.40±0.36[c]	1.58±1.16[b]	2.27±0.93[b]	20.75±2.04[c]
p	**<0.001**	**0.048**	**0.002**	**<0.001**

*: The same letter indicates similarities in measurements between groups, while different letters indicate differences between groups. Mean±SD. p: P values for intergroup comparison. One-way analysis of variance test. CMAP: Compound muscle action potential; DL: Distal latency; DMSO: Dimethyl sulfoxide; DMSO+NI: DMSO + nerve injury; GBP: Gabapentin; RAPA-H: Rapamycin high dose; RAPA-L: Rapamycin low dose.

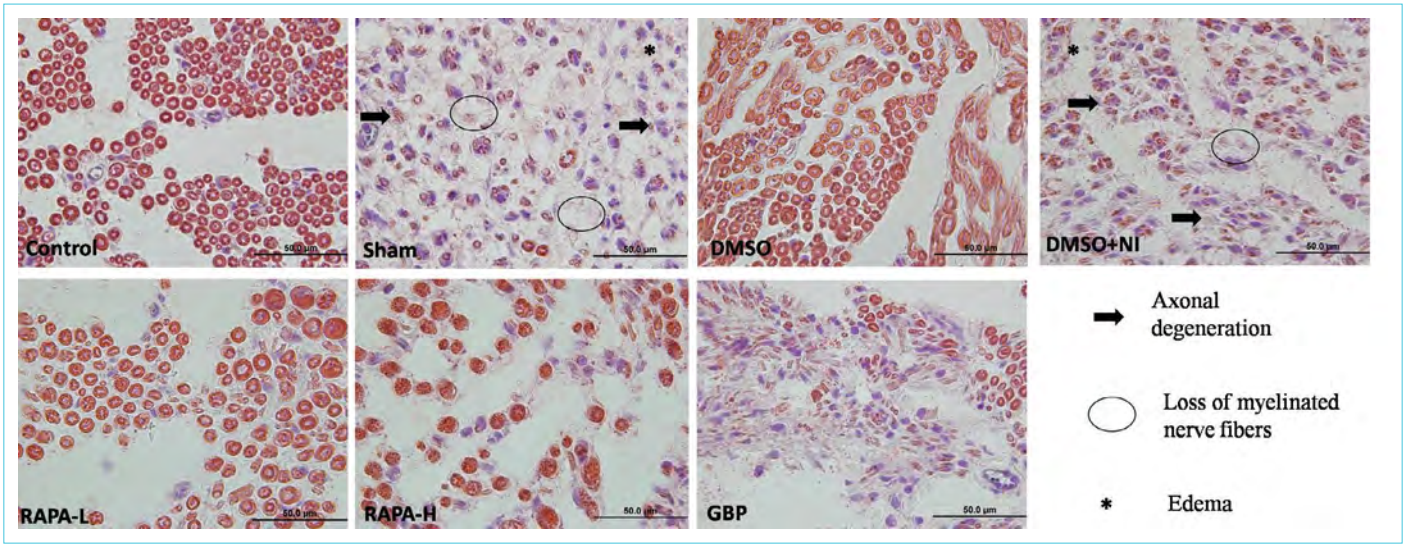

Figure 1. Representative photomicrographs of sciatic nerve sections from the study groups (Oil Red O staining, 100x)
DMSO: Dimethyl sulfoxide; DMSO+NI: DMSO + nerve injury; GBP: Gabapentin; RAPA-H: Rapamycin high dose; RAPA-L: Rapamycin low dose

mean value in the Sham, RAPA-L, RAPA-H, GBP, and DMSO groups was similar, and the mean value in the RAPA-H and GBP treatment groups was very similar. The DMSO group value was close to that of the Control, Sham, and RAPA-L groups.

A threshold of significance was detected between the groups in the mean CMAP amplitude value (p=0.048) (Table 1). The Control and DMSO group results were similar. The Sham and DMSO+NI group values were similar and significantly lower than those of the Control and DMSO groups. The mean RAPA-L, RAPA-H, and GBP group values were closer to that of the Control group but similar to that of the Sham group. There was no statistically significant difference between the mean RAPA-L, RAPA-H, and GBP group values.

A statistically significant difference was also observed between the groups in the CMAP area value (p=0.002) (Table 1). The Control group value was significantly higher than that of the Sham or DMSO+NI group. The mean Control group value was similar to that of the DMSO group. The mean RAPA-L, RAPA-H, and GBP group value was higher than that of the Sham group, but close to that of the Control group. However, there was no statistically significant difference between the mean RAPA-L, RAPA-H, or GBP group value and that of the Control or Sham group (Table 1).

Examination of the conduction velocity values revealed a significant difference in the mean value between the groups (p<0.001) (Table 1). The mean Control group value was similar to that of the DMSO group, but was significantly higher than that of the Sham or DMSO+NI group. The mean RAPA-L, RAPA-H, and GBP group value was similar to that of the Sham group.

Histological Results

Oil Red O-stained SN cross-sections were examined under a light microscope. Images of the SN samples from the Control group, the damage groups, and the GBP, RAPA-L and RAPA-H treatment groups are shown in Figure 1. Histological analysis of control group SN sections revealed an SN with normal appearance consisting of numerous axons bound together by connective tissue and surrounded by Schwann cell sheaths. SN sections from the damage-induced groups exhibited histopathological changes such

Table 2. Comparison of axon diameter, axon number, and neurofilament and S100 intensity values between groups

	Axon diameter	Axon number	NF	S100
Control	6.07±1.78[a]	273.00 (181.00–301.00)[d]	77.80±7.50[ab]	76.62±5.15[ab]
Sham	3.00±1.49[b]	78.00 (18.00–123.00)[a]	73.38±4.41[a]	74.09±4.56[a]
RAPA-L	4.10±1.13[c]	116.00 (62.00–178.00)[ab]	81.57±10.39[b]	78.26±4.27[bd]
RAPA-H	4.06±1.60[c]	179.00 (112.00–224.00)[bc]	73.97±4.66[a]	68.78±4.13[c]
GBP	3.91±2.00[c]	193.00 (179.00–203.00)[cd]	78.00±4.95[b]	80.41±5.95[d]
DMSO	2.49±0.94[d]	201.00 (171.00–254.00)[cd]	76.47±3.45[ab]	76.06±4.72[ab]
DMSO+NI	5.89±1.67[a]	122.00 (99.00–147.00)[ab]	73.53±4.88[a]	77.62±4.57[ad]
p	**<0.001**	**<0.001**	**<0.001**	**<0.001**

*: The same letter indicates similarities in measurements between groups, while different letters indicate differences between groups. Mean±SD and median (25th–75th percentile). p: P values for comparison between groups. The Kruskal-Wallis test and one-way analysis of variance were used for numerical variables. DMSO: Dimethyl sulfoxide; DMSO+NI: DMSO + nerve injury; GBP: Gabapentin; NF: Neurofilament; RAPA-H: Rapamycin high dose; RAPA-L: Rapamycin low dose

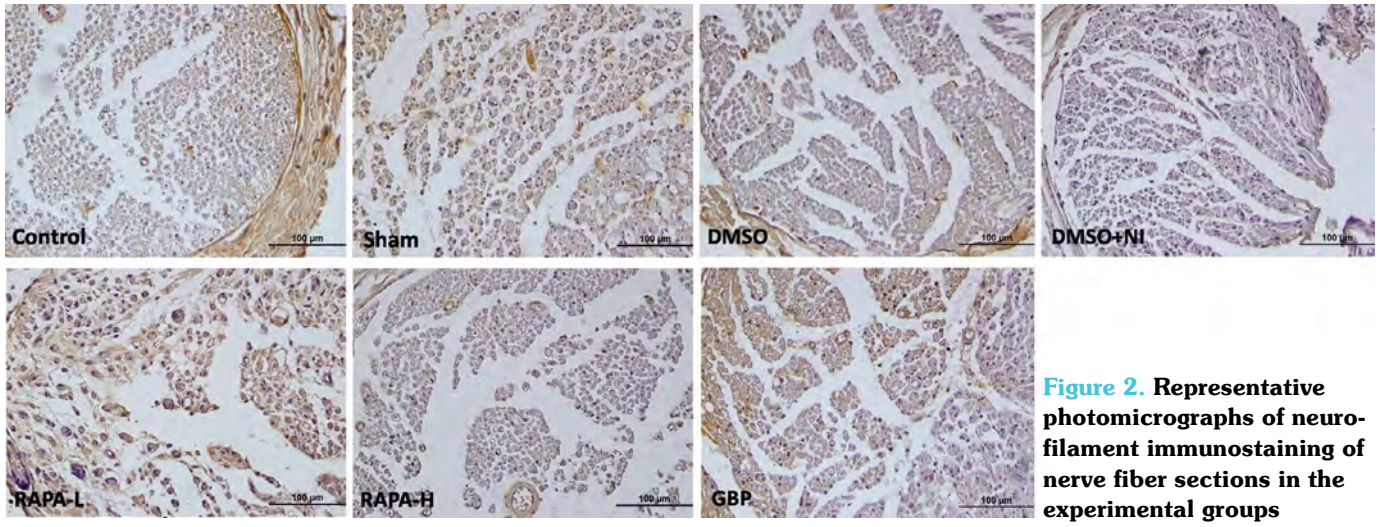

Figure 2. Representative photomicrographs of neurofilament immunostaining of nerve fiber sections in the experimental groups

as axonal degeneration (Fig. 1, arrow), loss of myelinated nerve sheaths (Fig. 1, circle), and edema (Fig. 1, star). The histological appearance of the SNs from the RAPA-L group was similar to that of the Control group. SN sections from the RAPA-H group exhibited a similar appearance to those from the RAPA-L group, but the histopathological changes in the RAPA-L group were better than those of the RAPA-H group. Histopathological findings in the SN of the GBP group were moderate compared with those of the damage-only group.

Histomorphometric analysis was performed following the imaging procedure for all of the SN specimens, including those from the Control group. Relationships between the histologically detected axon number and diameter were determined in the experimental groups (Table 2).

Analysis of the mean group axon diameter revealed significant variation ($p<0.001$) (Table 2). Significant decreases were seen in the Sham and DMSO+NI groups compared with the Control group. The findings in the DMSO group were similar to those of the Control group. Although the axon diameter in the treatment groups (RAPA-L, RAPA-H, and GBP) was similar, each group exhibited a statistically significant increase compared with the Sham and DMSO+NI groups. The RAPA-L group results were similar to those of the Control and DMSO groups. The axon diameter in the DMSO+NI group was significantly less than that of the Sham and treatment groups.

Analysis of the number of axons obtained with Oil red O staining also revealed a significant variation between groups ($p<0.001$, Table 2). The Sham and DMSO+NI groups differed significantly from the Control group, a difference associated with decreases in the Sham and DMSO+NI groups. The DMSO group results were similar to those of the Control group. Analysis of the axon count in the treatment groups (RAPA-L, RAPA-H, and GBP) revealed a higher axon count in the RAPA-L group than in the Sham group, although the difference was not statistically significant. While the axon number increased in the RAPA-H group compared with the RAPA-L group, the difference was not statistically significant. The axon number in the GBP group was significantly greater than that of the RAPA-L group. The axon count in the RAPA-H and GBP groups was similar, but was significantly higher than that seen in the Sham group. The axon number in the RAPA-H and GBP groups was similar to that of the Control and DMSO groups.

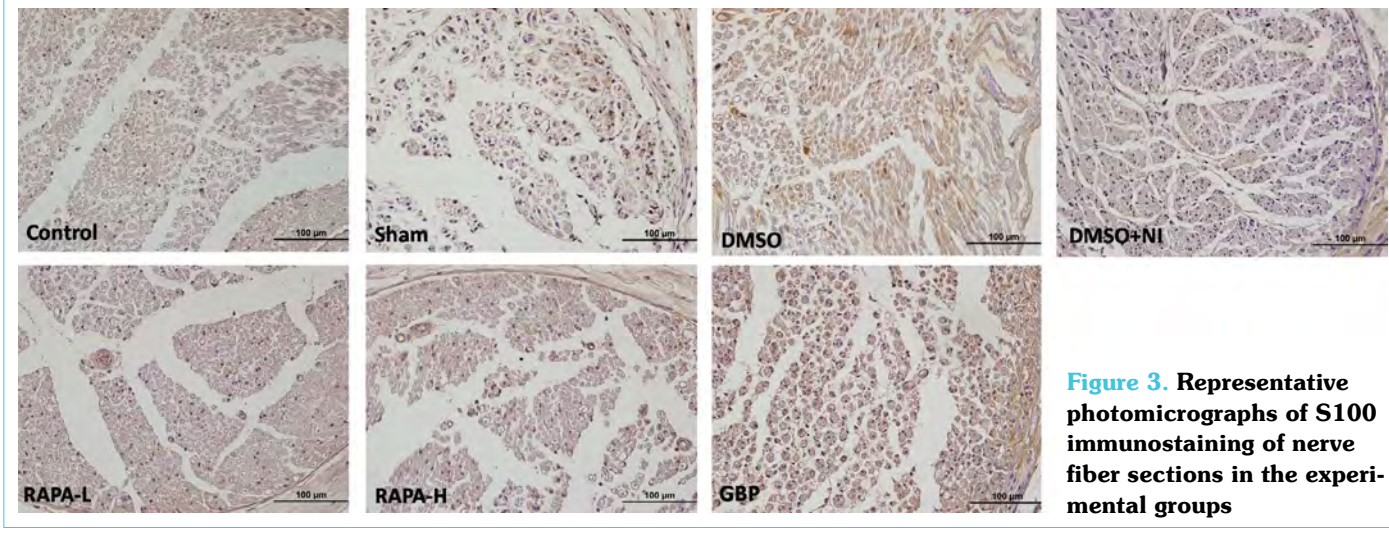

Figure 3. Representative photomicrographs of S100 immunostaining of nerve fiber sections in the experimental groups

Immunohistochemical Findings

SN fibers were visualized via immune staining using the specific markers for NF and S100 (Fig. 2, 3). A significant variation was observed between the experimental groups in terms of the mean NF immunoreactivity intensity ($p<0.001$) (Table 2). The highest NF value was detected in the RAPA-L and GBP groups. While the mean NF value was similar in the RAPA-L and GBP treatment groups, the results from the RAPA-H group were similar to those of the Sham group. The results from the DMSO and Control groups were similar to each other and to the other groups. The Sham, RAPA-H and DMSO+NI group results were all similar. The RAPA-L and GBP group results differed significantly from those of the Sham, DMSO+NI, and RAPA-H groups.

A significant variation was observed between the groups in terms of S100 immune intensity ($p<0.001$) (Table 2). No significant difference was determined between the Control, DMSO, Sham, and DMSO+NI groups. The S100 intensity was similar in the RAPA-L and GBP groups. The S100 intensity in the RAPA-L and GBP groups was significantly higher than that of the Sham group. The S100 intensity was lower in the RAPA-H group than in all the other groups.

DISCUSSION

Traumatic PNI is an important public health issue, as it can lead to important functional loss and permanent disability (1). Despite advanced microsurgical repair techniques, complete functional healing is not achieved in the majority of patients with PNI (1). Numerous drugs have been used as pharmacological treatment in both nerve injury models and neuropathic pain models in the literature (1, 10–12).

GBP and pregabalin are frequently used as first-line medications to treat cancer- and chemotherapy-related pain, trigeminal neuralgia, diabetic neuropathic pain, and post-herpetic neuralgia (10–12). An intraperitoneal GBP dosage of 90 mg/kg for 30 days in the GBP group was used in this study based on the information provided in the literature (13–15).

In the PNS, Schwann cells play an important role in various processes, such as the repair of degeneration, remyelination, and axonal growth (1). The mTOR pathway is a cellular signaling pathway involved in a variety of significant physiological functions, such as cell growth and proliferation, protein synthesis and metabolism, and autophagy (16).

The effect of the mTOR pathway on the regeneration of neuronal axons in the PNS remains unclear. Huang et al. (4) found that in contrast to the PNS, in the CNS, the mTOR pathway has an important role in regulating the regenerative capacity of neurons, and that mTOR activity exhibited different effects in the CNS and the PNS.

Ding et al. (17) evaluated the effect of slow-release RAPA-coated bionic peripheral nerve scaffold (tissue scaffold) on post-injury regeneration in rat SN injury. The histological examination revealed axon regeneration in terms of mean nerve fiber diameter and mean myelin sheath thickness in their examination of nerve conduction and CMAP using electrophysiology. The authors concluded that RAPA significantly reduced the inflammatory response in the injured region.

Huang et al. (18) used RAPA at a dosage of 1 mg/kg in rats with induced SN injury and reported that RAPA treatment increased the number of autophagosomes and LC3-II expression and reduced the number of apoptotic cell numbers in the lesion. They observed that autophagy modulation in PNI might be an effective pharmacological approach for nerve regeneration and may contribute to the reacquisition of motor function.

Liu et al. (19) reported that low-dose RAPA (1.53 nmol/L) increased Schwann cell migration at least as much as 100 μmol/L (high-dose) FK506. Hadamitzky et al. (20) defined a low dose in their research as 3 mg/kg. Liu et al. (21) used RAPA at a dose of 2, 4, and 6 mg/kg. Based on the literature, in the present study, we defined 3 mg/kg as a low dose and 6 mg/kg as a high dose when evaluating the dose-related effects of RAPA.

Electrophysiological tests are currently the most sensitive and specific tests to evaluate traumatic PNI (1, 22, 23). Karşıdağ et al. (22) observed that while histological parameters such as axon number and diameter describe the quantity and quality of axons, they do not provide sufficient information about functional status. They

stated that electrophysiological studies can provide useful data immediately following PNI and in the healing period, including parameters such as amplitude, latency, and conduction velocity (22) (23). The present study also investigated both histomorphological and electrophysiological findings in rats with a SN injury. The DL, CMAP amplitude, CMAP area, and conduction velocity values were analyzed using electrophysiology (Table 1).

The general electrophysiological findings of this study indicated that the CMAP amplitude and area, and conduction velocity in the Sham group were lower than those in the Control group (Table 1). This finding demonstrates that the experimental damage model was successfully established. The DL, CMAP amplitude and area, and conduction velocity values were similar between the treatment groups (RAPA-L, RAPA-H, and GBP). The highest conduction velocity among the treatment groups, and the closest to the Control group, was observed in the RAPA-L group. This finding suggests that RAPA-L and RAPA-H treatment yielded similar results to GBP treatment, and that low-dose RAPA treatment in particular may be considered an alternative to GBP.

Electrophysiological findings; morphometric findings, such as axon diameter and count; and anti-S-100, as an immunohistochemical marker, have been used to evaluate SN damage induced in rats (24). NFs are essential for the radial growth and structural stability of myelinated axons and for electrical signals that are passed along axons to reach maximum velocity. NFs increase conduction velocity in large myelinated fiber (25).

When the histological findings in this study were evaluated using a light microscope, the histological appearance of SN cells from the RAPA-L group was close to that of the Control group. The RAPA-H group SN sections exhibited a similar appearance to that of the RAPA-L group, although the histopathological findings were better in the RAPA-L group. In the GBP group, the SN histopathological findings were moderate compared with those of the damage group.

Analysis of histomorphometric findings revealed that they were similar between the treatment groups (RAPA-L, RAPA-H, and GBP) and statistically significantly higher than those seen in the Sham group. The RAPA-L group had the highest axon diameter values among the treatment groups. Similarly, the axon number was markedly lower in the Sham group than in the Control group, while the axon number in the RAPA-H and GBP treatment groups was significantly higher than that of the Sham group. The best axon number among the treatment groups, and the value closest to that of the control group, was seen in the GBP group, while the lowest was observed in the RAPA-L group. The RAPA-H group results were similar to those of the GBP group in terms of both axon diameter and number (Table 2).

The S-100 protein level is known to decrease in the event of nerve damage and can be illustrated using immunohistochemical staining (26, 27). Since S100 is a Schwann cell marker in peripheral nerve tissue and S100-negativity in damaged nerves is a finding of nerve damage, S100-positivity is regarded as a positive marker of nerve regeneration (27). S100 expression as a Schwann cell marker has been evaluated in many studies. It has been reported that Schwann cell regeneration plays a critical role in post-injury nerve regeneration, and that S100 protein signal expression is basically present in the myelin sheath (24, 28, 29). Kato and Satoh (30) used the immunoassay method to analyze S100 and enolase levels in an induced SN-damage model in rats. Measurements were performed during the damage period and 4-9 weeks after nerve healing, and were higher after healing (0.63 µg/mg vs. 0.36 µg/mg). This suggests that S100 positivity may be a marker of healing in nerve fibers.

In terms of the general immunohistochemical analysis findings in this study, the NF and S100 immunoreactivity intensity revealed similar expression in the Control, Sham, DMSO, and DMSO+NI groups. The NF and S100 immunointensity of the RAPA-L group was similar to that of the GBP group. The NF expression in the RAPA-H group was similar to that of the Sham group, while the RAPA-L and GBP group values were closer to that of the control group. The lowest S100 immunointensity value was obtained in the RAPA-H group; the results of the RAPA-L and GBP group were similar. The S100 immunointensity in the RAPA-L group was closer to that of the Control group (Table 2). Combined analysis of the histological and light microscopy findings showed that low-dose RAPA yielded results similar to GBP treatment, suggesting that it may be suitable alternative therapy.

In conclusion, analysis of electrophysiological and histological findings indicated that low-dose RAPA treatment yielded promising results in rats with an induced SN injury and it therefore may be an alternative to GBP in cases of peripheral nerve damage.

Acknowledgements: This study was supported by the Erciyes University Scientific Research Unit under project no. TSA-2018-7853. We are grateful to the Erciyes University Scientific Research Unit for its support. We also wish to thank Erciyes University Pharmacy Faculty Pharmaceutical Technology Department Research Assistant Sedat Ünal for his contribution to the preparation of the effect agent formulations, and to the director and staff of the Erciyes University Experimental Research and Application Center (DEKAM) for their constant support during the experimental research.

Ethics Committee Approval: The Erciyes University Animal Experiments Local Ethical Committee granted approval for this study (date: 13.12.2017, number: 17/136).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – – MC, DGC, SK; Design – MC, HAÇ, DGC; Supervision – AHY, MC, DGC; Materials – HAÇ, GÖÖ, AHY; Data Collection and/or Processing – AHY, GÖÖ, MFY; Analysis and/or Interpretation – GEZ; Literature Search – MC, MFY, DGC; Writing – MC, DGC, AHY, GEZ; Critical Reviews – MC, DGC, SK.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: This study was supported by the Erciyes University Scientific Research Unit under project no. TSA-2018-7853.

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