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HEAD AND NECK

Effects of chitosan and platelet-rich plasma on facial nerve regeneration in an animal model

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Abstract

Purpose There is still no widely-accepted local agent proven to be effective in nerve regeneration. We aimed to investigate the effects of chitosan gel and platelet-rich plasma

Materials and methods Electrophysiological measurements were performed before and immediately after injury. The injured nerves were covered with spongostan impregnated with the following agents: Group 1 (Control Group): Saline at a dose of 50 µl; Group 2: Chitosan (CHT) at a dose of 50 µl; Group 3: PRP at a dose of 50 ml; and Group 4: a solution of CHT with PRP (1:1). The final measurements were performed after 3 weeks and the injured nerve of each rat was removed.

Results There were statistically-significant differences between the groups regarding the measurements of the after-treatment values of stimulus threshold ($p < 0.05$). The best improvement in electrophysiological measurement and histopathological evaluation was found in Group 4 (CHT-PRP).

Conclusion Chitosan gel has a positive effect on nerve healing and applying it along with PRP can enhance the effect of chitosan.

Keywords Chitosan · Platelet-rich plasma · Facial nerve · Regeneration · Rat model

Introduction

The facial nerve stimulates the muscles that control the facial movement. Facial expression is very important both aesthetically and functionally. Permanent or transient facial paralysis may occur in various pathological conditions [1, 2]. This undesirable condition often leads to asymmetry of the face, a decrease in the patient's ability to transmit emotions, and severe mood disorder. Following Bell's palsy (idiopathic), the second most common cause of facial paralysis is trauma [3]. Although many methods have been used to manage traumatic facial nerve injury, functional recovery is often poor [4].

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Platelet-rich plasma (PRP) is prepared by centrifugation of the patient's own blood [5]. PRP consists of various neurotrophic factors such as neurotrophin-3 (NT-3), angiopoietin-1, glial cell line-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF) [6]. The role of these factors in nerve regeneration has been investigated and their positive effects on nerve healing have been emphasized [6–9].

Chitin is a biopolymer commonly found in the exoskeletons of arthropods, the shells of crustaceans, and insect cuticles [10, 11]. Chitosan (CHT) is obtained by the partial deacetylation of chitin. Chitosan has been used in gels, micro- or nanoparticles, and films [12]. The most basic and cost-effective form of CHT is the gel form obtained after dissolution in acid [13]. Chitosan has been frequently used in cell culture, tissue engineering, and gene delivery [14]. Chitosan exhibits anti-inflammatory effects by reducing neutrophil infiltration and levels of tumor necrosis factor alpha (TNF-α) and interleukin-1b (IL-1b) in serum [15]. In some studies, antioxidant properties of CHT and its beneficial effects on wound healing have been demonstrated [15, 16].

The aim of this study is to investigate the effectiveness of the gelatin sponge impregnated with CHT-gel and PRP in facial nerve regeneration using a rat facial nerve model. This enriched sponge containing properties of neuromodulator, neuroprotection, stem cell, and growth factor can easily be prepared and applied. Assessment of nerve regeneration was performed through electrophysiological measurement and histopathological evaluation.

Materials and methods

The study was approved by Ethics Committee for Animal Experiments. Twenty-four healthy male Wistar albino rats, each weighing 250–300 gr and aged 6 months, were used in the study. All animals were housed in an air-conditioned room at constant temperature (18–20 °C) with a 12-h light/dark cycle and given food and water ad libitum throughout the experiment. No experimental animal loss occurred in the study groups.

Preparation of PRP

Platelet-rich plasma was obtained through a simplified closed centrifugation system according to the method described by Kovacs et al. [17]. Five rats were sacrificed serving as a source of blood. From the heart of each of these rats, 5 ml of fresh blood treated with 10% sodium citrate was obtained in a sterile tube. After obtaining the blood, a two-step centrifugation (200 g/min for 10 min and 300 g/min for 15 min) was performed.

Preparation of CHT gel

Chitosan from shrimp shells (75% deacetylated, at around 200cP viscosity) was purchased from Sigma Aldrich, Germany. Glacial acetic acid was obtained from Fischer Scientific. 200 mg of chitosan powder was dissolved in 6 ml of glacial acetic acid (0.05% v/v) with vigorous stirring. This solution was kept at room temperature overnight and then brought to pH 6–7 with sodium hydroxide. The final solution was sterilized by filtration through a polytetrafluoroethylene (PTFE) membrane filter (0.22 µm, 47 mm). 50 µl of chitosan formulation was absorbed into the spongostan (0.5 × 0.5 × 0.1 cm, Spongostan®; Ethicon Inc., Somerville, NJ, the USA).

Experimental methods

The rats were randomly divided into four groups to include six rats in each group. The rats were anesthetized with intraperitoneal injections of ketamine at a dose of 100 mg/kg and chlorpromazine at a dose of 3 mg/kg. The regions corresponding to the trace of the right facial nerve were shaved after administration of anesthesia and then painted with povidone-iodine. A 2 cm horizontal skin incision was performed from the anterior part of the external auditory meatus toward the level of the first whisker at the corner of the mouth. The buccal branch of the facial nerve was exposed after passing the skin and subcutaneous tissues and the first measurement was performed in this way (before injury). Then, the buccal branch of the facial nerve of each rat in all groups was traumatized by a vascular clamp with a single click that continued for 20 s. After the trauma, nerve stimulus thresholds were determined in these groups again from the proximal part of the traumatized section by using the same method mentioned above (after injury). The same standard procedures were performed in all 24 rats. Immediately after the second measurement, damaged nerve segments were covered with spongostan impregnated with the following agents: Group 1 (Control group): Saline at a dose of 50 µl; Group 2: CHT at a dose of 50 µl; and Group 3: PRP at a dose of 50 µl. For Group 4, PRP was prepared by dissolving CHT gel in glacial acetic acid in a ratio of 1:1. While in an ice bath, PRP was added dropwise to the chitosan gel solution in a CHT to PRP molar ratio of 1:1 and with isotonicity close to that of extracellular fluid. A formulation of 50 µl of CHT plus 50 µl of PRP was absorbed into the Spongostan®.

In all groups, the incision site was closed appropriately with sutures after surgery. Monofilament polydioxanone sutures (7–0) (PDS II; Ethicon, Germany) were then used in the subcutaneous tissue near the destroyed section of the facial nerve to mark the area for the follow-up surgery. At the end of the third week, the buccal branch of the facial

nerve was found under anesthesia in all groups and stimulus thresholds of the buccal branch of the facial nerve were determined by means of nerve integrity monitoring using the same method (after treatment). Then, the damaged nerve segments of all animals were removed for histologic evaluation.

Electrophysiological assessment

A nerve integrity monitor (Medtronic NIM-Response 3.0 System) was used for electrophysiological monitoring. One of the intramuscular needle electrodes of the nerve integrity monitor was placed in the orbicularis oris muscle and the grounding electrode was placed in the sternocleidomastoid muscle (Fig. 1). Nerve stimulation was performed from the proximal part of the traumatized section. Nerve stimulus thresholds beginning from 0.05 mA of all animals in each group were determined by means of nerve integrity monitoring. The intensity of the stimulus was gradually increased until contraction in the facial expression muscles and wave form on the screen of the device were observed. Measurements were performed three times: before injury (BI), after injury (AI), and after treatment (AT).

Histopathological evaluation

All rats were examined 3 weeks post-surgery and anesthetized by the same technique. The previously-cut skin was incised in the same region and the destroyed site was identified by locating the previously-implanted sutures. The nerve was dissected from the adjacent tissues and released. The buccal branch of the facial nerve including regions 5 mm distal to the destroyed region was then excised. Tissue samples were fixed with 10% neutral formaldehyde. After routine tissue processing, samples were embedded into paraffin.



Fig. 1 Buccal branch of facial nerve (→), parotid gland (- - - →) and placement of electrodes

4 µm serial sections were stained with hematoxylin eosine. Blinded examinations were conducted by two histologists via DM4000 Leica Germany Image Analysis Systems. Vacuolisation and axonal degeneration were scored from 1 to 4 (1: minimal, 2: mild, 3: moderate, 4: severe). Nuclei and axons per area (mm^2) were assessed via ImageJ software, National Institutes of Health (NIH) [18].

Statistical analysis

Statistical analyses were made by the use of IBM SPSS Statistics for Windows, version 20.0 (Armonk, NY: IBM Corp.). Continuous variables were tested for normality by the Kolmogorov–Smirnov test. Normally distributed data are presented as mean ± standard deviations. The rates and proportions of discrete variables were calculated using the Chi-squared test. For data not normally distributed, median with data range (minimum to maximum) was used. The Wilcoxon test was used to compare the change in latency and amplitude. One-way ANOVA was used to compare normally-distributed variables. The Kruskal–Wallis test is used for comparing ordinal and non-normal variables for more than two groups. The independent samples t-test and Mann–Whitney *U* test were used to test the significance of pairwise differences using Bonferroni corrections to adjust for multiple comparisons.

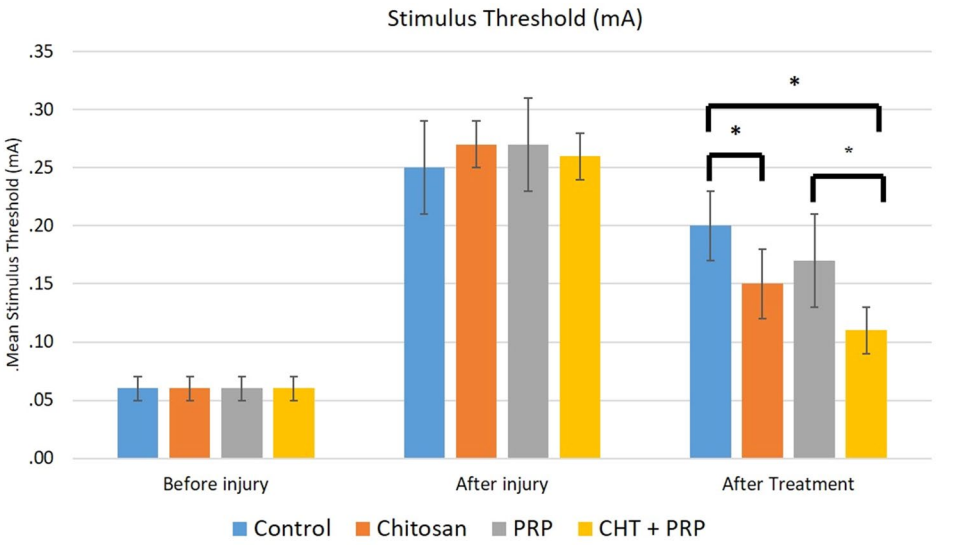
Results

Electrophysiological results

Subjects in each experimental group were evaluated separately using a standard method. All the results and changes regarding nerve stimulus thresholds are shown in Fig. 2. When the groups were compared with each other in terms of the stimulus threshold values determined before injury (BI), no statistically-significant difference was found ($p > 0.05$). In addition, there was no statistically-significant difference between the groups regarding the measurement of after injury (AI) stimulus threshold values ($p > 0.05$) (Fig. 2).

After treatment, no statistically-significant difference was determined between the measurements of AI and AT in the control group (p; $p > 0.05$). There is a significant difference between the measurements of AI and AT in the groups CHT, PRP, and CHT + PRP ($p < 0.05$).

When the groups were compared with each other after treatment, there was statistically-significant difference between the groups regarding the measurements of after-treatment stimulus threshold values. There were statistically-significant differences between group 1 (Control) and group 2 (CHT) ($p = 0.03$), group 1 (Control) and group 4 (CHT + PRP) ($p < 0.01$), group 3 (PRP) and group 4



	Before injury	After injury	After Treatment
Control	0.06±0.01	0.25±0.04	0.2±0.03
Chitosan	0.06±0.01	0.27±0.02	0.15±0.03
PRP	0.06±0.01	0.27±0.04	0.17±0.04
CHT + PRP	0.06±0.01	0.26±0.02	0.11±0.02

Fig. 2 Distributions of measurements of stimulus thresholds according to the groups. Statistically-significant differences between groups were signed with stars ($p < 0.05$)

(PRP + CHT) ($p = 0.01$). The best improvement in electrophysiological results was found in the CHT + PRP group (Fig. 2).

Histopathologic results

The facial nerve and surrounding muscle tissue were observed. Axonal degeneration and vacuolization were prominent in Group 1 (Control) and minimal in Group 4 (CHT + PRP) ($p = 0.010$ for axonal degeneration and $p = 0.012$ for vacuolisation) (Fig. 3). Inflammatory cell infiltration was assessed around this material. But infiltration did not spread in most of the cases. For the control group, cellularity was not as high as it was in each of the experimental groups (Fig. 4). Axon number per area also increased in the experimental groups. Group 4 had statistically-significant higher amount of axon than Group 1 and Group 3 each had ($p = 0.013$ and $p = 0.030$, respectively) (Fig. 5).

Discussion

The present study has two major findings: (1) The sponge impregnated with chitosan gel and PRP showed the best improvement in electrophysiological measurements. (2) Chitosan gel with PRP group (Group 4) were effective for reducing axonal degeneration and vacuolisation. Cellularity and axon/area ratio were the highest in this group. Chitosan and PRP could increase the regeneration ability of the nerve.

The facial nerve controls the facial muscles responsible for crucial aesthetic and functional tasks. Besides, it is responsible for hearing, taste sensation, and saliva production [3]. Facial paralysis may occur in various conditions such as surgery and iatrogenic or idiopathic diseases [3, 4, 19]. Oxidative stress following the axonal injury is considered to be the main mechanism leading to the neuronal damage. During post-traumatic regeneration, inflammation can cause fibrosis and scar formation in the nerve tissue [19–21]. Agents with antioxidant and anti-inflammatory properties may increase peripheral nerve regeneration after trauma. Therefore, the effectiveness of many different agents such as aminoguanidine, melatonin, Vitamin E, calcium channel inhibitor, and *N*-acetylcysteine have been investigated for the treatment of traumatic peripheral nerve injury [2, 22, 23]. Moreover, treatment methods that exhibit stem cell function and induce growth factors have been used in nerve healing [5–9, 14]. However, there is still no accepted treatment method and effective and safe agents for nerve healing are required. Thus, experimental studies to provide better functional and cosmetic recovery in the regeneration of the traumatic peripheral nerve are in progress.

Due to their strong anti-inflammatory and antioxidant properties, corticosteroids are the most commonly used agents for traumatic facial nerve palsy. In addition, they are able to reduce edema and nerve compression

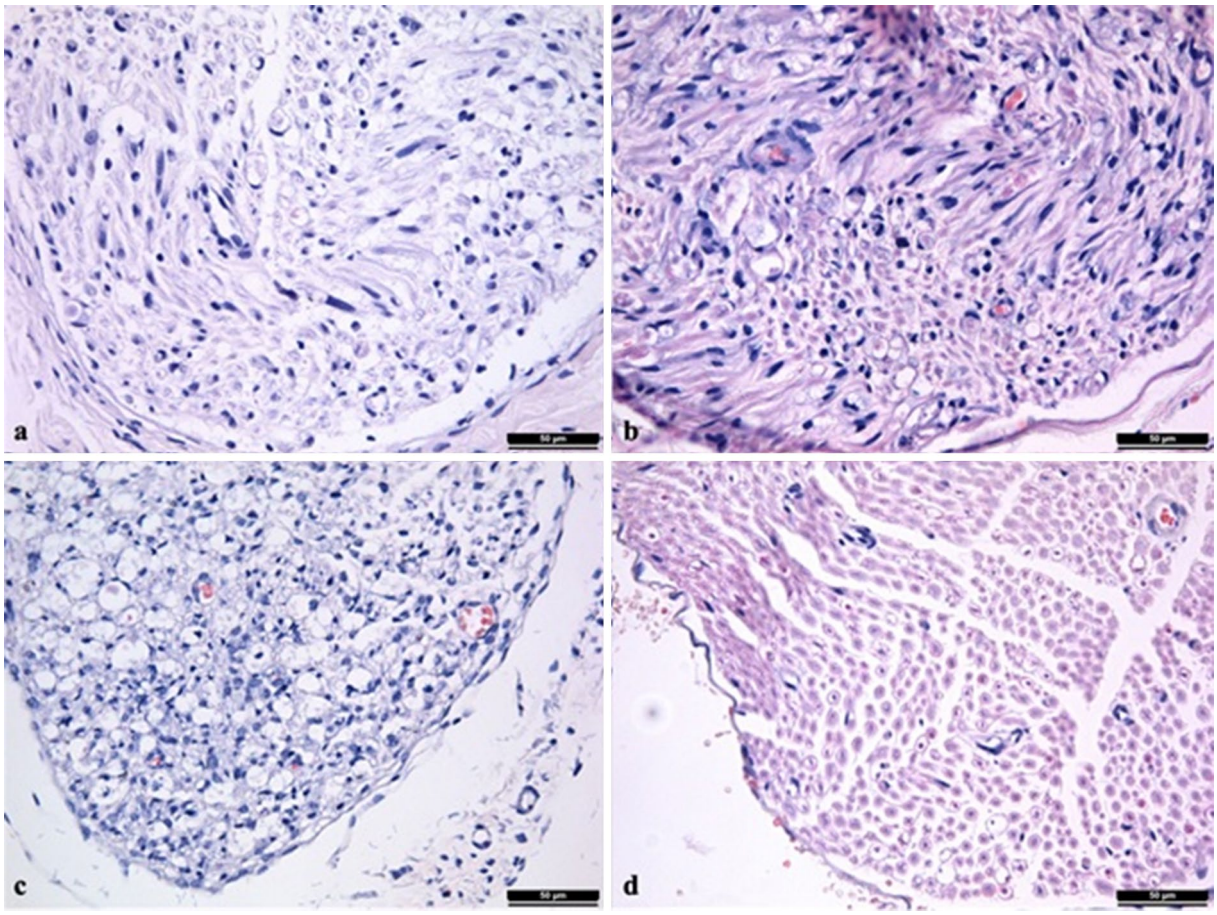


Fig. 3 Transverse sections of distal ends of facial nerves, hematoxylin and eosin stain. (**a** Control Group, **b** CHT Group, **c** PRP Group, **d** CHT + PRP Group)

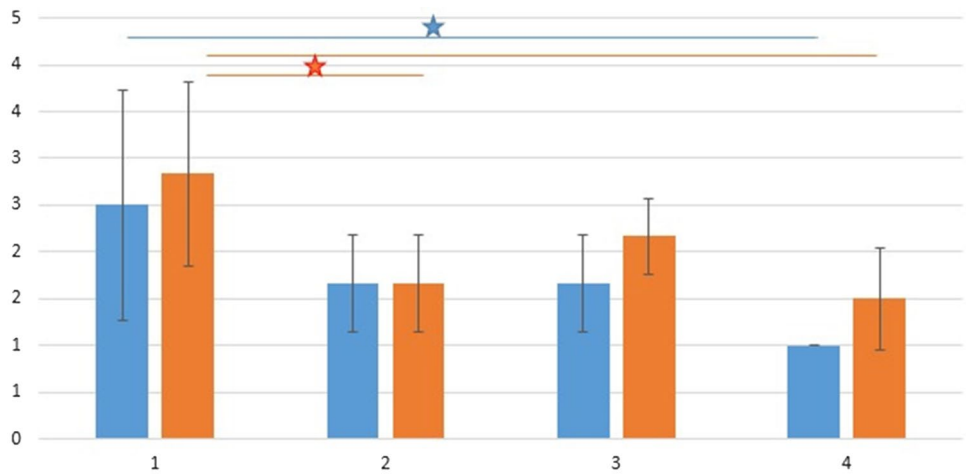


Fig. 4 Graph of axonal degeneration (blue bar) and vacuolisation (orange bar) (Mean ± SD). Group numbers were shown on the x-axis. (1: Control Group, 2: CHT Group, 3: PRP Group, 4: CHT + PRP Group). Statistically-significant differences between groups were signed with stars ($p < 0.05$)

by reducing capillary permeability [20]. Reduced fibrosis and oxidative damage lead to an increase in axonal regeneration and myelin sheath thickness [15]. However, some studies have shown that corticosteroids do not have a beneficial effect on nerve healing, not even on increased degeneration [2, 23]. Since systemic corticosteroids have marked side effects such as hyperglycemia and hypertension, numerous agents have been tested by comparing them with corticosteroids in nerve healing [7, 20, 23, 24]. Although there are handicap and confusion about systemic

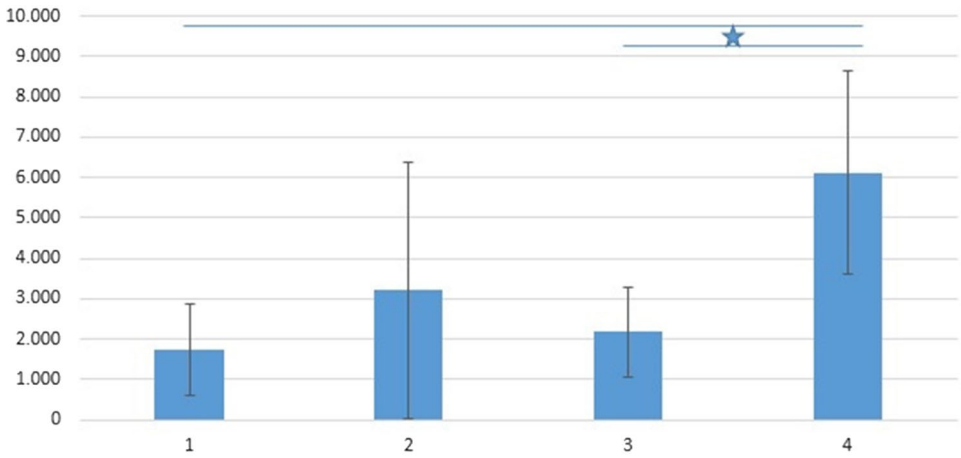


Fig. 5 Graph of axon/area ratio (Mean ± SD). Group numbers were shown on the *x*-axis. (1: Control Group, 2: CHT Group, 3: PRP Group, 4: CHT + PRP Group). Statistically-significant differences between groups were signed with stars ($p < 0.05$)

therapy in nerve healing, corticosteroids are still the most widely used agents for systemic treatment [24].

In our opinion, local therapy for traumatic nerve injury is important because it has minimal side effects and keeps systemic therapy in reserve for a synergistic effect. Traumatic nerve injury is not a rare problem in otolaryngology-head and neck surgery because of the intense nerve network in the head and neck region and facial nerve trauma, in particular, may lead to catastrophic consequences [25, 26]. The most common surgical trauma concerning the facial nerve occurs during the operation of the parotid gland. Identifying and exposing the facial nerve is an important surgical step that should be applied in almost all parotid gland surgeries and nerve trauma encountered during surgery is observed most frequently at this stage. This trauma can be in the form of cutting, stretching or crushing of the nerve. In this study we preferred to stimulate the crush injury model, in which it was much easier to standardize the damage in all experimental animals. Serious functional and cosmetic problems that affect half of the face may appear in case of any damage in the facial nerve [25].

Except for the case of sudden nerve damage, some techniques of facial nerve micro-anastomosis such as end-to-end, hypoglossal-facial, and cross-face facial nerve are also performed in otolaryngology for facial reanimation [27]. Agents shown to be effective can be applied locally to these anastomosis regions to enhance nerve recovery.

Platelets contain various growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-B, and vascular endothelial growth factor. These factors play diverse biological roles. It has been demonstrated that neurons express PDGF receptors and PDGF-B is a mitogen and survival factor for Schwann cells [28]. Moreover, increased PDGF-B expression in peripheral neurons has been seen after peripheral nerve injury and it has been concluded that PDGF-B plays a significant role in peripheral nerve regeneration [29, 30].

Chitosan stimulates the CCL2 expression by down-regulating the miR-327 of the Schwann cells, resulting in an increased macrophage accumulation in the damaged region [31]. Moreover, anti-apoptotic effects of chitosan on the Schwann cells occurred by means of decreasing caspase-3, -9, and Bax activities and increasing Bcl-2 activities have been observed. He et al. [32] analyzed the antioxidant effect of chitosan and observed that chitosan led to a reduced activity of malondialdehyde as well as an increase in superoxide dismutase activities. Decreased oxidative damage has a protective effect on the Schwann cells and affects nerve healing positively. In another experimental study on rabbits, daily chitosan administration was shown to be effective in nerve healing. The number of myelinated nerve fibers regenerated, the myelin sheath thickness, as well as the compound muscle action potential (CMAP) as the parameter for electrophysiological recovery, were found to be significantly higher in the chitosan-administered group. It was concluded that chitosan not only accelerated the peripheral nerve regeneration but also provided a neuroprotective effect [33].

The use of chitosan as a scaffold for nerve repair has yielded positive results in experimental studies. Recently, chitosan hollow nerve guides have been approved for clinical use in case of deficiency at the donor site [33, 34]. Moreover, the effects of neurotubes enriched with certain substances, e.g., laminin, growth factors (GDNF), or cells (bone marrow stem cells—BMSCs), on peripheral nerve regeneration have been investigated [7, 35, 36]. However, in sudden nerve injuries during surgery and in the most common nerve anastomosis techniques such as end-to-end, hypoglossal-facial, and cross-face facial nerve, a nerve conduit is not generally necessary because it is easy to obtain autogenous graft in the head and neck region [25, 26]. There is a specific need for an effective and easily-accessible agent to apply locally onto the damaged region. Thus, we investigated the effect of gelatin

sponge impregnated with chitosan gel and PRP on peripheral nerve regeneration using a rat facial nerve model. Functional recovery is often poor not only in facial nerve injury but also in all peripheral nerve injuries. Different peripheral nerves are at risk of injury due to surgical or iatrogenic trauma in almost all surgical departments.

Conclusion

Since they cause minimal side effects and the possibility to use systemic treatments to have a synergistic effect is held in reserve, the local agents effective in nerve regeneration is of high importance. It has been shown in this study that chitosan gel has a positive effect on nerve healing and applying it along with PRP can intensify the effect of chitosan. This enriched sponge with its minimal side effects may be an efficient and simple instrument for nerve regeneration.

Author contributions MMŞ: Study concept and design, experiment practice and administration of agents, drafting of the manuscript. MÇ: Study concept and design, statistical analysis, drafting of the manuscript. SKD: Study concept and design, technical support, statistical analysis. EÖ: Histologic evaluation, technical support. Mİ: Preparation of agents, experiment practice and administration of agents, technical support. EU: Statistical analysis, drafting of the manuscript, technical support. ÇE: Histologic evaluation, critical revision of the manuscript for intellectual content. MY: Study concept and design, critical revision of the manuscript for intellectual content. EA: Study concept and design, preparation of Agents, experiment practice and administration of agents, critical revision of the manuscript for intellectual content.

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Availability of data and materials All data are presented in this manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by Ethics Committee for Animal Experiments.

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