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ORIGINAL STUDIES

Chitosan oral administration stimulates regeneration after experimentally induced peripheral nerve injury

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Abstract

Background. Peripheral nerve injuries represent a challenging medical problem regarding rehabilitation and finding an optimal treatment method. Currently, various drugs are used for the symptomatic treatment of peripheral nerve injuries (such as pain medication, non-steroidal anti-inflammatory drugs, vitamins etc.) with inconsistent or short-term results, without providing an efficient recovery. Recently, the research has focused on different natural substances, such as chitosan, for the treatment of peripheral nerve injuries.

Aims. The present research analyzed the effects of chitosan oral administration on an experimentally induced peripheral nerve injury.

Methods. In the present applicative study, a peripheral nerve injury was induced on sixteen white male Wistar rats, divided into two equal groups. The effects of chitosan were examined during 21 days, compared to the control group, by assessing the following parameters: sciatic functional index (SFI), total body weight of the animal, pain-like behavior, serum nerve growth factor (NGF) and interleukin-6 (IL-6) levels, and also by histological studies.

Results. The obtained results were statistically evaluated using different methods (*t*-test, Bonferroni correction, GraphPad Software, ANOVA, Mann–Whitney U test), with the *p*-value significance level set at $p<0.05$. The animals treated with chitosan had a statistically significant functional improvement, compared to the control group regarding all investigated parameters and it was confirmed by the histological studies.

Conclusions. The present research suggests that chitosan administered orally can become an optimal conservative treatment method for peripheral nerve injuries, but more studies are needed to confirm these results.

Keywords: functional rehabilitation; peripheral nerve injury; chitosan.

Abbreviations: IL-6 - interleukin-6; NGF - nerve growth factor; PNI - peripheral nerve injury; SFI - sciatic functional index.

Introduction

Peripheral nerve injuries (PNI) are, in many cases, difficult to treat, especially from the rehabilitation point of view, and patients undergo long-term difficulties in their daily life activities due to different degrees of motor impairment, chronic or even cortical pain, sensitivity disorders and also psychological problems.

Nowadays, for complete nerve injuries, surgery represents the elective treatment method (Chen et al., 2007), but when it is not possible, conservative treatment is prescribed, usually involving pain medication and a physical rehabilitation program. However, rehabilitation results are inconsistent, incomplete and unpredictable in most cases that involve motor impairment (Jung et al., 2014). Therefore, finding a viable conservative treatment

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option, especially for these types of patients, represents an important research aspect.

After the onset of a peripheral nerve injury, Schwann cells (SCs) deliver neurotrophic factors and attract macrophages to the injury site (Vargas & Barres, 2007; Menorca et al., 2013), like nerve growth factor (NGF), which preserves the regenerating microenvironment for axonal elongation (Li et al., 2020). From the neurotrophin family, NGF is essential for synapse maturation and plasticity, axon targeting and neuron growth (Autry & Monteggia, 2012), stimulating axon regeneration and enhancing electrophysiological and histomorphological parameters after nerve injury (Kemp et al., 2011). In oral administration, NGF stimulates peripheral nerve regeneration (Kemp et al., 2011) by reducing the remyelination time and by enhancing the myelinated nerve fiber diameter (Li et al., 2020).

Another factor implicated in nerve regeneration is interleukin-6 (IL-6), a glycoprotein with pro-inflammatory activity, which increases the activation of T-cells and acts as a neurotrophic factor for both dopaminergic and cholinergic neurons, resulting in protection of the neurons and configuration of pain (Fregnan et al., 2012).

Over recent years, different natural compounds have been studied for the treatment of PNI, such as chitosan, obtained by deacetylation of chitin, part of the crustacean exoskeleton. Studies showed that chitosan protects the neurons, preventing Schwann cell apoptosis without any inflammatory responses (Baldrick, 2010). Recent studies demonstrated that chitosan, as part of different nerve tubes, enhances the number of axons (Chen, 2019) and provides good peripheral nerve recovery, especially regarding small nerve defects (Dietzmeyer et al., 2020). The chitosan-based nerve tube (Reaxon® Nerve Guide developed by Medovent GmbH, Mainz, Germany) was produced in 2014 and has been used with good results in the treatment of small nerve defects in experimental studies, being currently evaluated for human use (Bąk et al., 2017).

Boecker et al. noticed in their review the stimulatory effects of chitosan nerve tubes on Schwann cells and axonal regeneration, the neuroglial affinity and reduced toxicity (Boecker et al., 2019). Previous studies showed that chitosan restored axonal excitability and function, decreased the appearance of post-operative neurinoma, increased cell affinity for neurons (Boecker et al., 2019), provided a substrate for SC survival and oriented growth (Yuan et al., 2004), stimulated neuronal cell survival and differentiation (Freier et al., 2005; Simoes et al., 2011).

Moreover, animals treated with chitosan nerve tubes presented an increased number of activated SCs and axons at 21 days after the induced PNI (Haastert-Talini et al., 2013). Several studies showed that oral administration of chitosan improved functional rehabilitation (Boecker et al., 2019), without an inflammatory effect and with low toxicity (Baldrick, 2010).

Since most of the researches were performed on PNI using chitosan nerve tubes, we aimed to evaluate the effects of oral administration of this compound.

Hypothesis

The study hypothesis was to observe if oral chitosan can provide peripheral nerve regeneration, by assessing the parameters described below.

Materials and methods

Research protocol

a) Period and place of the research

The study was conducted in October-November 2020, at the Department of Physiology of "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania.

b) Subjects and groups

Sixteen white male Wistar rats, with a weight of 140-310 g, aged 16-20 weeks (provided by the Iuliu Hatieganu University of Medicine and Pharmacy's Research Base, Cluj-Napoca, Romania) were used. They were randomly divided into two equal groups: a control group - right sciatic nerve peripheral injury without treatment, and an experimental group - right sciatic nerve peripheral injury treated with chitosan.

The study complied with the ethical standards regarding animal research: the principle of the 3Rs (reduce, refine, replace), and was approved by the University Ethical Board and the Veterinary and Food Safety Direction (project authorization no. 204/10.03.2020). The animals were monitored daily and no animal had to be withdrawn from the study, as no animal exhibited any signs or symptoms of distress or disease.

c) Applied tests

Chitosan was purchased from Sigma-Aldrich (St. Louis, California, USA), with a medium molecular weight between 190,000-310,000 Daltons, the molecular weight at which chitosan is easily absorbed and incorporated. The solution administered to the animals was elaborated by dissolving chitosan in a NaCl solution 0.9%, 1 mL solution containing 0.0145g chitosan.

The experimental group received 2.5 mg/kg chitosan solution, by gavage, daily, starting the next day after the peripheral nerve injury induction until day 21. The control group received the same daily dose, by gavage, of a simple NaCl solution 0.9%. The preparation and administration method was similar to that described by the authors of a previously published study (Pop et al., 2021).

Before inducing the PNI, the animals were anesthetized with intraperitoneal ketamine 40 mg/kg and xylazine 8 mg/kg. PNI was induced using a method previously described by the authors (Pop et al., 2021): a skin incision of approximately 2.5 cm at the right femoral eminence of all animals was made, and with a non-resorbable 5.0 nylon surgical wire, a 3 mm segment of the right sciatic nerve was compressed, for 15 seconds, and strangulated at 1-1.2 cm proximal to the nerve trifurcation. The sciatic nerve was chosen for its accessibility and for the standardized evaluation of functional regeneration that is currently used (de Medinaceli et al., 1982).

The sciatic functional index (SFI) score measurement, a standardized method elaborated by Medinaceli et al. in 1982 and modified by Bain et al. in 1989, represents the mark left by the animal's posterior feet, impregnated with blue ink, when the animal moves in a controlled environment (glass tunnel). The following formula was used to calculate the SFI score for the normal foot (N) and the experimental foot (E): SFI= -38.3*PLF+109.5*TSF+13.3*ITF–8.83 (Bain et al., 1989), with the following parameters: TS (toe spread) = distance between fingers

1-5; ITS (intermediary toe spread) = distance between fingers 2-4; PL (print length) = plantar print length. Next, the following factors were calculated: PLF (print length factor) = (EPL-NPL)/NPL, TSF (toe spread factor) = (ETS-NTS)/NTS, ITF (intermediary toe factor) = (EIT-NIT)/NIT (Bain et al., 1989).

The SFI score was calculated for all animals at T0 (prior to PNI occurrence), T1 (7 days after PNI), T2 (14 days after PNI) and T3 (21 days after PNI), and the values were interpreted considering Medinaceli's criteria, in Table I (de Medinaceli et al., 1982).

Table I.
Types of functional rehabilitation regarding the sciatic functional index (SFI) (score after Medinaceli, 1982).

SFI Score	Functional Rehabilitation Degree
12 to −12	Excellent
−13 to −37	Good
−38 to −62	Medium
−63 to −87	Non-satisfactory
−88 to −137	Complete deficit

Pain-like behavior in rodents can be evaluated using the Randall-Selitto test (analgesiometry), developed in 1957, and the currently most utilized method for assessing the presence of pain in animals, by applying a mechanical stimulus and observing the animal's behavior (withdrawal of the foot or tail) (Decosterd & Woolf, 2000). The evaluation followed a previously described method by the authors (Pop et al., 2021), where a maximum mechanical force of 300 g was applied to all animals, both on the normal foot (N) and the experimental foot (E), in the same place (between the tip of the cone-shaped and the plane surface of the foot), and the animal's behavior was assessed and noted by the researcher. The analysis was performed before the nerve injury induction and at 7, 14 and 21 days after the occurrence of PNI, using a bench-top Ugo Basile (Gemonio, Italy) analgesia-meter.

Right sciatic nerve samples from both groups were harvested at day 21 of the study, after euthanasia. The samples were isolated and fixed in 10% neutral formalin solution for 48 hours. Sections were cut at 7 µm (Reichert microtome, Austria) after paraffin embedding and mounted on glass slides. Xylene was used for dewaxing and subsequently, the nerve samples were rehydrated and stained with methylene blue (Merck, Darmstadt, Germany). The sciatic nerve samples were analyzed with an incorporated camera optical microscope (Optika 383-LD2, Ponteranica, Italy).

Studies have indicated that body weight analysis can represent a general health indicator for animals, which can be associated with the presence and intensity of pain, which can influence appetite (Hogan et al., 2004; Turner et al., 2019). All animals were weighed at the beginning of the study (T0) and at 21 days after peripheral nerve injury (T3), and the values were compared statistically.

At T0 (prior to the sciatic nerve lesion), at 7 days after the lesion (T1) and at 21 days after the nerve injury (T3), blood samples were taken and analyzed for the identification of NGF and IL-6 levels (ELISA kit, Sigma Aldrich, Darmstadt, Germany), using a Sunrise microplate ELISA reader (Tecan, Grödig city, Austria) and an Asys microplate ELISA washer (Atlantis, Austria).

d) Statistical processing

The mean value, standard deviation, median, and inter-quartile range (Q1–Q3, the range between the 25th percentile and the 75th percentile) were used to describe the quantitative data. Friedman test was used for not normally distributed data. Pairwise comparisons (Wilcoxon signed-rank tests) were performed with Bonferroni correction, to compare the measurements from week 0 with each of the other weeks. To verify if there was a significant difference between the chitosan treatment and control groups for the non-normally distributed variables, Mann-Whitney U test was applied. Normally distributed data between two groups were compared using t-test for paired samples, t-test for independent samples, and Levene test for variances. A p-value equal to or lower than 0.05 was considered statistically significant. Data were analyzed using IBM SPSS software, v25 (manufactured by IBM). The obtained NGF and IL-6 values were statistically analyzed with GraphPad Prism version 5.03 for Windows, GraphPad Software (San Diego, California, USA), two-way ANOVA followed by Bonferroni post-tests, setting the threshold significance level at $p < 0.05$.

Results

Chitosan treatment efficiency by evaluating the sciatic functional index score (SFI)

A Friedman test was run to determine if there were differences in SFI scores between T0, T1, T2 and T3. SFI was statistically significantly different at the various time points for the chitosan group, $\chi 2(3)=11.25$, $p=0.010<0.05$. Next, pairwise comparisons were performed with Bonferroni correction to compare the measurements from week 0 with each of the other weeks; the significance level was set at 0.0167. The median SFI score at T0 was -4.08 (within the limits of excellent functionality), at T1 the median SFI score decreased insignificantly to -4.60 after the intervention, then the median SFI score decreased at T2 and slightly increased at T3, but the differences were statistically significant only between the T0 and T3 scores (Fig. 1).

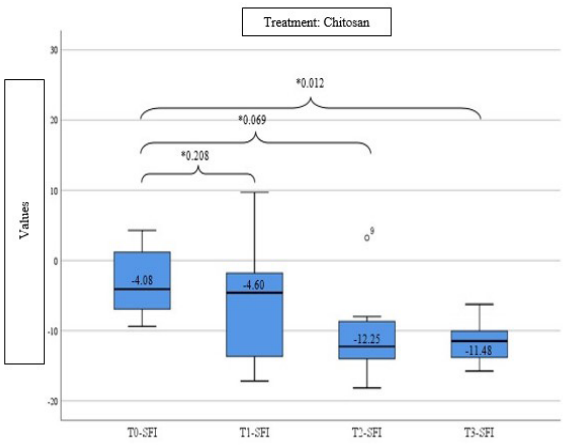

Fig. 1 – SFI score distribution (median) and evolution in time (*Wilcoxon signed-rank tests: *p*-values) for the chitosan treatment group.

A Friedman test was run to determine if there were differences in SFI scores between T0, T1, T2 and T3. SFI was statistically significantly different at the various time points for the control group, $\chi 2(3)=14.55$, $p=0.002<0.05$. Pairwise comparisons were performed with Bonferroni correction to compare the measurements from week 0 with each of the other weeks; the significance level was set at 0.0167. Post hoc analysis revealed statistically significant differences in the median SFI scores at T0 (-4.59) compared to T1 (-70.07), T2 (-64.69) and T3 (-120.55), respectively (Fig. 2). The results for the control group were previously published by the authors (Pop et al., 2021).

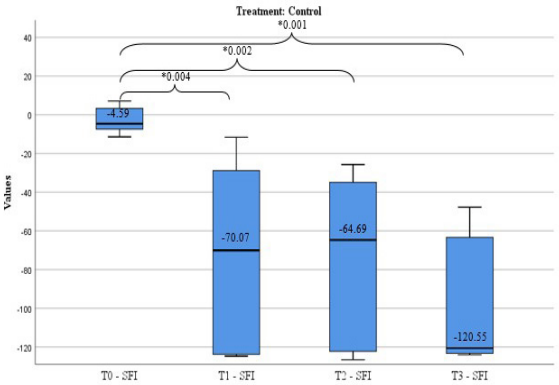

Fig. 2 – SFI score distribution (median) and evolution in time (*Wilcoxon signed-rank tests: *p*-values) for the control treatment group.

By comparing the evolution in time of the SFI score of the two groups (Fig. 3), a significant difference was observed, as the chitosan treatment group presented superior functional rehabilitation compared to the control group (according to Medinaceli's criteria presented in Table I).

Chitosan treatment efficiency by evaluating pain-like behavior

Comparing the pain scores of the control foot (healthy foot) showed no significant differences between the mean pain scores of the chitosan group and the control group for T0, T1, T2 and T3, respectively (*T*-tests for independent samples: $p>0.05$). When comparing the pain scores for T0 of the experimental foot, the chitosan treatment group mean (195.63) was not statistically significant compared to the control group mean (191.88), $t = 0.460$, $p = 0.652>0.05$. A comparison of the pain scores for T1, T2 and T3 indicated that the chitosan group had a statistically significant mean increase compared to the control group, $p < 0.001<0.05$ (Table II).

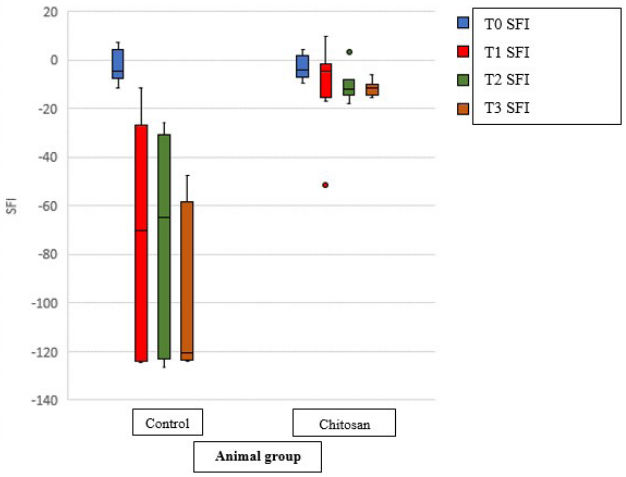

Fig. 3 – Comparative analysis of the SFI score between the control group and the chitosan treatment group (dynamic evaluation).

Figs. 4 and 5 show the comparative analysis between the two groups regarding pain-like behavior; a statistically significant difference was observed. The evolution of the chitosan group was favorable, the animals presenting better pain tolerance compared to the control group. Moreover, at 21 days after nerve injury, the chitosan group values were similar to those recorded at T0.

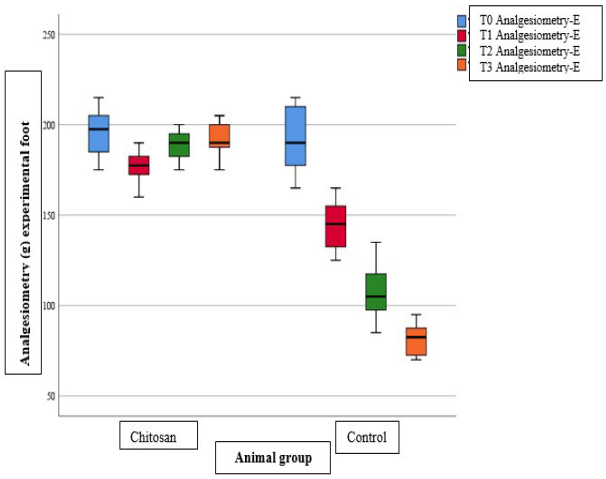

Fig. 4 – Comparative analysis of pain level between the CP group and the control group for the experimental foot.

Table II

Comparison of mean pain scores between the two treatment groups for the experimental foot.

Comparison between groups (experimental foot)	Time	Chitosan treatment		Control treatment		Independent t-test
		Mean	SD	Mean	SD	p-value
Pain scores	T0	195.63	13.63	191.88	18.50	0. 652
	T1	176.88	9.23	144.38	14.00	<0.001
	T2	188.75	8.34	107.50	15.58	<0.001
	T3	191.88	9.98	81.25	9.16	<0.001

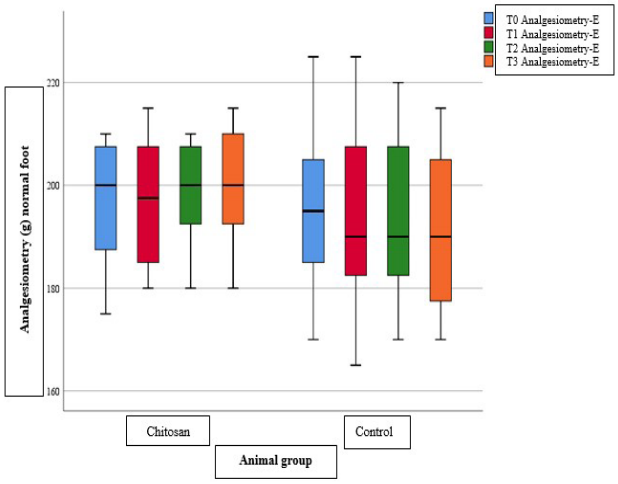

Fig. 5 – Comparative analysis of pain level between the CP group and the control group for the normal foot.

Dynamic evolution of the body weight as a possible tool to evaluate the efficiency of chitosan treatment

When comparing the weight for T0 and then for T3, the chitosan group mean was not statistically different compared to the control group mean, $p > 0.05$ (Table III). Comparing the weight scores for the chitosan treatment group revealed that the T3 mean was significantly higher than the T0 mean ($p=0.001<0.05$). Comparing the weight scores for the control group revealed that the T3 mean was significantly lower than the T0 mean ($p=0.001<0.05$). The comparative analysis of the two groups is shown in Fig. 6.

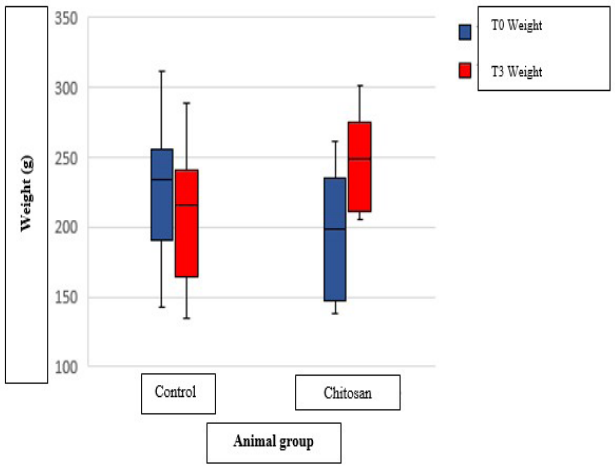

Fig. 6 – Comparative analysis of body weight evolution between the chitosan treatment group and the control group.

Chitosan treatment efficiency – histological studies

The histological analysis of right sciatic nerve samples from both groups showed that the control group presented various areas of disorganization of the myelin sheath. These areas are described as ovoid shaped vacuoles that seem to have determined the degeneration of the axon (Fig. 7). These modifications can cause changes in the myelin sheath thickness and structure, associated with a poor recovery outcome. The right sciatic nerve of the animals treated with chitosan had an almost normal or minimally altered structure (Fig. 8), in which the frequency of the ovoid formations was significantly lower compared to the control group.

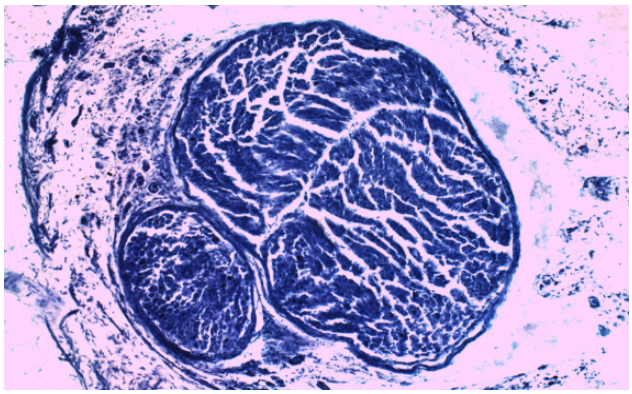

Fig. 7 – Right sciatic nerve section – control group.

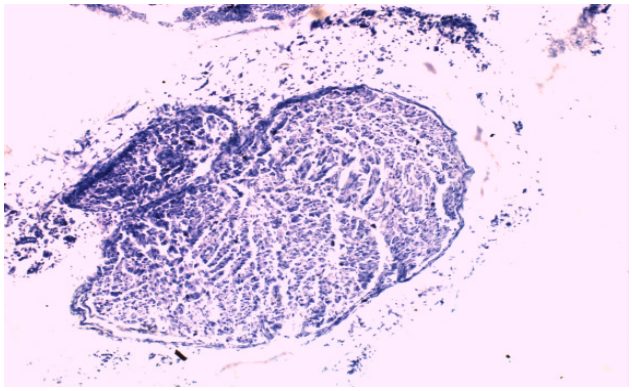

Fig. 8 – Right sciatic nerve section - chitosan treatment group.

Chitosan treatment efficiency – dynamic analysis of serum NGF and IL-6 levels

The dynamic analysis of serum NGF and IL-6 levels showed no statistically significant differences between the two groups (Figs. 9, 10). Nonetheless, the chitosan group had marginally constant higher values of serum NGF between the levels recorded at the beginning of the experiment and those registered at 21 days compared to the

Table III

Comparison of mean weight between the two treatment groups.

Comparison between groups	Time	Chitosan treatment		Control treatment		Independent t-test
		Mean	SD	Mean	SD	p-value
Weight (g)	T0	196.25	45.07	228.50	50.56	0.204
	T3	246.75	35.48	209.62	49.03	0.105

control group. The serum NGF levels of the control group were increased at seven days after the sciatic nerve injury, and subsequently, the NGF values followed a constantly decreasing tendency until day 21.

There were no significant differences between the two groups regarding serum IL-6 levels during the study. However, for both groups there was a constantly increasing tendency of IL-6 levels, as more time passed from the nerve injury onset.

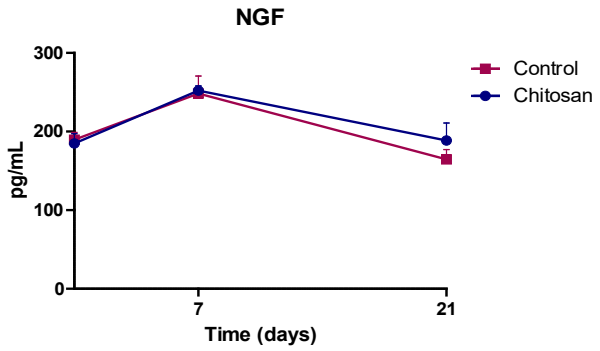

Fig. 9 – Serum NGF levels for the chitosan treatment group and the control group.

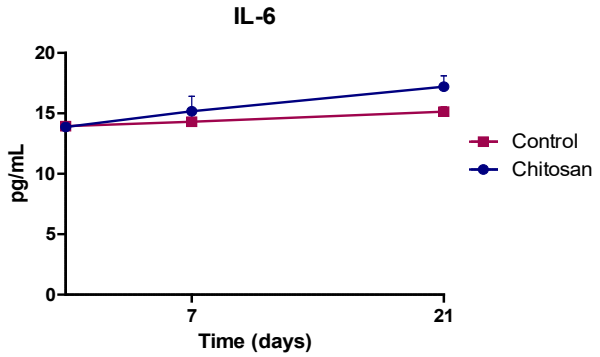

Fig. 10 – Serum IL-6 levels for the chitosan treatment group and the control group.

Discussions

The chitosan treatment group presented excellent functional rehabilitation at the end of the study, according to Medinaceli's criteria (Table I), as well as good pain tolerance and an increased body weight. Serum NGF and IL-6 levels were not statistically different compared to the control group, but the NGF levels of the chitosan treated group tended to remain slightly increased, suggesting that chitosan might be able to stimulate NGF secretion. Literature data showed significantly lower serum NGF levels for diabetic neuropathy patients compared to healthy patients, suggesting that the NGF results of the chitosan group in the present study can be associated with a positive peripheral nerve rehabilitation outcome (Giannaccini et al., 2017). In addition, Rui et al. reported in their study that NGF had neurotropism, promoted neurogenesis and its expression started within the same day after a PNI, accelerating the growth and myelination of the new cells (Li et al., 2020). These results were correlated with histological findings and therefore, the study hypothesis was confirmed: chitosan solution, administered orally, daily can represent an optimal treatment method for peripheral nerve injuries.

Like the present study, previous researches revealed the stimulating and regenerating action of chitosan on Schwann cells and axons, pointing out that chitosan had reduced cellular toxicity and did not promote any inflammatory response (Assa et al., 2017). These findings are consistent with our results, which showed that the serum IL-6 levels of the chitosan treated group were not significantly modified compared to the control group, indicating that chitosan administration does not cause notable inflammation. On the other hand, literature data suggest that the increase of IL-6 in both nerve cells and glia enhances the gene expression of regeneration-associated genes and multiple growth factors, playing an important role in nerve regeneration (Fregnan et al., 2012). Therefore, the increasing tendency of serum IL-6 levels can be interpreted as a sign of an enhanced nerve regeneration process.

The animals that received chitosan gained weight (significantly compared to the control group), results that correlate with other studies that observed a higher weight of muscles on the healthy side compared to the muscle weight of the injured site in animals treated with chitosan nerve guides (Meyer et al., 2016). In our study, we appreciated that the total body weight could represent a possible tool to assess the animal's general behavior and health. The weight loss of the control group could have been caused by the presence of motor impairment, resulting in a possible poor food reach. On the other hand, the animals of the control group could have also suffered from decreased appetite, in correlation with the intensified pain-like behavior that was observed.

Similarly to our study findings, other literature data revealed that chitosan administered orally for incomplete nerve lesions induces an excellent functional rehabilitation (Shakhbazau et al., 2012), making chitosan a potential treatment alternative for PNIs.

Although the present study results are encouraging, there were some limitations. The number of animals used, chosen in consideration of the ethical principles, can be considered low. Nonetheless, despite this low number, the results of the study were statistically significant. The number of animals could be increased in future researches, to observe if the initial results can also be obtained with a higher number of animals. Another limitation could be the subjective manner in assessing the SFI score and pain-like behavior, but, on the other hand, both methods are standardized and have been used in animal research for many years now. Therefore, chitosan administered orally could become a viable treatment option for peripheral nerve injuries, but more studies are needed in order to confirm this hypothesis.

Conclusions

1. Regarding the studied parameters, chitosan oral treatment seems to enhance peripheral nerve rehabilitation.

2. The results showed statistically significant differences between the chitosan treated group and the control group in relation to the sciatic functional index, total body weight, pain-like behavior, with a good rehabilitation outcome for the chitosan treated group, results that were confirmed by histological images.

3. No statistically significant differences were observed between the groups regarding the serum levels of NGF and IL-6, with a slightly increasing tendency of NGF and IL-6 values at the end of the study for the chitosan group, suggesting that the administration of chitosan might stimulate NGF secretion and the nerve regeneration process, with no statistically significant inflammation.

4. The results of the research confirm the hypothesis that orally administered chitosan could provide peripheral nerve regeneration and contribute to finding an efficient conservative treatment for peripheral nerve injuries.

Conflict of interests

None declared.

Acknowledgments

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