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Research article

Evaluating Hepatic Toxicity of Chitosan and Chitosan-Functionalized Iron Oxide Nanoparticles in a Rat Model of Peripheral Nerve Injury — Relevance for Rehabilitation Medicine

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Abstract: (1) Background: Peripheral nerve injuries (PNI) can generate important medium- and long-term disability, as patients mostly complain about associated pain, sensibility and, or motor deficit, and even psychological manifestations. Chitosan and different nanoparticles types were previously used in several studies as treatment of peripheral nerve injuries. The present study aimed to assess the hepatic responses at oral administration of simple chitosan solution and of magnetic iron oxide nanoparticles functionalized with chitosan (CMNPs) solution in an experimentally induced peripheral nerve injury. (2) Methods: Chitosan or CMNPs were oral administrated, for 21 days, to animals with peripheral nerve injury. The treated groups were compared with a control group (peripheric nerve injury without any treatment). The hepatic toxicity of administered solutions was analyzed histologically, through transmission electron microscopy (TEM) and through the oxidative stress parameters, in comparison with the control group. (3) Results: Liver tissue histological evaluation showed non-significant degeneration of hepatocytes in Chitosan group and, in CMNPs group, slight periportal inflammation. TEM investigation revealed nuclear and mitochondrial polymorphism and lipid accumulation in hepatocytes in Chitosan group, and in CMNPs group, irregular nucleus profiles and increased glycogen storage in cytosol. Oxidative stress analysis showed antioxidant hepatic effect of both treatments. (4) Conclusions: Compared to control group, both treatments produced significant increases of hepatic antioxidant protection, probably induced by chitosan properties. Histological modifications of the liver were minimal for both treatment groups. TEM investigation showed unspecific alterations of the hepatocytes structure.

Keywords: chitosan; iron oxide magnetic nanoparticles; peripheral nerve injury; toxicity

1. Introduction

Peripheral nerve injuries (PNI) can produce disability on medium and long term, clinical problem that represents a challenge from the rehabilitation point of view.

Depending on the site and on the severity of the lesion but also on the occurring mechanisms, the patients can present pain, motor deficit, sensibility and even psychological disorders [1].

PNI are mostly caused by trauma, but also non-traumatic compression or different diseases like diabetes mellitus, could initate this pathological problem. Nowadays, PNI treatment is mainly symptomatic, regarding pain management. Depending on the pathological problem, PNI treatment can also include physical therapy or surgical intervention. The current treatment management plan has limitations: systemic side effects, limited drug penetration into affected nerve and frequent administration needs. Moreover, current treatments do not completely assess PNI symptoms or causes, reason why the latest research focused on developing an alternative drug delivery systems designed to address these shortcomings.

Recent studies analyzed the regeneration of peripheral nerves using different compounds like chitosan or some types of nanoparticles. Magnetic nanoparticles proved to be efficient transport systems for various therapeutic agents such as chitosan. A major component of crustacean exoskelet is chitin and its deacetylation produces chitosan, a compound that may block the apoptosis of Schwann cells, can increase axons number and diameter, and can mitigate the inflammatory reactions [2]. Chitosan may enhance functional rehabilitation, being involved in axon regeneration with low toxicity and without inflammatory reactions after oral administration [3]. Magnetic nanoparticles functionalized with chitosan were studied in oncological therapy and their ability to avoid the immune system was demonstrated [4].

Superparamagnetic iron oxide nanoparticles coated with mannose reduced the reactive oxygen species (ROS) around the injured nerves, both in vitro and in vivo, along side with interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) mitigation in macrophages localized near the sciatic nerve, consequently alleviating peripheral neuropathic pain [5].

Cerium oxide nanoparticles neutralized the reactive oxygen species, enhancing nerve regeneration and protecting neuronal viability [6]. Nanoparticles can also guide axons along predetermined trajectories. For example, graphene oxide nanosheets and methacrylated gelatin hydrogel scaffolds amplifly neurite length and can correct 10 mm sciatic nerve defects in rats, while improving gastrocnemius muscule regeneration [5]. Moreover, nanoparticles enhance myelin sheath reconstruction, providing a nurturing environment for Schwann cells and myelin regeneration [5]. Polymeric nanoparticles containing thiolated trimethyl chitosan can secrete growth factors and modulate cellular behavior, facilitating gene delivery to peripheral neurons, re-establishing functional neural conduits after intramuscular administration [7].

Hanwright et al. combined biodegradable nanoparticles with insulin-like growth factor 1 (IGF-1) within dextran sulfate polyelectrolyte complexes, which enhanced forepaw grip strength recovery in the muscles atrophied by denervation, reducing senescence in Schwann cells and stimulating neuromuscular reinnervation [8]. Nanoparticles represent potent drug carriers also due to their capacity for site-specific delivery, minimizing undue impact on healthy tissue. From the peripheral nerve injuries point of view, the delivery of neuroactive agents to the target sites represents a very important aspect. Lopes et al. constructed a neurotropic nanoparticle based on polyethyleneimine, which, after subcutaneous injection, facilitated neuron-specific transfection, with no histological adverse effects [9].

The need for developing effective drugs, target-specific and with fewer side effects in PNI management stimulated our research team to investigate the effects of an oral treatment based on chitin on nerve regeneration, pain alleviation, and side reactions.

Biomedical applications of nanoparticles were investigated in several studies which evaluated their potential side effects, disclosing mostly hepatic and renal toxicity. Different studies reported the most frequent adverse effects of iron oxide magnetic nanoparticles: inflammation, ulceration, reduced body weight, neural-behavioral alterations. The toxicity of nanoparticles may be correlated with their chemical

composition, administered dose, immunogenity, half-life and a certain organ-specific toxicity [10].

Nanoparticles are widley used in industry, the metallic nanoparticles are present in textile and footwear industry, cosmetics (shampoos, creams, toothpaste), plastic utensils and many others. Therefore, researchers studied the possible side effects of metallic nanoparticles, which may affect biological systems at the cellular level [11] through different mechanisms that are not fully understood.

Several studies analyzed chitosan and nanoparticles toxicity regarding oxidative stress parameters. Oxidative stress represents an imbalance between free radicals and antioxidants. Oxygen reactive species are produced in mitochondria during cellular metabolism, under the control of enzymatic and non-enzymatic systems, in physiological conditions.

Oxidative stress represents the main cause of various liver diseases such as hepatitis, liver failure, alcoholic liver disease, and others [12]. MNPs can accumulate in the liver, disturbing the activities of antioxidant enzymes and causing oxidative stress. Mice exposed to silver NPs (AgNPs) presented elevated hydrogen peroxide levels, altered liver enzymes, elevated plasma fibrinogen levels and decreased serum aspartate aminotransferase (AST) activity [13].

Despite other findings, some studies revealed that MNPs can produce reactive oxygen species (ROS), generating oxidative stress due to their increased chemical reactivity, high surface area-to-volume ratio and because of their small sizes. Studies reported that MNPs can interfere with the functionality of various components (proteins, nucleic acids, fats) responsible for ROS production, causing oxidative stress [14]. The inflammation produced by MNPs can affect the liver, like gold NPs that activate hepatic macrophages, inducing immune hepatitis and liver dysfunction [15].

Literature data described the side effects of MNPs on different cellular organelles of the hepatocytes. MNPs appear to alter the internal structure of the cellular membrane, interfering with its permeability and fluidity, causing inactivation of different receptors, and inducing hepatotoxicity. MNPs can enter the hepatocyte nucleus altering the DNA, with effects on gene expression [11]. MNPs may initiate mitochondria modifications, leading to hepatocyte death through oxidative stress, enzymes alterations or electron transport chain deterioration [11]. Moreover, MNPs seem to interfere with the activity of the hepatocyte endoplasmic reticulum (ER), a structure involved in protein and lipid metabolism, steroid hormone production or in calcium storage, affecting in this way the liver detoxification function. [11].

The oral administration of silver nanoparticles decreased the hepatic antioxidant protection in rats [16]. The same effect on oxidative stress in the liver was observed after the exposure to zinc oxide NPs (ZnONPs), process that caused necrosis and apoptosis of hepatocytes [11].

Literature data tried to explain the interactions between hepatocytes and nanoparticles and the mechanism of uptake and elimination. The uptake and retention rate is correlated with the nanoparticle dimension, electric charge, and chemical structure [17]. Kupffer cells can internalize the nanoparticles through multiple scavenger receptors, using different mechanisms (macropinocytosis, caveolin-mediated endocytosis, endocytotic pathways) [17]. A study that evaluated the uptake mechanism of 20 nm and 60 nm superparamagnetic iron oxide nanoparticles (SPIONs) by human macrophages revealed that 60 nm SPIONs showed a sixty times higher take-over than 20 nm SPIONs, using different receptors for endocytosis [18]. Hepatobiliary system or the Kupffer cells sequestration are involved in nanoparticles effects [17]. Nanoparticles with sizes lower than 150 nm can extravasate into the space of Disse [17].

The objective of the present study was to evaluate the potential adverse effects of chitosan and iron oxide magnetic nanoparticles functionalized with chitosan on the liver tissue using histological studies, transmission electron microscopy investigation and oxidative stress parameters analysis, in an experimental model of an induced scitatic nerve lesion, in order to determine if oral chitosan or CMNPs could represent a potential safe treatment for PNI.

2. Results

2.1. Histological investigation

The hepatic tissue in Control group showed normal histological aspect, with discrete sinusoidal dilation (Figure 1).

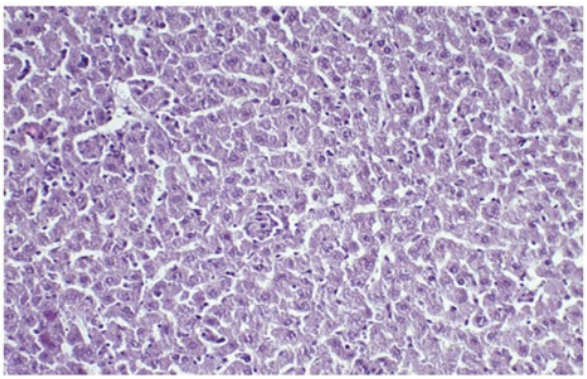
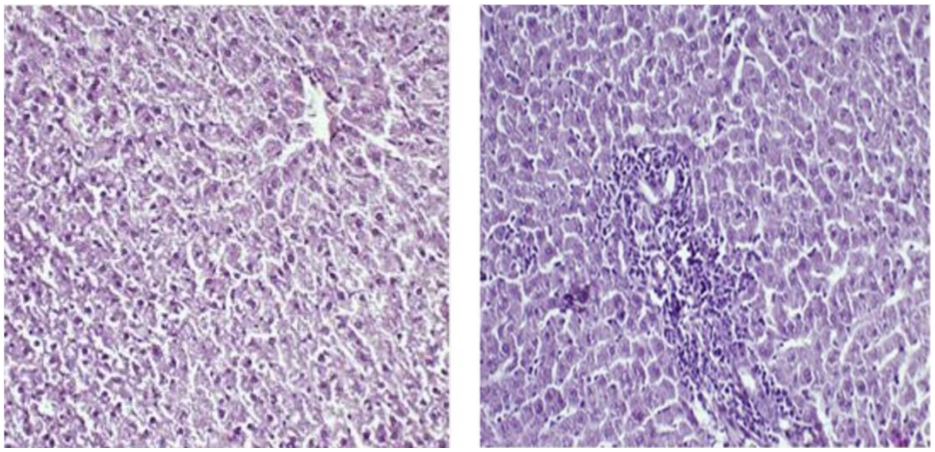


Figure 1. Histological examination of the hepatic tissue in Control group

Histologically, hepatic tissue in Chitosan group presented hepatocytes degeneration (Figure 2a), whereas in CMNPs group, significant periportal inflammation was noted (Figure 2b).



a **b**

Figure 2. Histological examination of hepatic tissue in **(a)** Chitosan group and **(b)** CMNPs group.

2.2. TEM investigation

In Control group, TEM investigation revealed normal aspect of hepatocyte nucleus but the endoplasmic reticulum (smooth and rough) was dilated and mitochondria were polymorphous. Numerous lipid droplets were seen in the cytoplasm, the lysosomes and peroxisomes (not shown) had typical aspect and number, while the glycogen granules were very rare (Figure 3).

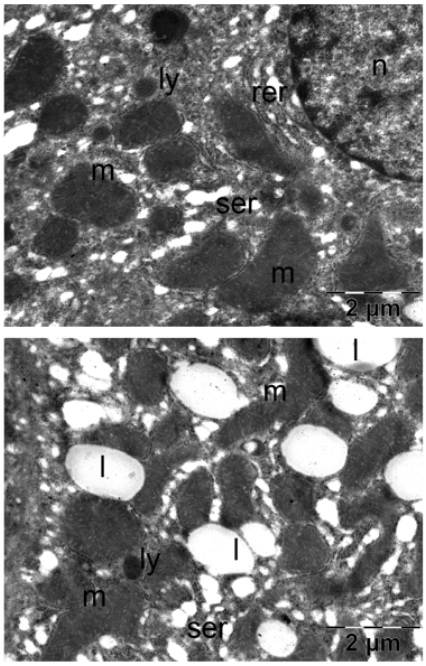
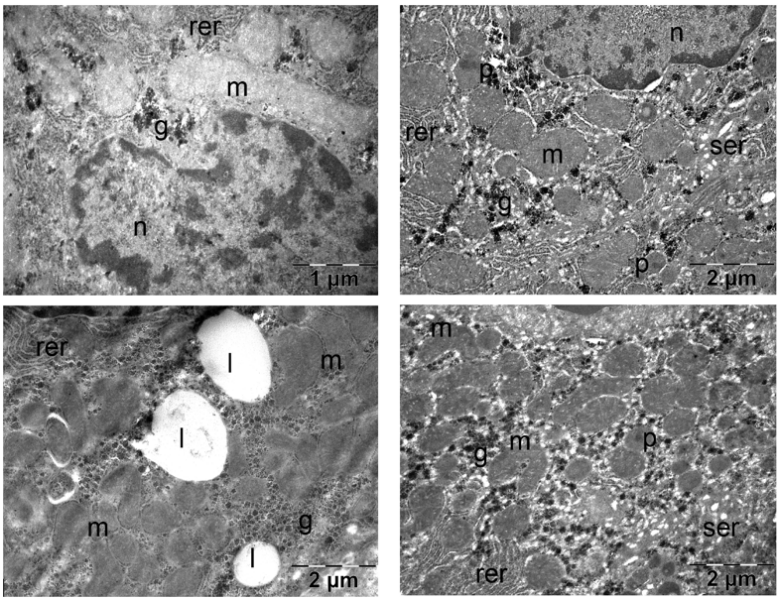


Figure 3. TEM investigation of hepatic tissue in Control group (l, lipid droplet; ly, lysosome; m, mitochondrion; n, nucleus; rer, rough endoplasmic reticulum; ser, smooth endoplasmic reticulum).

In Chitosan group, polymorphic nuclei and mitochondria were noted inside the hepatocytes, with moderate accumulation of large lipid droplets and high amounts of glycogen in cytoplasm. Peroxisomes and lysosomes were in usual number, and both types of endoplasmic reticulum displayed usual ultrastructural aspect (not shown) (Figure 4a).

TEM examination of hepatic tissue in CMNPs group showed mainly the irregular contour of the nucleus and the presence of polymorphous mitochondria. Peroxisomes and lysosomes were in normal number, and the both forms of the endoplasmic reticulum displayed normal ultrastructural aspect. The glycogen was present in high amount (Figure 4b).



(**a**) (**b**)

Figure 4. TEM investigation of hepatic tissue in **(a)** Chitosan group and **(b)** CMNPs group sections.(g, glycogen; l, lipid droplet; m, mitochondrion; n, nucleus; p, peroxisome; rer, rough endoplasmic reticulum; ser, smooth endoplasmic reticulum).

2.3. Hepatic Oxidative Stress Evaluation

Compared to Control group, both treatments (chitosan and CMNPs solutions) did not modified significantly the malondialdehyde (MDA) levels in hepatic tissue (Figure 5a). GSH(reduced glutathione)/GSSG (oxidized glutathione) ratio revealed significant increases in Chitosan ($p<0.05$) and in CMNPs ($p<0.01$) groups, compared to Control group, showing that both treatments had a hepatic antioxidant effect (Figure 5b).

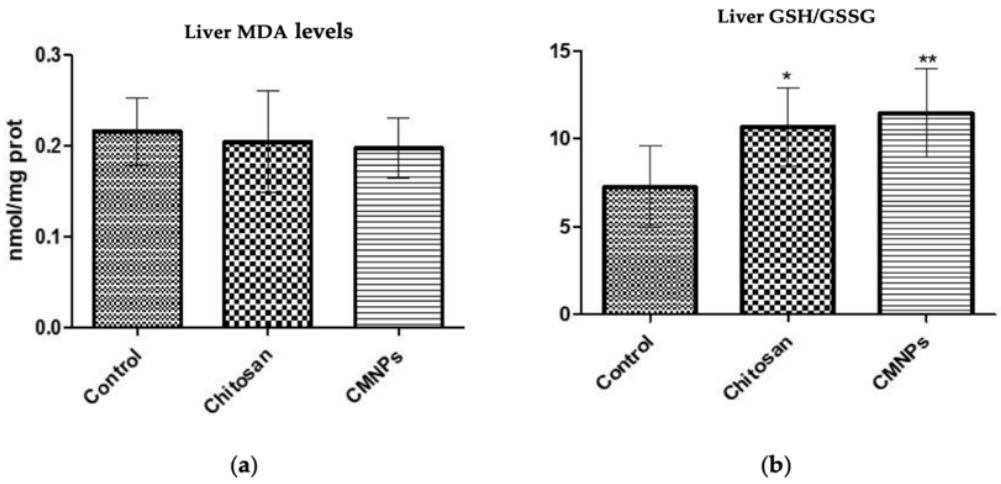


Figure 5. Liver malondialdehyde (MDA) levels (**a**) and GSH/GSSG ratio (**b**) in groups of rats with peripheral nerve injury (*p<0.05, **p<0.01 compared to Control group).

The levels of reduced glutathione (GSH) in hepatic tissue were increased significantly ($p<0.01$) by CMNPs solution administration, in comparison with Control and Chitosan groups (Figure 6a). Oxidized glutathione (GSSG) levels decreased significantly in Chitosan ($p<0.001$) and CMNPs ($p<0.05$) groups, compared to Control group (Figure 6b).

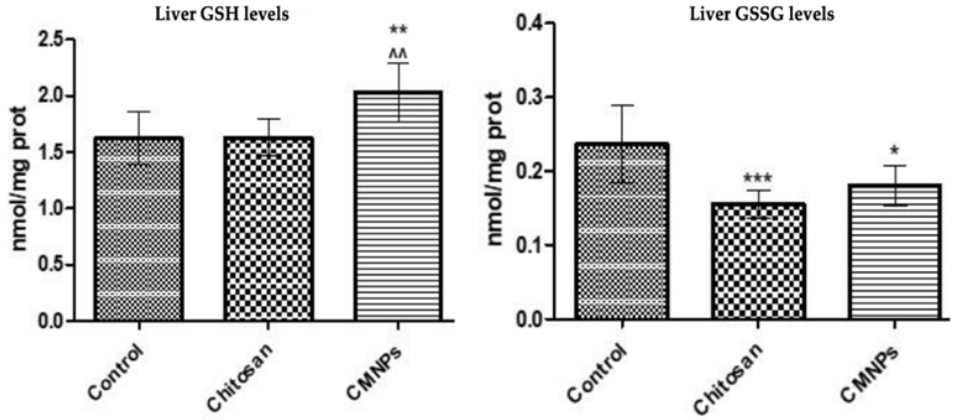


Figure 6. Liver reduced glutathione (GSH) (**a**) and oxidized glutathione (GSSG) (**b**) levels in groups of rats with peripheral nerve injury (*p<0.05, **p<0.01, ***p<0.001 compared to Control group; ^^p<0.01 compared to Chitosan group).

2.4. Chitosan Magnetic Nanoparticles Characterization

CMNPs characterization was previously published by the authors in a study that evaluated the regenerative effect of CMNPs as a treatment for peripheral nerve injuries [19]. For the characterization, FTIR (Fourier-transform infrared spectroscopy), transmission electron microscopy (TEM) and magnetometry were used. FTIR confirmed

the functionalization with chitosan of the magnetic nanoparticles and TEM revealed the spherical shape of CMNPs, with a diameter between 29 nm and 32 nm. Magnetometry confirmed the superparamagnetic behavior of CMNPs, the magnetite nucleus having a diameter under the critical domain dimensions [20].

3. Discussion

Multiple biopharmaceutical and biomedical applications of chitosan represented a focus in recent research due to chitosan's biocompatibility and relatively reduced toxicity. Chitosan enters the cells, interacts with nucleic acids and interferes with protein synthesis. This compound's toxicity may be corelated with its molecular weight and administrated dose [21]. On the other hand, chitosan showed hepatic protective effects in acetaminophen-induced hepatotoxicity, and beneficial impact in renal insufficiency treatment [22,23].

Concerning chitosan functionalized magnetic nanoparticles, these particles can cross the gastrointestinal tract, pulmonary alveoli and the nasal cavity, reaching the systemic circulation and causing various noxious reactions. Several studies showed that chitosan nanoparticles may produce hepatic injuries and their general toxicity is higher than that of simple chitosan solution [24]. Researches that used cellular lines for toxicity evaluation of chitosan nanoparticles demonstrated that these particles can penetrate the cell, affecting the integrity of cellular membrane [24].

In the present study, histology examination revealed modifications of liver tissue in both treated groups, severe alterations being recorded after CMNPs solution administration, results that are consistent with literature data, which described histopathological alterations (hepatic congestion, local hemorrhage, cellular degeneration, apoptosis and necrosis) [25].

TEM investigation of liver tissue revealed accumulation of glycogen after both treatments: simple chitosan solution or CMNPs solution. These findings were similar with other studies results in which ZnONPs determined hepatocyte necrosis, congestion, and glycogen clumping, with significant increased ALT (alanine transaminase) and AST values, suggesting severe damage [11]. Histological analysis of fish liver exposed to AgNPs (concentration of 1.5 mg/L) and copper nanoparticles (CuNPs 0.15 mg/L) revealed dilated sinusoids and nuclear necrosis. In the same study, ultrastructural results showed mitochondrial edema, dilated and loosened endoplasmic reticulum, accumulation of lipid droplets and glycogen depletion [26]. By comparison to the present study, our TEM images also encountered lipid accumulation but with a higher amount of glycogen.

Hepatic oxidative stress analysis revealed that neither of the applied treatments produced oxidative stress compared to the control group, both treatment options showing a hepatic antioxidant effect. These results correlate with other studies' findings, which reported the antioxidant effect of chitosan nanoparticles on rat microglial cell lines [27]. The neuroprotective effect of chitosan is realized by a "sealing" mechanism of the injured cellular membrane, which can explain its antioxidant action [28].

Other studies reported the hepatoprotective effect of chitosan and of CMNPs when liver toxicity was induced by emamectin benzoate (EMB), a chemical compound that increased lipid peroxidation, decreased antioxidant protection and produced several histopathological changes in the liver. [29].

Another study concluded that chitosan nanoparticles protected the liver against induced hepatocarcinoma. It appears that treatment with chitosan nanoparticles significantly increased the antioxidant protection and ascorbic acid, while reducing lipid peroxidation. Chitosan nanoparticles also reduced the levels of low-density lipoprotein and very low-density lipoprotein cholesterol, concluding that due to their antioxidant and anti-lipidemic properties, chitosan nanoparticles may have a protective effect on hepatocytes [30].

Curcumin/chitosan coated silver nanoparticles presented antifibrotic effects by preserving the liver structure and function during fibrosis progression, effects that are opposite to those produced by the treatment with naked AgNPs or only with curcumin

[31]. Moreover, ursolic acid niosomes coated with chitosan prevented liver damages induced in mice, decreasing the seric levels of AST and ALT, mitigating the inflammation of the bile duct and improving the hepatocytes' nuclei aspect [32].

Chitosan derivatives may have beneficial role in cancer, producing apoptosis of cancer cells and reducing the oxidative stress in healthy cells [33]. Chitosan-glutathione nanoparticles did not have significant effects on chondrocytes viability, in a study realized in vitro [34], research that has shown some of the limitations of chitosan use.

Overall, the results of the present study showed a potential antioxidant effect of chitosan and chitosan magnetic nanoparticles, similar with other literature data in which chitosan is presented as a wound healing promoter [34]. However, the complexity of tissues' responses to chitosan and CMNPs imposes the researchers to continue the investigation through many diverse studies. Moreover, the study has some limitations, like the relatively small number of animals used for the experiment, that can impaire a strong statistical conclusion. From ethical principles, the lowest number of animals that can give significant results was chosen. The female rats must be also investigated in a similar study, to evaluate the antioxidant role of estrogens in this type of therapy. The period of time of the future studies must be longer to investigate the side effects in different organs after chitosan or CMNPs solution administration.

4. Materials and Methods

4.1. Study Design

The experimental-induced right sciatic nerve injury, the treatment protocol and the preparation of the chitosan functionalized magnetic nanoparticles were previously published by the authors in a study that evaluated the regenerative effect of CMNPs [13]. The study included 24 healthy, white male Wistar rats, 20 weeks of age (provided by the "Iuliu Hatieganu" University of Medicine and Pharmacy's Research Base, Cluj-Napoca, Romania), divided into three equal groups (N=8), which received oral treatment for 21 days, as following: group 1 (Control) with peripheral nerve lesion (PNL), without treatment/placebo (2.5 mg/kg/day of simple NaCl 0.9% solution); group 2 (Chitosan) with PNL treated with 2.5 mg/kg/day of chitosan dissolved in NaCl 0.9% solution (1 mL of solution containing 0.0145 g of chitosan); group 3 (CMNPs) with PNL treated with 2.5 mg/kg/day of CMNPs dispersed in NaCl 0.9% solution (1 mL of solution containing 0.0145 g of CMNPs). An experimental design was used in order to closely observe the administrated treatment effects and possible adverse reactions on a living organism, for future applications in humans.

Oral administration of the used substances mentioned above was preferred for the novelty that it implies, as current PNL treatment is surgical when nerve repair is targeted and only symptomatic when administrated orally. Therefore, the authors wanted to evaluate a possible oral treatment capable of peripheral nerve repair and a proper rehabilitation outcome. All the substances were administrated by gavage, without previous anaesthesia. The dosage of the administrated substances regarded the literature data of different treatment methods that used chitosan or that evaluated nanotoxicity [35,36].

The experiment respected the ethical principles required of the 3Rs (reduce, refine, replace) and was carried out after receiving "Iuliu Haţieganu" University of Medicine and Pharmacy's Ethical Board and the Veterinary and Food Safety Direction approval (project authorization no. 204/10.03.2020). Concerning ethical guidelines, the study included a minimal number of animals, just above the statistical processing and analysis limit. During the experiment, the animals were closely monitorized for any symptoms, signs or diseases (major distress or pain, infection of the lesion site, death) that would have attracted the removal from the study. All the animals survived and completed the experiment. The animals were overlooked by specialized personnel, able to rapidly oberve any sign of distress and to properly interven if needed. All the animals received the same

type and amount of food, at regular hours and had fresh water at their disposal. At the end of the experiment, ketamine 10% (5 mg/100gbw) and xylazine hydroxichloride 2% (100 mg/100gbw) were used to induce in rats deep anesthesia, to collect the liver for histological analysis, transmission electron microscopy investigation and oxidative stress parameters evaluation.

4.2. Preparation and Characterization of Chitosan and Magnetic Nanoparticles Functionalized with Chitosan

Chitosan was provided from Sigma Aldrich and CMNPs characterization was performed using FTIR (Fourier-transform infrared spectroscopy), transmission electron microscopy (TEM) and magnetometry. CMNPs were prepared using a method published in the literature [37,38]. The morphology of the magnetic nanoparticles was analyzed by TEM with Hitachi HD-2700 scanning transmission electron microscope (Hitachi High-Tech, Krefeld, Germany), at room temperature with a vibrating-sample magnetometer manufactured by Cryogenics (Cryogenic Limited, London, UK). FTIR spectroscopy was used to confirm the synthesis of magnetic nanoparticles functionalized with chitosan. The results of the preparation and characterization of simple chitosan and CMNPs were published by the authors in a previous study [19].

4.3. Evaluation of Hepatic Oxidative Stress Parameters

The oxidative stress in liver tissue was evaluated through the reduced glutathione (GSH), oxidized glutathione (GSSG) and malondialdehyde (MDA) levels. MDA is used as a marker for the presence of oxidative stress, as its production increases directly with the levels of free radicals [39]. Glutathione is a natural antioxidant which removes oxygen reactive species, its level being an indicator of oxidative stress [40].

Lipid peroxides and aldehyde dosage is the most frequent used method to assess oxidative stress [41]. For the present study, lipid peroxides' determination was realized by fluorescence, as the resulted MDA forms a fluorescent adduct with the thyobarbituric acid. For dosage, the samples were boiled for 1 hour with a 10 mM 2-thyobarbituric acid solution, in 75 mM dipotassium phosphate, pH 3. Next, the samples were cooled down suddenly, the reaction product was extracted in n-butanol, and the concentration was determined in organic phase, after the separation by centrifugation. Emission intensity was measured at 534 nm, using a Perkin Elmer (Boston, USA) spectrofluorometer, with a sincron fluorescence technique at a wavelength between excitation and emission ($\Delta\lambda$) of 14 nm. MDA concentration (nmol/mg protein) was established based on a calibration curve realized with known MDA concentrations processed in the same way.

Determination of GSH and GSSG was performed after a known method [42]: 50 μL of tissular homogenate with 450 μL solution of 10% m-phosphoric acid were centrifuged for 10 minutes at 1000 rotations/minute. For the determination of GSH, 0.1 mL of the obtained supernatant were diluted with 1.8 mL phosphate buffer 0.1 M (pH 8), containing 5 nmols/L EDTA, after which 0.1 mL o-ftalaldehyde solution was added into methanol (1 mg/mL). After incubation for 15 minutes, the emitted fluorescence was measured at 420 nm with an excitation of 350 nm. Estimation of GSSG was conducted in 250 μL supernatant, which was incubated for 30 minutes with 40 nmol/L N-ethylmaleimide. Then, 0.65 mL NaOH 0.1 N were added and the same procedure as for GSH was done, with the difference that instead of the phosphate, 0.1 mL of reaction mix between 1.8 mL NaOH 0.1 N and 0.1 mL o-ftalaldehyde solution were added. Concentration values for GSH and GSSG were calculated using calibration curves obtained from known concentrations of GSH and GSSG, respectively.

4.4. Histopathology Analysis

Liver samples were taken at the end of the experiment from all animals, fixed in 10% neutral formalin solution for 48 h and embedded in paraffin. After that, sections were cut at 7 μm with a Reichert microtome (Vienna, Austria) and mounted on glass slides. Liver sections were dewaxed in xylene, rehydrated, and methylene blue (Merck, Darmstadt, Germany) staining was used for the histological exam. The histopathological changes were assessed with an incorporated camera optical microscope (Optika 383-LD2, Ponteranica, Italy).

4.5. TEM Analysis

Liver samples of 1 mm^3 collected from the three groups were prepared for TEM according to the chemical fixation and embedding protocol (Hayat, 2000) [19,43]. They were prefixed for 2 h in 2.7% glutaraldehyde and postfixed for 1.5 h in 1.5 % OsO_4, dehydrated with ethanol series (30 minutes each), and infiltrated and embedded with Embed 812 epoxi resin. From the polymerized blocks, sections of 60-70 nm were cut with a LKB ultrotome (Bromma, Sweden), contrasted 15 min with 13 % uranyl acetate and 5 minutes with 2.8 % lead citrate, and examined on a Jeol JEM-100CX II transmission electron microscope (Jeol, Tokyo, Japan) equipped with a Mega View G3 camera (Emsis, Münster, Germany) [19].

4.6. Data Analysis

Statistical analysis was realized using GraphPad Prism version 5.03 for Windows program, GraphPad Software (San Diego, California, SUA) with one-way ANOVA, post-test Tukey (Tukey multiple comparisons). The statistically significant level was established at a value of $p < 0.05$.

5. Conclusions

Hepatic toxic histological modifications produced by chitosan and chitosan functionalized magnetic nanoparticles consisted mostly in inflammatory changes. None of the two treatments produced significant alterations of oxidative stress parameters, with a tendency at an antioxidant hepatic effect. Even though the results are promising and may be the base of a treatment for peripheral nerve regeneration with minimal hepatic side effects, more studies are required for a more precise conclusion and to expand the research to human testing. Moreover, research must focus on liver-nanoparticles interactions, studies which are necessary in future orientation of treatment directions.

Author Contributions: Conceptualization, N.L.P., A.N., D.R.M. and R.O.; methodology, N.L.P., A.E.U.-C., A.F., V.A.T., R.M.; software, A.N., A.E.U.-C., D.R.M; validation, N.L.P., D.R.M. and R.O.; formal analysis, N.L.P., A.N., D.R.M., A.E.U.-C., A.F.; investigation, N.L.P., R.M., N.D.; resources, N.L.P., A.N., A.E.U.-C., A.F., V.A.T., N.D.; data curation, N.L.P., R.M., A.E.U.-C.; writing—original draft preparation, N.L.P., A.N., A.E.U.-C., A.F., V.A.T.; writing—review and editing, D.R.M., R.O.; visu- alization, N.L.P.; supervision, D.R.M., R.O.; project administration, N.L.P.; funding acquisition, N.L.P. All authors have read and agreed to the published version of the manuscript.

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