BIOCOMPATIBILITY STUDIES

An alternative to nerve repair using an antioxidant compound: a histological study in rats

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Abstract The fascicular composition and organisation of the inferior alveolar nerve (IAN) were determined to confirm the microarchitecture of the IAN bundles into each of the mandibular teeth, including the composition of the mental nerve. The aim of this study was to evaluate peripheral nerve repair after the application of an antioxidant compound to the damaged nerve tissue to elevate the concentration and bioavailability of elements capable of favouring tissue repair. Twenty-five Wistar rats were divided into groups: The Control 1 (Ctl 1) (n = 5) animals had the ischiatic nerve exposed with no suture injury and were sacrificed at 30 days post-operatively. The Control 2 (Ctl 2) (n = 10) animals had the ischiatic nerve exposed, and the nerve was injured using suture in three distinct regions. In the experimental (Exp) animals (n = 10), an antioxidant organic compound was applied to the nerve injury site. The animals with nerve injury (Ctl2 and Exp group) were sacrificed at 15 and 30 days post-operatively. The histological analysis showed less degeneration in the Exp group at 15 and 30 days post-operatively. Nerve neoformation forming a connection between the distal and proximal suture sites was observed in the experimental group. This study presented an alternative to nerve repair using an antioxidant compound.

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1 Introduction

The timely repair of peripheral nerve injuries has been the sine qua non for the successful recovery of nerve function, especially since Seddon's extensive experience with treatment of missile injuries to the extremities during and after World War II. Seddon's comment, "If a purely expectant policy is pursued, the most favourable time for operative intervention will always be missed." is as pertinent today as it was more than 60 years ago. As in all other causes of nerve injury, the treatment of a patient with a dental implant-associated nerve injury is dependent on the correct diagnosis of the injury and timely management.

The perioperative administration of supportive medications has been advocated for patients undergoing procedures such as dental implants, mandibular osteotomies, and removal of the lower third molars, which are associated with a risk of nerve injury. There is conflict in the literature between those who recommend starting corticosteroids preoperatively and others who advise waiting postoperatively for several days

before initiating administration. Many surgeons routinely give a single preoperative intravenous dose of a steroid (dexamethasone or hydrocortisone). Whether it is beneficial to initiate corticosteroid or anti-inflammatory medications after a nerve injury has occurred is questionable.

Manipulation of the nerve can generate a severe inflammatory reaction in the nerve, which has been likened to a chemical burn with dense scarring, accompanied by considerable pain [1].

Regeneration of the peripheral nervous system is possible, although appropriate surgical interventions are needed for the recovery of a nerve that is completely sectioned (Sunderland's fifth-degree peripheral nerve injury) [2]. Autologous nerve grafts yield the best results when a transection of the peripheral nerve occurs [3, 4], but this procedure presents limitations, such as: (I) increase of morbidity at the donor site, (II) scarcity of donating sites of nerve, (III) possible structural differences between the donor and the recipient site, and (IV) sensation loss at the donor site [5, 6]. Alternative therapies such as autologous stem-cell transplants, tube techniques with various materials, and trophic material applications have been investigated to optimise the repair of damaged peripheral nerves [6, 7].

Rapid progress in the area of tissue engineering has encouraged researchers to propose therapeutic strategies for repairing peripheral nerves using biopolymers and neurotrophic factors. [8, 9]. A scaffold of poly-(2-hydroxyethylmethacrylate) and poly-(L-lisine), with different concentrations of neurotrophins, might orientate the axonal growth after damage to the peripheral nerve [10]. Carbon nano-tubes are used as a structure capable of controlling the liberation of neurotrophins, aiding in the process of repairing the peripheral nerve [11].

Another approach for nerve repair to control redox balance because the relationship between injury damage and cellular adaptation is extremely fragile [12]. Despite the simplicity of this concept, its application requires a broad knowledge of metabolic variables and the particularities of each tissue and its interaction with the tissue/cells that surround the process of repair. The application mode might interfere with the metabolism of other systems. The antioxidant overdosing approach is commonly used [13, 14], but its clinical applications are limited [15].

Our strategy for nerve repair is to combine the approaches mentioned above with antioxidants to promote protection and tissue repair on a scaffold that gradually releases its content around the injured nerve. Our strategy utilises the oxidative mediation with a local application. A biodegradable scaffold was developed, containing an antioxidant compound and mineral salts with the function of mediating the process of in situ nerve repair.

The objective of this study was to evaluate the effect of the local application of an antioxidant compound on the repair of damaged nervous tissue.

2 Materials and methods

### 2.1 Antioxidant compound physical and chemical properties

The antioxidant compound was composed of: β-carotene, α-tocopherol, B complex vitamins, selenium salts, zinc salts, magnesium salts, phosphor salts, glutamic acid, soy lecithin, hydrolysed collagen, glycosaminoglycan sulphate and chondroitin sulphate ($\stackrel{\circ}{3}$-NERVE, JHS-biomaterials, Sabará, Brazil).

The mixing ratio was previously determined. The amounts of selenium, zinc and magnesium varied from 10 to 15 % of the total weight of the active component. Selenium varied from 20 to 30 % of the relative weight compared with zinc and magnesium, which represented 1.8 and 1.2 %, respectively. β-carotene and α-tocopherol varied between 15 and 20 %, with α-tocopherol having 64 % of β-carotene. The B complex varied from 20 to 30 % of the total weight of the active components, with vitamin B1 and B6 having 30 % of the complex and glutamic acid having 50 % of the vitamin B6. Vitamin D (calciferol) was added to the final process with maximum of 40 parts per million of the final weight. Hydrolysed collagen, glycosaminoglycan sulphate and chondroitin sulphate are agglutinating agents, and they were added in proportions of 60, 20 and 20 %, respectively, until the material had an adequate consistency. The final consistency of the mixture was similar to epoxy resin, making its local application easier.

2.2 Cytotoxicity assay

The antioxidant compound cytotoxicity was verified by an in vitro test in accordance with the International Standardization Organization (ISO) and previous publications by the neutral red uptake methodology [16–18]. This method consists of putting the dilutions of the sample extract in direct contact with the cell culture over 24 h and evaluating the cytotoxic effect by the cellular viability. Positive and negative controls were used to confirm good assay performance.

The sample and control extracts were prepared by immersion of the samples in cell culture Eagle medium (MEM) supplemented with 5 % foetal calf serum (FBS) over 24 h at 37 ± 1 °C in a proportion of 0.5 cm^2 or 0.1 g per mL of cell culture medium. The samples were subjected to sterilisation by γ radiation (20 kGy). The positive control was a natural rubber
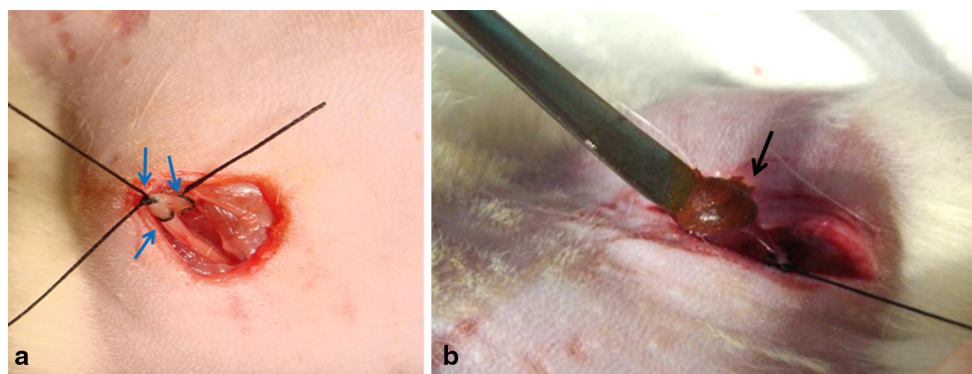
Fig. 1 Ischiatic nerve injury in three different regions of the nerve using suture (*arrow*) and the antioxidant compound application (**b**)

latex film, and the negative control was Al_2O_3 because the antioxidant compound is similar to a ceramic compound. The cell line NCTC clone 929 of mouse connective tissue from the American Type Culture Collection bank (Manassas, VA 20110 USA) was used. The cells were seeded at approximately 5×10^4 cells per each of 96 wells of a microplate. The serially diluted sample and control extracts (100; 50; 25; 12.5 and 6.25 %) were pipetted into each well in triplicate. This microplate stayed in an incubator at 37 °C with a humidified atmosphere with 5 % CO_2 for 24 h. After 24 h, the extract was replaced by 50 µg neutral red per mL of MEM solution, and the microplate was maintained at 37 °C for 3 h to allow for neutral red uptake by living or non-injured cells. The medium in the microplate was discarded, and the plate was washed twice with phosphate-saline buffer (PBS) at pH 7.4 and with wash solution (1 % $CaCl_2$ in 0.5 % formaldehyde). In each well, 0.2 mL of extraction solution (0.5 % acetic acid in ethanol) was added to provoke neutral red liberation by cellular lysates. The optical density (OD) of the microplate was measured in a Spectrophotometer Sunrise, Tecan, at 540 nm.

2.3 Animal surgery

Twenty-five male Wistar rats with weights of approximately 350 g were divided into 3 groups of 5 animals each for each time: Control 1 (Ctl 1), Control 2 (Ctl 2) and experimental (Exp) groups. The Ctl 1 animals were euthanised 15 days after the surgical procedure, and the Ctl 2 and Exp groups were sacrificed 15 and 30 days after the surgical procedure. The rats were kept in an animal house with a controlled temperature of 21 °C. The lighting system followed an international standard of 12 h of artificial light and 12 h of dark with the use of timers. This study was approved by the Ethics Committee in Animal Experimentation CEPA-IPEN/SP (Permit Number: 049/09). All surgeries were performed under sodium pentobarbital anaesthesia, and all efforts were made to minimise suffering.

The animals were previously anaesthetised with intramuscular ketamine (0.1 mL/kg) and xylazine (0.1 mL/kg). The right ischiatic nerve was exposed after a parallel incision to the muscles fibres of the biceps femoris muscle of the right thigh of the rats. The nerve injury was induced by suturing three distinct regions of the nerve, with approximately 3 mm intervals for each suture knot with nylon [19] (Fig. 1a). In the next image (Fig. 1b), it is possible to observe the placement of the antioxidant compound in the local surgical damage.

2.4 Histological analysis

The animals were sacrificed 15 and 30 days after the surgery, and the tissues were collected and processed for histological analysis. Paraffin blocks were sectioned with a thickness of 3 µm and stained with 1 % toluidine blue. Images were obtained using a light microscope and analysed in a computer with the software *Image-ProPlus* 4.1 for Windows (Media Cybernetics, Silver Spring, USA).

3 Results

3.1 Cytotoxicity assay

The cytotoxic effect was evaluated by the cell viability percentage, which was calculated by the average of the optical density of each extract concentration in relation to the cell control, which was considered 100 %. A graph with the cell viability against the extract concentration was made to determine the cytotoxicity index ($IC_{50 \%}$). The $IC_{50 \%}$ is the extract concentration that produces 50 % of death or lysis in the tested cell population. In the graph (Fig. 2), the positive control showed a $IC_{50 \%}$ of approximately 53 %. The antioxidant compound demonstrated no cytotoxic effect, as observed by the cell viability curve similarity to the negative control, which is known as non-cytotoxic compound.

3.2 In vivo study

Macroscopically, the antioxidant compound favoured the process of nerve repair 30 days after its application around a nerve injury with obvious contrast to controls. A disorganisation of the structures was observed in the control group (Fig. 3a). The experimental group with the antioxidant compound presented integrity of the nerve strand and a neoformation under the suture wire (Fig. 3b).

Histologically, in the control group (Ctl 1) was possible to observe the occurrence of signs of nervous degeneration (Fig. 4), which were possibly derived from the local inflammatory process from the surgical intervention of manipulation of the ischiatic nerve.

In the histological samples from group Ctl 2, obtained after 15 days after the surgery, the nerve strand presents, on the region were the suture was performed, an intense nerve degeneration and substitution with adipose/

connective tissue, with the formation of vacuoles and fibrotic tissue encapsulating the suture wire (Fig. 5a). The presence of foreign-body giant cells and a disarray of the morphological structure of the ischiatic nerve were observed, indicating the maintenance of a chronic inflammatory process and ultimately resulting in local degeneration of the nerve. In the experimental group, it was possible to observe the neoformation of the nerve despite the signs of morphological alterations indicating damage to the nerve strand (Fig. 5b). Foreign-body giant cells were observed, indicating the persistence of an inflammatory process in the region.

In the samples collected after 30 days, it was possible to observe a complete degeneration in the control group (Ctl 1), with the substitution of the nerve tissue by adipose tissue and the presence of only a few basement membranes of Schwann cells (Fig. 6a). The experimental groups presented nerve neoformation, with the presence of a well-formed epinerve, separating the neoformed strand or
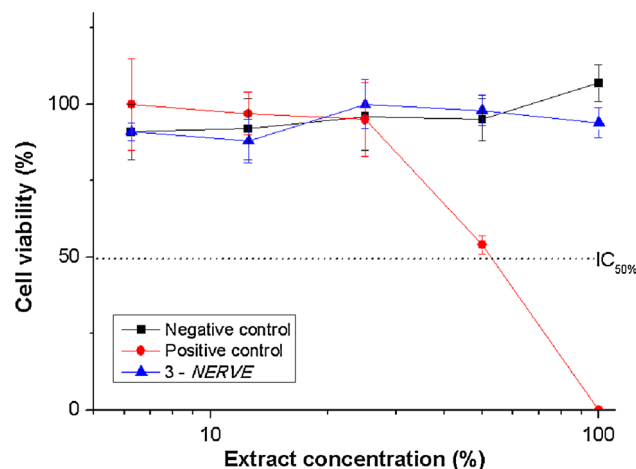

Fig. 2 Cell viability test of the organic compound using the neutral red uptake technique. The sample showed similar behaviour to the negative control and was non-cytotoxic. The positive control had an IC50 of 53 %, i.e., the extract of natural rubber latex in a concentration of 53 % defrauded 50 % of the cell population in the test
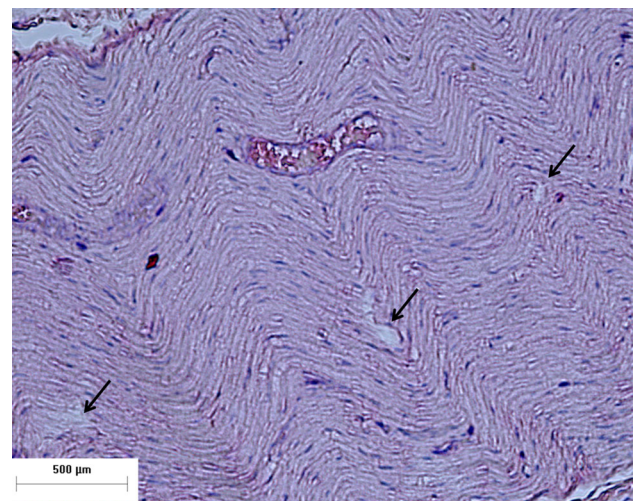

Fig. 4 Signs of morphological alterations of the ischiatic nerve in the control group (Ctl 2) 30 days post-operatively (*arrows*). H & E staining

Fig. 3 The surgical site was re-entered 30 days post-operatively. Intense nerve degeneration was observed in the control (Ctl 2) group (**a**). In the experimental group (Exp), less degeneration was observed and a "bridge" formed between the distal and proximal portions of the nerve strand over the wire suture (*arrows*)
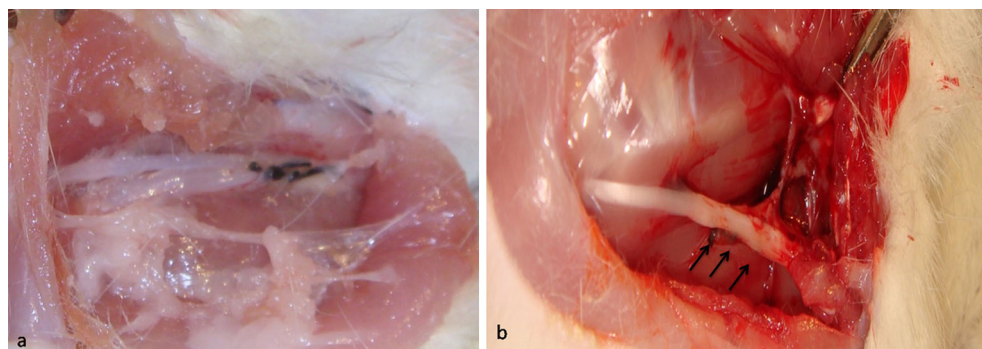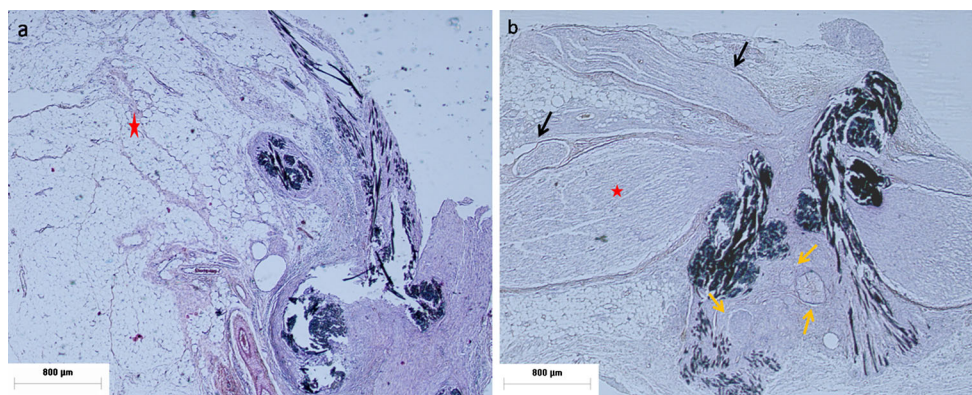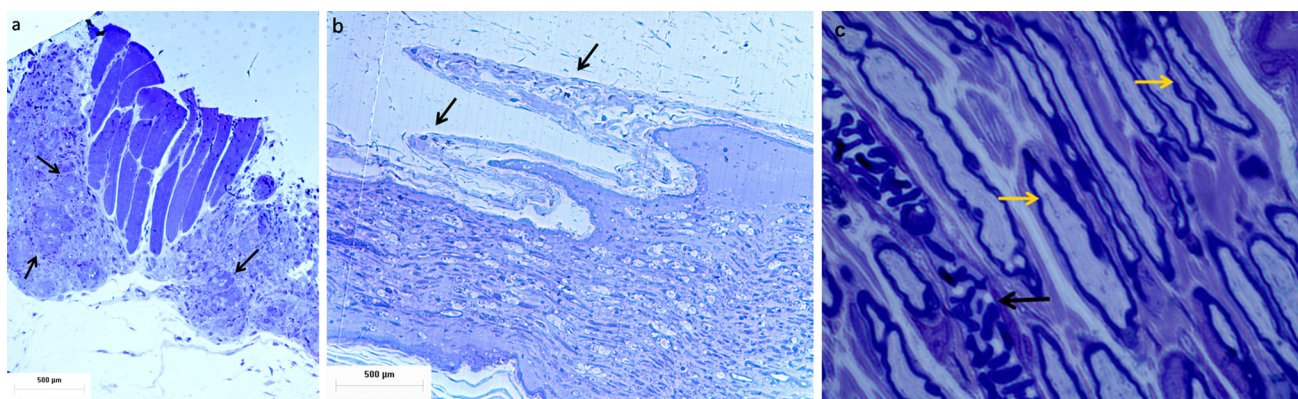
Fig. 5 Histological images of the nerve injury site 15 days post-operatively. In the control group (Ctl 2), a marked degeneration of the distal region of the ischiatic nerve (*red star*) was observed (**a**). The presence of sutures used to perform the injury are shown. In the experimental group (Exp), preservation of the distal region of the nerve strand (*red star*) was observed, with an apparent neoformation of the nerve in the region (*black arrows*) (**b**). The presence of foreign-body giant cells (*yellow arrows*) phagocytising remaining the suture are shown. H & E staining (Color figure online)

Fig. 6 Histological images of the nerve injury site 15 days post-operatively. In the control (Ctl 2) group, the presence of connective tissue inside the nervous strand is shown. Foreign-body giant cells (*arrows*) are present, indicating a chronic inflammatory process and degeneration of the nerve strand (**a**). In the experimental group (Exp), a nerve growth cone was observed (*arrows*), despite an intense degeneration in the main body of the nerve strand (**b**). In **c** the *inset* showing in higher magnification the narrower myelin and blood vessels. Toluidine blue staining

secondary strand from the main strand (Fig. 6b). The secondary strand presented blood vessels and a narrower myelin sheath than normal (ratio strand/sheath), denoting an immature nerve strand (Fig. 6c).

Under higher magnification, it was possible to visualise the main nerve strand that presented morphological changes from the surgical intervention, with an indication of Wallerian degeneration in the axons, and the myelin of the axons from the secondary strand showed no morphological alterations. In the control (Ctl 2) group, an intense degeneration of the nerve strand with the maintenance of the basement membrane of Schwann cells (Fig. 7a) was observed. The experimental animals presented organisation of the secondary nerve strand, parallel to the main strand, showing the formation of a well-organised epinerve separating the main strand from the secondary strand (Fig. 7b).

4 Discussion

In the present study, it was observed that an antioxidant compound promoted neoformation of the nerve after injury of the ischiatic nerve. Macroscopically, nerve tissue neoformation between the distal and proximal region of the injured nerve was observed in the experimental group (Exp). A disarray of the nerve-vascular strand was observed in the control group (Ctl 1). Our hypothesis was based on three different affirmations, in addition to findings already described by Sen [12]: (I) After a trauma, many cells return to their embryonary state; Robbins [20]: (II) Many cytokines expressed in the beginning of the process of repair are present during organogenesis; and Tosh and Slack [21]: (III) the healing of damaged tissue is defined in the early stages of the process of repair. In the beginning of
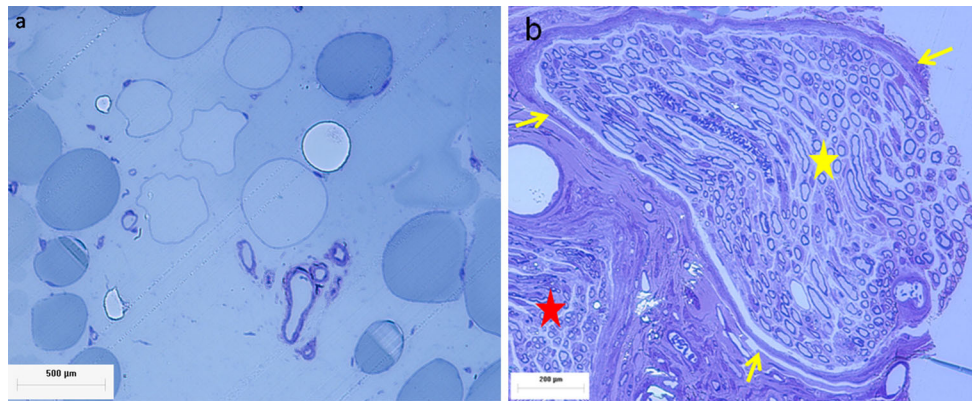
Fig. 7 Histological images of the nerve injury site 30 days post-operatively. In the control (Ctl 2) group, an intense degeneration of the nerve chain including without maintaining the basal membrane of Schwann cells (**a**). The experimental animals presented organisation of a secondary nerve strand (*yellow star*) parallel to the main strand (*red star*) (**b**). The formation of a well-organised epinerve (*yellow arrows*) separating the main strand from the secondary strand is shown. Toluidine blue staining (Color figure online)

the process of repair, there is a great cellular and tissue potential that proportionate favourable conditions to the repair of the damaged tissue, which are linked directly to the time and extension of the damage [22]. An early intervention might improve the process of tissue repair.

Our strategy was to develop a biomaterial that locally provides functional microelements with the ability to mediate the repair process. The gradual liberation of antioxidants and a metabolic substrate capable of interfering positively in the cellular activity and protein synthesis might aid in the process of repairing nervous tissue. The histological results observed in this experiment indicated nerve neoformation, mainly at 30 days post-operatively (Fig. 6b). The control group without the application of an antioxidant organic compound (Fig. 6a), showed significant degeneration with invasion of the connective tissue and foreign-body giant cells and complete degeneration of the region distal to the nerve injury at 30 days (Fig. 7a).

The antioxidant organic compounds are composed of substances that favour the process of nerve repair. We can succinctly exemplify a possible form of action by two different pathways: 1- oxidative protection, 2- supply of metabolic substrate. The vitamin components of β-carotene, α-tocopherol, B complex and calciferol (vitamin D) have direct or indirect protective functions against reactive oxygen species (ROS). β-carotene and α-tocopherol act against lipid peroxidation and damage to the cellular DNA [23, 24]. The B complex is involved in the production of metabolic substrates connected to the production of α-amino acids and α-acetic acids, substrates for the formation of proteins and ATP, which in turn indirectly protects the cells from the action of reactive oxygen species and limits the cellular damage [25, 26]. The D vitamin has been associated with an increase in the synthesis of anti-inflammatory/repairing cytokines such as TGF-β and IL-8

and a reduction of TNFs-α, limiting the local inflammatory process [27].

The inorganic compounds of zinc and selenium are part of prosthetic groups of the Superoxide dismutases (SOD) enzymes that catalyse the dismutation of superoxide into oxygen and hydrogen peroxide. They are an important antioxidant defence mechanism in nearly all cells exposed to oxygen and glutathione and act as enzymatic co-factors in the cellular protection [28–32]. There is a search for a path of cellular protection, proportionated by the oxidative agents by tocopherols, without interfering with the production of free radicals that signal the neo-vascularisation of the damaged tissue [33, 34]. By another path, a larger local available concentration of amines from the B group, which is related to the synthesis of α-amino acids and α-acetic acids (proteins and ATPs), can favour the repair of damaged tissue. This results in reducing the cellular damage through antioxidants and through another path supplying metabolic substrates capable of sustaining the metabolism of the local cells.

Further evaluations should be performed to investigate the effects through in vivo electrophysiological studies that are important to determine the potential clinical impact. However, in vivo electrophysiological studies in mice are rare, because of the delicate preparations required and the difficulties in keeping anaesthetized mice alive long enough [35]. In addition, other important assessments are being carried out in new studies by immunocytochemistry and ultrastructure to validate the mechanism of antioxidant action as pharmacodynamics of compound.

The components of the hydrolysed collagen, glycosaminoglycan and chondroitin sulphate are linked to the formation of the application matrix. This matrix facilitates its use in surgical procedures, making the material plastic, and allows for the easy manipulation for this experiment.

Multiple strategies, such as the induction of growth factors or the inhibition of deleterious factors, have been related to the protection of the peripheral nerve, with the objective of enhancing the repair of these structures, but all of these have important limitations, which are mainly related to the time after the initial trauma, including this proposal, which indicates the early use of the organic material ℥-NERVE. We believe that, depending on the level of degeneration of the distal stump, only the use of autogenous grafts might have some result. The use of the organic early can reduce the damage to the peripheral nerve bundle, reducing the extension of nerve damage and its consequences.

5 Conclusions

The current study presented effective repair of the ischiatic nerve in Wistar rats by forming a new connection between the distal and proximal portions of the injured nerve strand using an antioxidant compound in the early phase of the injury. These findings might represent the beginning of a study that can represent an alternative treatment for peripheral nerve injury. Further studies are needed to validate these findings.

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