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A Romanian therapeutic approach to peripheral nerve injury

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Abstract

The study of nerve regeneration and functional recovery of the injured peripheral nerves represents a worldwide subject of clinical and scientific research. Our team aimed to obtain the first guide for nerve regeneration, bioartificial and biodegradable, using exclusively Romanian resources and having the advantages of price and quality, over the imported nerve conduits already used in clinical practice. First steps of this project consisted in obtaining the prototype of nerve guide conduit and its' testing *in vitro* and *in vivo*. Tests of physicochemical characterization, FTIR (Fourier Transform Infrared) spectrometry, thermal analysis (differential calorimetry, thermogravimetry), electron microscopy, water absorption and enzymatic degradation of the obtained prototype were followed by *in vivo* testing. The first results, obtained on a group of Brown Norway rats who suffered experimental lesions of 1 cm at the level of left sciatic nerve, which have then been repaired using the Romanian conduit prototype, are favorable in terms of biocompatibility, biodegradable capacity and support of nerve regeneration.

***Keywords*:** nerve regeneration, lesion of sciatic nerve, collagen nerve conduit.

Introduction

The study of nerve regeneration and spontaneous or therapeutic functional recovery of the injured peripheral nerves represents an area of practical, but also scientific interest, because of the complications generated and the high costs associated to this pathology [1].

The microsurgical techniques currently used to correct peripheral nerves injuries in humans are the microsurgical suture of nerve ends (termino-terminal neurorrhaphy) without tensioning the proximal and distal nerve segments [2] and, when the distance between axonal ends is too big to realize a non-tensioned suture, the use of autogenic grafts – originating from sensitive nerves whose sacrifice does not generate major functional impairments [3]. Since the number of nerves available to be sacrificed, in order to be used as autogenic grafts, are limited and the functional results of the grafting techniques are limited [4, 5], tubular structures (nerve conduits) have been created from synthetic or natural materials, non-resorbable or biodegradable [6], with role in guiding axonal growth, minimizing the invasion of fibrous tissue into the injured site and assuring an optimal microenvironment for axonal regeneration [3].

The intense preoccupation of the scientific community to develop new therapeutic strategies to promote the nerve regeneration [7], and the appearance in 2007, in Romania, of the first variants of imported nerve conduits at very high prices (EUR 600–1000 for 3 cm of conduit) led to the development of a project whose aim was to obtain the first guide for nerve regeneration, bioartificial and biodegradable, using exclusively Romanian resources and having the advantages of quality and price, over the imported conduits already used in clinical practice. The project is developed by a partnership between the Experimental Microsurgery Laboratory, "Carol Davila" University of Medicine and Pharmacy, Bucharest, from the Emergency Hospital Bucharest, and the Romanian National Leather and Footwear Institute, Bucharest.

For this goal, a research protocol has been elaborated, comprising four main phases:

1. Manufacturing several batches of nerve conduits, with different physicochemical properties;
2. *In vitro* testing of biocompatibility and biodegradable capacity of these bathes of conduits;
3. *In vivo* testing on animal models, of the prototype of nerve conduits;
4. Morpho-functional analysis of the nerve regeneration obtained in animal models, through conventional grafting technique compared to already marketed collagen tubular guides and the prototype of nerve conduits selected following *in vitro* and *in vivo* tests during the preceding stages.

This article will present the results of the first three stages of the above research protocol.

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Materials and Methods

Materials that compose the nerve conduit and the technical production protocols have been selected so that the manufacturing process can be facile, reproducible, with precise manipulation of physico-chemical properties and low production costs, while the complexity of the implantation techniques remains minimal.

We have chosen to produce a collagenic nerve conduit [8]. The raw material to produce type I collagen has been the raw veal skin (the dermis of animals under 2-year-old), composed of weakly reticulated collagen. The veal skin was previously analyzed for bovine spongiform encephalopathy, according to Council Directive 93/42/EEC. In order to confer mechanical resistance to the tubular structures, it has been used the technology of collagen extraction in fibrillar form [9].

Used *in vivo*, the collagen biomaterials are exposed to enzymatic attack; to avoid this process, the structures are stabilized by reticulation. Chemical reticulation by glutaraldehyde (GA; producing additional chemical bonds between molecules and/or fibrils of collagen) assured thermal, enzymatic and mechanical stability to the conduits and reduced their immunogenicity [10]. Reticulation occurred at 4^0C, in 24 hours. After reticulation, the collagen gel has been lyophilized (quick freezing at -40^0C, followed by drying) in order to obtain a spongious (microporous) structure, with properties similar to extracellular matrix. Lyophilization took 24 hours and was realized using Delta LSC 2-24 (Martin Christ, Germany) lyophilizer. After lyophilization, the conduits with three-dimensional structure have been immersed for maturation, several days, in a buffer solution of phosphates, pH 7.4, afterwards being dried at 25^0C in a Venticell drying oven with Hepa filter.

Following the above physicochemical processes, two batches of nerve conduits resulted, with different reticulation grades, lengths and inner and external diameters. These have been *in vitro* tested for physico-chemical characterization, such as Fourier Transform Infrared (FT-IR) spectrometry, thermal analysis, electron microscopy, water absorption and enzymatic degradation. A JASCO FT-IR-4200 spectrometer has been used at a resolution of 4 cm^{-1}, making 30 acquisitions for every sample and the bands characteristic to type I collagen: amide I, 1600–1740 cm^{-1}; amide II, 1485–1590 cm^{-1}; amide III, 1190–1300 cm^{-1}; amide A, around 3300 cm^{-1}; amide B, around 3070 cm^{-1} [11].

Electron microscopy was performed using Hitachi S-2600N microscope with Energy-dispersive X-ray spectroscopy detector, at resolution up to 4 nm and 50× augmentation.

Enzymatic degradation has been investigated by monitoring the samples' weigh loss, depending on the time of exposure in type I collagenase solution, *Clostridium histolyticum*. Collagen conduits have been weighted wet and afterwards introduced in a buffer solution of phosphate and collagenase (1 μg/mL) at pH 7.4, incubated at 37^0C. At regular time intervals, conduits have been weighted. Water absorption was calculated after the conduits being hydrated with water, at 37^0C, for 24 hours, referring the difference between the matrix saturated with water and the initial mass of the dry matrix to the initial mass of dry matrix. Thermal analysis was realized with a Caloris heated micro-table, at a temperature range of 22–100^0C, in order to identify the temperature where take place the conformational transition of molecules from triple helix to statistic coil.

After the above described tests, the optimal composition of nerve type conduits was selected and it was produced a third batch, characterized afterwards by the mentioned physicochemical tests.

The testing of biocompatibility of the nerve conduit prototype was realized by colonization with cells lines HTB11 and HTB14, purchased from ATCC (American Type Culture Collection). The colonization was realized in the culture environment DMEM (Dulbecco's Modified Eagle Medium), with incubation at 37^0C for seven days. After this interval, cells have been washed, stabilized with 2% paraformaldehyde, cryoprotected, stained with Hoechst 33258 and analyzed with a Nikon microscope with epifluorescence.

The biocompatibility and biodegradable capacity of the prototype batch have been *in vivo* tested on a number of eight male Brown Norway rats, aged between 10 and 16 weeks (13 weeks median), weighting between 250–300 g. All animals have been treated accordingly to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [12] and the Romanian legislation [13]. The used rats have been purchased from the Biobase of the "Cantacuzino" Romanian National Institute of Research and Development in Microbiology and Immunology, Bucharest. In order to experiment on animal models, we obtained the approvals of the Ethic Commissions of the "Carol Davila" University of Medicine and Pharmacy, Bucharest, and of the "Floreasca" Emergency Hospital, Bucharest.

To every animal included in the study, the left sciatic nerve has been sectioned, in the middle region, creating a defect of 1 cm length [14]. The bioartificial nerve conduits have been moistened with saline solution before being implanted into the rats' bodies. Conduits slightly longer than the realized nerve defects have been chosen. In order to fix the nerve conduit to the two sectioned sciatic nerve ends, a nylon 8/0 suture has been used, with suture in two points for each end of the nerve.

Before making the last suture point, the neural regeneration chamber represented by the interior of the conduit has been filled with saline solution, in order to prevent the formation of a blood cloth that could block the axonal fibers from the proximal neural end to their way to the distal neural end. At every 30 days, for a total period of 90 days, two rats have been sacrificed for macroscopic and microscopic analysis of the injured area.

Results

The physicochemical characteristics of the initial batches of nerve conduits were as follow:

▪ Batch No. I: nerve conduits with collagen concentration of 1.5%, reticulated with 0.25% glutaraldehyde (reported to the collagen concentration), inner diameter 2.5 mm, external diameter 2.6 mm, and variable length (1–1.2 cm);

▪ Batch No. II: nerve conduits with collagen concentration of 2%, reticulated with different concentrations of glutaraldehyde (0–1%), inner diameter 2.5 mm, external diameter 2.7 mm, and variable length (1–2.5 cm).

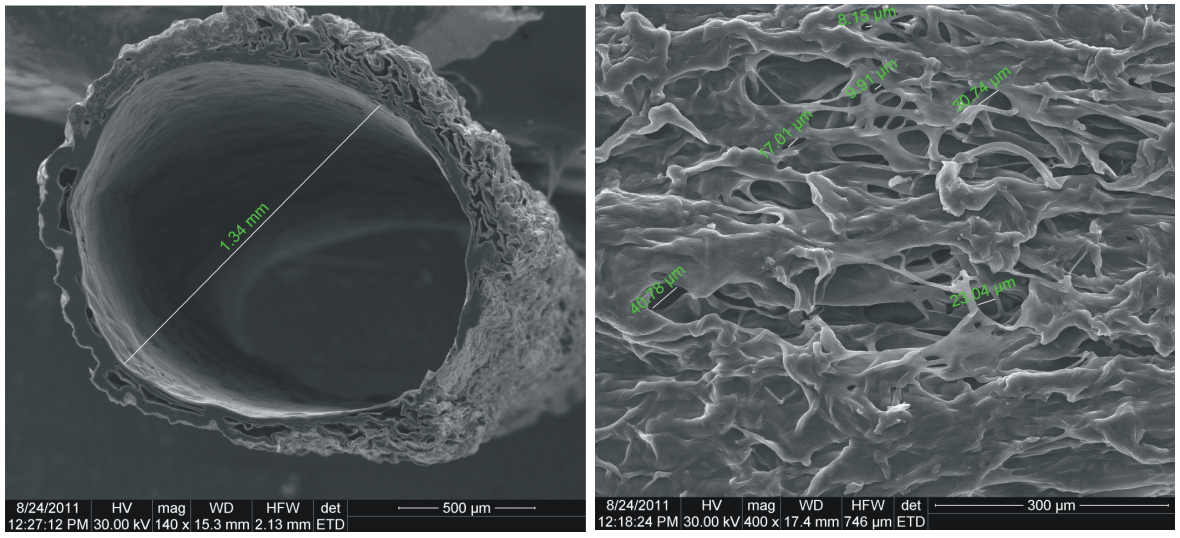
FT-IR analyses showed that, during the process, collagen remained with triple helix structure. Depending on the concentration of the reticulating agent, the nerve conduits presented different thermal behaviors, the increase of the reticulation grade producing both the increase of the denaturation temperature and melting temperature. The water absorption of conduits in 24 hours decreases as the reticulation grade rises. The stability to enzymatic degradation increases with the grade of reticulation.

The results of electron microscopy show that the sample reticulated with 0.8% GA presents the most uniform microporous structure. Thus, it resulted a prototype batch of nerve conduits that will be tested in order to be produced at a larger scale.

The prototype batch is characterized by a concentration of 2% collagen and reticulation with 0.8% glutaraldehyde, variable dimension of the inner diameter (0.54; 0.7; 0.86; 0.15 and 2.5 mm) and variable lengths (1–1.5 cm). The denaturation temperature is 94.8^0C (compared to 83^0C for reticulation with 0.2%); the conduits degrade in proportion of 54% in the collagenase solution and absorb water in proportion of 80%. All these data demonstrate a good stability of this nerve conduit (Figure 1).

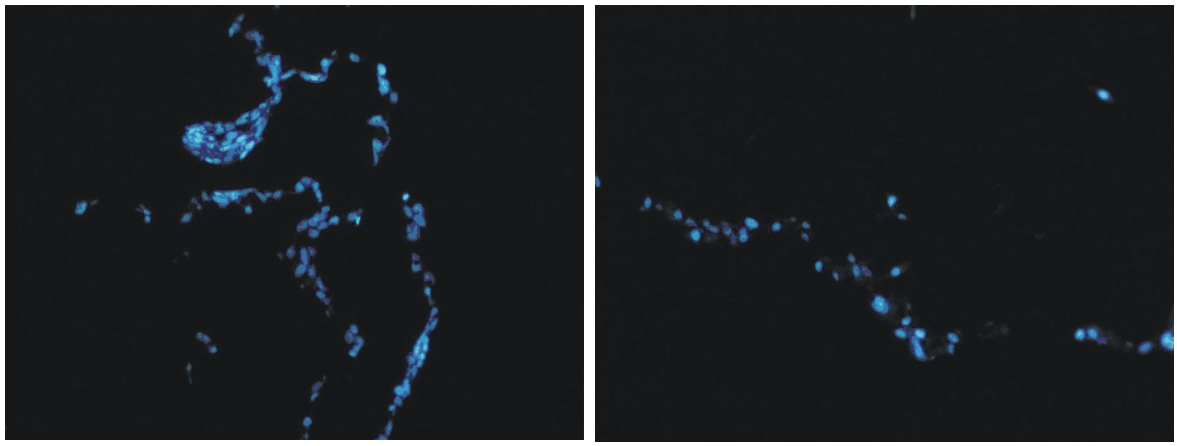
According to the biocompatibility tests, the prototype of nerve conduit supported the adhesion and growth of neuronal cells HTB11 and HTB14 (Figure 2).

The results of *in vivo* tests showed that after 30 days from implantation, the nerve conduits maintained the structural integrity in proportion of 100%. At 60 days from implantation, also there was structural integrity, without signs of acute inflammation around the conduit. After the section of the conduit, optical microscopy identified axonal fibers that traversed the regeneration room. At 90 days, the structural integrity of the prototype nerve-guide conduit was maintained and there was no acute inflammatory tissue around the conduit (Figures 3 and 4).



(a) (b)

Figure 1 – ***Electron microscopy images of the nerve conduits prototype: (a) Section of the nerve conduits; (b) The microporous structure of the nerve conduits.***



(a) (b)

Figure 2 – ***Cells cultures: (a) HTB14 and (b) HTB11 one week after the seeding on the nerve conduit. Hoechts stain.***

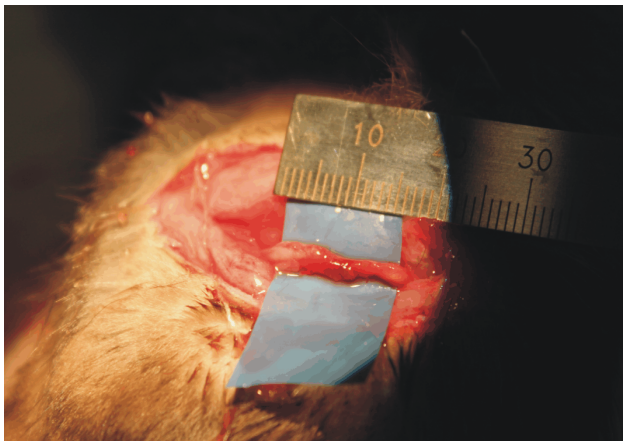


Figure 3 – ***Macroscopic aspect of the nerve conduit, at 60 days from implantation.***

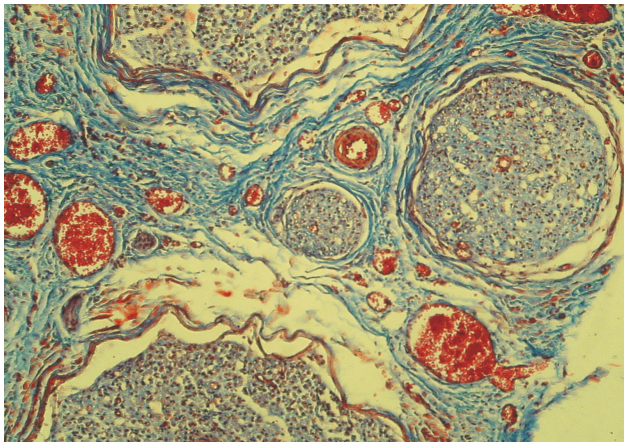


Figure 4 – ***Aspect of optical microscopy (Masson's trichrome stain, ob. 10×). Nerve fibers traverse the regeneration room, at 60 days from implantation.***

Discussion

The essential properties of a nerve conduit are the biocompatibility and biodegradable capacity. A non-biodegradable tube generates, over time, local inflammatory reactions, making necessary a new surgical intervention for removal [4]. A too rapid degradation of the tubular wall will lead to the exposure of the incompletely regenerated nerve and formation of fibrous tissue. Products that result from the biodegradation of the nerve conduits should not be toxic, carcinogenic, mutagenic or induce immune reactions, including allergies [15].

The mechanical properties (flexibility, mechanical resistance, elasticity) as well as the dimensions (tubular wall thickness, tubular length, luminal tubular diameter) influence the result of nerve regeneration [16–18]. For example, a tube too rigid will generate chronic compression, limiting the growth of nerve fibers and producing inflammatory reaction in the surrounding tissues [9].

With respect to the above characteristics, collagen has been chosen to produce the nerve conduits [19]. Collagen, the principal protein of human and animal connective tissue, presents a good biocompatibility, being biodegradable, without releasing substances with toxic potential [20].

At the same time, collagen can form structures with good mechanical resistance, but with permeability favorable to nerve regeneration. The haptotactic properties of the collagen assure the proliferation and alignment of Schwann cells and angiogenesis. Although 28 types of collagen exist, the most widely spread is type I collagen [6].

The realization of the nerve conduit "NeuraGen (R)" – a collagen conduit realized by Integra LifeSciences Corporation and approved for human use by the *Food and Drug Administration* (USA) and the *European Commission* – required more than 15 years of comparative studies on animals models and, afterwards, in human beings [21, 22]. Our results at *in vitro* and *in vivo* tests have been encouraging for continuing the study with comparative morpho-functional analysis, between the Romanian prototype and a collagen conduit already approved for human use, as well as with the comparison with the standard therapeutic method of grafting with autologous nerve graft. These studies on animal models (rats) are already in progress, with results favorable to the continuation of the study with the Romanian prototype of nerve guide conduit.

Conclusions

We consider that presented results and our following experiences will lead us to the production and patent of the first Romanian nerve guide conduit.

Acknowledgments

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