Available online at www.sciencedirect.com

SciVerse ScienceDirect

Procedia Engineering 59 (2013) 206 – 210

Procedia Engineering

www.elsevier.com/locate/procedia

3rd International Conference on Tissue Engineering, ICTE2013

Employment of a collagen conduit soaked in an angiogenic fraction derived from natural latex in the regeneration of sciatic nerve of rats

Marcos Vinícius Muniz Ganga[1], Valéria Paula Sassoli Fazan[1], Joaquim Coutinho-Netto[2], Carlos Henrique Rocha Catalão[1], Camila Araujo Bernardino[1], Rui Seabra Ferreira Junior[3], Luiza da Silva Lopes[1]*

[1] *Department of Surgery and Anatomy, School of Medicine of Ribeirao Preto, University of Sao Paulo (USP), Ribeirao Preto-SP, Brazil*
[2] *Department of Biochemistry and Immunology, School of Medicine of Ribeirao Preto, University of Sao Paulo (USP), Ribeirao Preto-SP, Brazil. In memoriam*
[3] *Center of Studies of Venoms and Venomous Animals (CEVAP), University of Sao Paulo State (UNESP), Botucatu-SP, Brazil*

Abstract

Peripheral nerve injuries are very frequent in medical practice and although the use of autografts remains the standard procedure to repair the gap between the proximal and distal stumps, alternative techniques have been proposed to avoid complications to the donor site and speed up the nerve regeneration process. A membrane produced from natural latex has been used successfully both experimentally (angioplasties, esophagus neoformation, reconstruction of the ocular conjunctiva) and clinically (myringoplasties, treatment of skin ulcers), showing angiogenic potential and leading to tissue neoformation. The purpose of this study is to evaluate the capacity of a conduit made with collagen and soaked in an angiogenic protein extracted from latex in accelerating and improving the regeneration after surgically sectioning the rat sciatic nerve. Adult Wistar male rats had the sciatic nerve sectioned under anesthesia with a subtraction of a 10mm nerve fragment. Then they received an autograft implant (inverted nerve fragment) or the interposition into the nerve gap of a tube made up of that collagen and soaked in an angiogenic fraction derived from natural latex. At the endpoint of the experiments, the animals were submitted to neurological function evaluation, and killed by an overdose of anesthesia and exsanguination. The implants (collagen conduit or autograft) and the tibialis and gastrocnemius muscles were removed, fixed and processed with embedding in resin. Cross-section of implants and muscles were performed and prepared in histological slides to observation under light microscopy. Functional recovery was correlated with histopathological analysis. Both

* Corresponding author. Tel.: +55-016-3602-4690; fax: +55-016-3633-0836.
E-mail address: luslopes@fmrp.usp.br

Selection and peer-review under responsibility of the Centre for Rapid and Sustainable Product Development, Polytechnic Institute of Leiria, Centro Empresarial da Marinha Grande
doi:10.1016/j.proeng.2013.05.112

showed a significant better performance in rats that received implants with the collagen conduit soaked in angiogenic fraction derived from natural latex.

Selection and peer-review under responsibility of the Centre for Rapid and Sustainable Product Development, Polytechnic Institute of Leiria, Centro Empresarial da Marinha Grande.

Keywords: sciatic nerve injury; nerve regeneration; biomaterials; angiogenic fraction from natural latex; *Hevea brasiliensis*.

1. Introduction

Traumatic injury to peripheral nerves is not an uncommon casualty and results from trauma due to vehicle accidents and less commonly from penetrating trauma, falls, and industrial accidents [1]. Even with advances in repair techniques and new technologies involved, the surgical results are often disappointing especially when there is total transected nerve or when the nerve injury resulted in substance loss between the two nerve stumps [2]. These facts led to the development of techniques using nerve grafts or conduits that bridge the ends and guide the growth of nerve fibers between the stumps, including the use of natural or synthetic materials [3-5]. Thus, a new protein composed by natural latex extracted from rubber tree *Hevea brasiliensis* was initially used as a biomembrane with characteristics that enabled cell adhesion and stimulation of various cell types involved in the healing process [6-7]. Further studies showed that the protein extracted from the rubber tree *Hevea brasiliensis* (P-1) has equivalent properties [8].

The aim of this study was to evaluate is to evaluate the capacity of a conduit made with collagen and soaked in an angiogenic protein extracted from latex (P-1) in accelerating and improving the regeneration after surgically sectioning the rat sciatic nerve.

2. Methods

2.1. Animals

All animals were treated in accordance with guidelines by the COBEA (Brazilian College of Animal Experimentation) and protocols were approved by the local animal ethics committee (CETEA - School of Medicine of Ribeirão Preto, University of São Paulo – USP), protocol # 080/2005. We used adult male Wistar rats that were bred locally. The rats were clinically healthy and weighed 250g (± 20g). They were distributed in 3 experimental groups, each group with an equal number of rats (n=5), anesthetized and operated with transplantation of autograft (group 1) or a conduit made with collagen and soaked (group 3) or not (group 2) in an angiogenic protein extracted from natural of *Hevea brasiliensis* latex (P-1). The endpoint of experiments was 8 weeks after the transplantation surgery.

2.2. Surgical procedures

Animals were anesthetized with 10% Ketamine 90mg/Kg (Ketamina®- Pfizer) associated with Xylazine 10mg/Kg (Rompum®- Bayer), administered via intraperitoneal injection. The right hind legs were shaved, the skin disinfected and aseptic techniques used to ensure sterility. After skin incision and dissection of the muscle planes, the sciatic nerve was identified, sectioned, and 10mm of nerve was removed. In the autograft rats the nerve was excised, inverted, and reimplanted between the proximal and distal stumps of the nerve. In the groups of collagen conduit the rats were transplanted with a 12mm conduit made of collagen soaked or not in a solution of a protein extracted from the rubber tree *Hevea brasiliensis* (10µg of P-1/monoolein gel), interposed between the proximal and distal stumps and sealed with a new fibrin sealant was developed by a group of researchers from the Center for the Study of Venoms and Venomous Animals, in Sao Paulo State, Brazil [9]. The conduit dressed the nerve stumps, leaving a 10-mm gap between nerve stumps. The muscle layer was re-approximated with 4-0 nylon

sutures, and the skin closed with 4-0 sutures. Each rat received one implant that was removed 8 weeks post surgery.

2.3. Functional analysis by walking track and footprints analysis

Before surgery, animals were trained once on a 1.5m long CatWalk runway (Noldus Information Technology, The Netherlands). Only animals able to make un-interrupted runs between 1.0 and 2.0s were selected to surgery. At the endpoint of experiments (8 weeks after surgery), at least three CatWalk runs were obtained per animal, as well as SSI data. Three CatWalk runs typically showing four step cycles each were analyzed for each animal and time point. Importantly, throughout the study, the animals were examined for signs of autotomy and contractures, which may strongly affect the animals' well-being and behavioural performances.

2.4. Histological processing and morphological analysis

Histopathological analysis was conducted on samples corresponding to the regions proximal upper stump, distal lower stump of the cut nerve, and the medial part of the collagen conduit content or autograft, and gastrocnemius and anterior tibial muscles. The animals were euthanized after a period of 8 weeks. The sciatic nerves were mounted on a piece of paper and immersed in a fixative solution (2% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer). After the fixation, the samples were postfixed in 0.5% osmium tetroxide, dehydrated, embedded in epoxy resin; 0.5μm semi-thin sections were cut on an ultramicrotome, and stained with 1% Toluidine Blue. The fragments of gastrocnemius and anterior tibial muscles were immersion fixed in 3% buffered paraformaldehyde, dehydrated in a sequence of alcohols and xylene, and embedded in paraffin. Sections (5μm thickness) were stained with Masson's trichrome staining.

The histological slides were digitized using a Carl Zeiss Axiophot microscope at 20x (muscles) or 40x-magnification (nerves) and Carl Zeiss Kontron 2.0 software. We evaluated the presence of regenerated nerve within the conduit and in the autograft, the conditions of the nerve segment before de proximal point of surgery, the presence of nerve regeneration in the distal portion of the implant, degree of myelination, and the health of muscles.

2.5. Data presentation and statistical analysis

All data are presented as mean ± standard error of the mean and were analyzed to confirm a normal distribution. Using one-way ANOVA followed by a Tukey post test comparison, data (mean ± SEM) were considered to be statistically significant when p-value was $p<0.05$. A non-parametric test was chosen when data did not pass normality test. Software used was GraphPad Software´s InStat, version 3.06 for Windows (San Diego, USA). The histological analysis embraces the presence or no of myelinated nerve fibers into the collagen conduit and the condition of muscle, including the fibrosis.

3. Results and discussion

3.1. Functional recovery by walking track and footprints analysis

The implanted animals (particularly that received collagen conduits soaked in an angiogenic protein extracted from natural of *Hevea brasiliensis* latex P-1) showed a tendency for auto-mutilation of their digits in the operated limb, making this analysis extremely difficult. Some animals from the autotransplanted nerve group (group 1) dragged their paws of the operated side. So, in most rats only the qualitative appearance of footprints was evaluated. The records showed better function of right hind paw in the group of rats that received collagen conduits soaked in an angiogenic protein extracted from natural of *Hevea brasiliensis* latex P-1, but the difference has not statistical significance.

The relation between right and left hind paws were considered to evaluate the stand (s), the print length (cm), the print area (cm^2) and the mean intensity of step. The results are showed on table 1.

Table 1. Results of CatWalk analysis (mean +/- SEM).

	Group 2	Group 3
Stand (RH/LH)	1.3107+/-0.0465	1.1952+/-0.1404
Print length (RH/LH)	1.5335+/-0.2292	1.2914+/-0.4715
Print area (RH/LH)	1.2107+/-0.2978	0.8826+/-0.5935
Mean intensity (RH/LH)	1.0161+/-0.1163	0.9301+/-0.0303

3.2. Morphological analysis

Histological aspects of proximal stump, new nerve, and distal stump in rats of group 2 (collagen conduit without P-1) and rats of group 3 (collagen conduit soaked in P-1) are represented in Figure 1. There was greater neural regeneration into collagen conduit soaked with P-1 group than in autotransplanted nerve group or collagen conduit without P-1, 8 weeks after surgery. Muscles of collagen conduit groups (without or soaked with P-1), are seen in figure 3, and show better quality of muscle fibers in rats of group 3 (with P-1). The muscles of autotransplanted or collagen conduit without P-1 groups usually had muscular cells atrophy and fibrosis surrounding them (Figure 2).

The results of functional tests used come in proving that the performance of the operated side hind paw is better in animals that have been transplanted with collagen conduits soaked in an angiogenic protein extracted from natural of *Hevea brasiliensis* latex (P-1). In the same animal group (3), the quality of regeneration was also slightly better, with higher density of myelinated nerve fibers, as well as with reduced atrophy and fibrosis of muscles.

Thus, an absorbable conduit engineered and soaked with P-1 can be considered as a potential conduit for the regeneration of injured peripheral nerves in cases of traumatic damages.

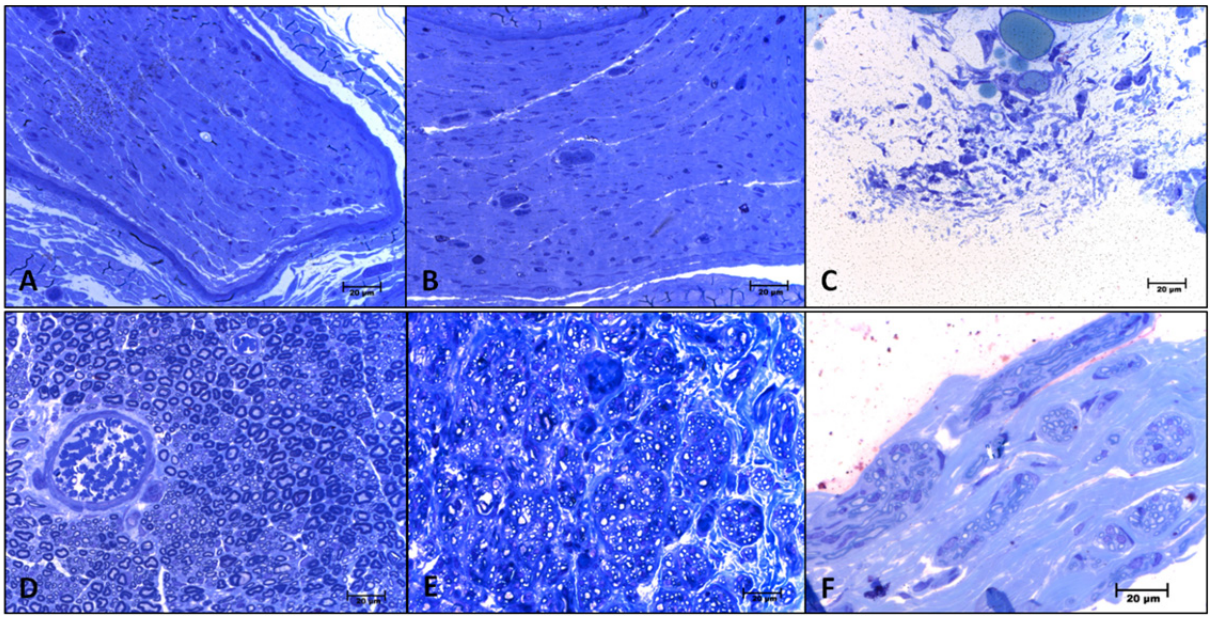

Figure 1. Light microscopy of semi-thin sections (0.5µm) stained with toluidine blue 8 weeks after the surgical procedure. Myelinated fibers are characterized by an oval-round white area surrounded by a blue band of myelin. (Upper line: A=proximal stump nerve, B=new nerve into collagen conduit, and C=distal stump nerve, Group 2=collagen conduit without P-1). (Lower line: D=proximal stump nerve, E=new nerve into collagen conduit, and F=distal stump nerve, Group 3=collagen conduit soaked in P-1). Rats implanted with collagen conduit soaked in P-1 show better quality of regenerated nerve. Toluidine blue stain. Objective magnification: 40x oil.

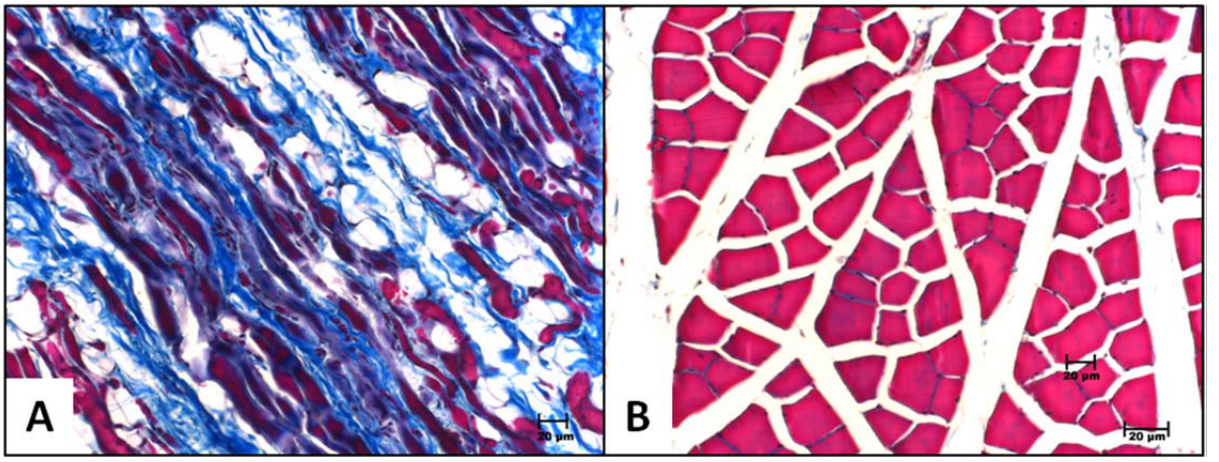

Figure 2. Photomicrographs showing harvested tibial muscle (A= group 2 rat=collagen conduit without P-1; B=group 3=collagen conduit with P-1), after embedding in paraffin. Rats implanted with collagen conduit without P-1 show muscle with atrophy and fibrosis (A). Masson's trichrome staining. Objective magnification: 20x.

Acknowledgements

The authors are grateful to Antonio Renato Meirelles e Silva (Laboratory of Applied and Experimental Neurology, Department of Neuroscience and Behavior Science, Scholl of Medicine of Ribeirão Preto, University of São Paulo) for his assistance with microscope photographs. Technical assistance of Daniel Mazzetto is acknowledged. Dr. Ganga held a Postgraduate scholarship from CNPq (National Council for Scientific and Technological Development) – Brazil.

References

[1] Robinson, L.R., 2000. Traumatic injury to peripheral nerves, Muscle Nerve 23, p. 863.
[2] Battiston, B., Geuna, S., Ferrero, M., Tos, P., 2005. Nerve repair by means of tubulization: literature review and personal clinical experience comparing biological and synthetic conduits for sensory nerve repair, Microsurgery 25, p. 258.
[3] Ayhan, S., Yavuzer, R., Latifoglu, O., Atabay, K., 2000. Use of the turnover epineurial sheath tube for repair of peripheral nerve gaps, Journal of Reconstrutive Microsurgery 16, p. 371.
[4] Bian, Y.Z., Wang, Y., Aibaidoula, G., Chen, G.Q., Wu, Q., 2009. Evaluation of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) conduits for peripheral nerve regeneration., Biomaterials 30, p. 217.
[5] Zhao, Z., Wang, Y., Peng, J., Ren, Z., Zhan, S., Liu, Y., Zhao, B., Zhao, Q., Zhang, L., Guo, Q., Xu, W., Lu, S., 2011. Repair of nerve defect with acellular nerve graft supplemented by bone marrow stromal cells in mice, Microsurgery 31, p. 388.
[6] Balabanian, C.A., Coutinho-Netto, J., Lamano-Carvalho, T.L., Lacerda, S.A., Brentegani, L.G., 2006. Biocompatibility of natural latex implanted into dental alveolus of rats, Journal of Oral Science 48, p. 201.
[7] Alves de Sousa, L.C., Ribeiro de Toledo Piza, M., Coutinho-Netto, J., Ruiz, D.B., Schmidt, V.B., 2007. Latex biomembrane: a new method to coat the open cavity in tympanomastoidectomies, Brazilian Journal of Otorhinolaryngology 73, p. 331.
[8] Issa, J.P., Defino, H.L., Pereira, Y.C., Netto, J.C., Sebald, W., Bentley, M.V., Iyomasa, M.M., Ervolino, E., 2012. Bone repair investigation using rhBPM-2 and angiogenic protein extracted from latex, Microscopy Research and Technique 75, p. 145.
[9] Barros, L.C., Ferreira Jr, R.S., Barraviera, S.R.C.S., Stolf, H.O., Thomazini-Santos, I.O., Mendes-Giannini, M.J.S., Toscano, E., Barraviera, B., 2009. A new fibrin sealant from Crotalus durissus terrificus venom: applications in medicine, Journal of Toxicology and Environmental Health, Part B: Critical Reviews 12, p. 553.