Histol Histopathol (2010) 25: 741-747

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Oxidized regenerated cellulose does not prevent the formation of experimental postoperative perineural fibrosis assessed by digital analysis

Pedro Hernández-Cortés[1], Magdalena Peregrina[1], Jose Aneiros-Fernández[2], Mohamed Tassi[2], Miguel Pajares-López[1], Miguel Toledo[1] and Francisco O'Valle[2]

[1]Department of Traumatology and Orthopaedic Surgery and

[2]Department of Pathology, IBIMER, "San Cecilio" Clinical Hospital and University of Granada, Spain

Summary. Introduction: It is difficult to prevent and treat intra- and peri-neural fibrosis after peripheral nerve surgery. Many authors have attempted to develop and verify the effectiveness of substances to decrease the formation of adherences in different tissues. Material and Methods: this study aimed to assess the effectiveness of a barrier of oxidized regenerated cellulose (ORC) to reduce adherence and perineural fibrosis in a model of surgical perineural induced fibrosis in rat sciatic nerve in 40 rats. After tissue aggression, the nerve of the right rear limb was wrapped in ORC and the left limb served as control. Animals were killed at 3 and 6 weeks, and nerves and muscle mass were extracted *en bloc*. Connective tissue was quantified by conventional histopathological techniques and Fibrosis HR® automatic image analysis. Results: No significant differences were found in intra- or peri-neural induced fibrosis between control nerves (6.88% and 8.90%, respectively) and treated nerves (6.57% and 9.90%) at 3 or 6 weeks (10.41% and 12.51% in controls; 11.85% and 15.72% in treated nerves). Inflammatory phenomena and granulomatous reactions were more frequent in treated animals. Conclusions: ORC conferred no advantage in prevention of nerve fibrosis and might have interfered with healing.

Key words: Fibrosis, Nerve, Neural scar, Digital analysis, Biomaterials, Rat

Offprint requests to: Dr. P. Hernández Cortés, Rector Marín Ocete 10, 5º C. Granada 18014, Spain. e-mail: hdezcp@hotmail.com

Introduction

It is difficult to prevent and treat intra- and peri-neural fibrosis after peripheral nerve surgery. Recurrence of carpal tunnel syndrome after surgical release of the median nerve occurs in 0.3-20% of cases (Steyers, 2002). Postoperative fibrosis and adherences are the main factors implicated in symptom recurrence (Steyers, 2002; Stütz et al., 2006). After fibrosis has developed, even meticulous neural decompression with surgical microscope often exacerbates symptoms, because every tissue manipulation is a new stimulus to induce further fibrosis (Millesi et al., 1993). Indeed, some patients may best be described as having a "Sisyphus syndrome", in which repeated surgery initially relieves symptoms that eventually return to haunt the patient and the surgeon, often associated with extraneural fibrosis (McCall et al., 2001).

Numerous complicated procedures have been proposed, including fascio-fat or muscle flaps, vein graft wrapping or even free vascularized flaps to reduce perineural scarring and postoperative fibrotic adherences, with very heterogeneous results (Rose et al., 1991; Tham et al., 1996; Guillemot et al., 1999; Dahlin et al., 2002; Goitz and Steichen, 2005).

Many authors have attempted to develop and verify the effectiveness of substances to decrease the formation of adherences in different tissues (Petersen et al., 1996; Oncel et al., 2004; Dam-Hieu et al., 2005; Ilbay et al., 2005; Massie et al., 2005; Ivanic et al., 2006). However, evaluation of their effectiveness is hampered by the lack of objective assessment methods to demonstrate small or moderate but possibly clinically relevant histopathological differences between the use and non-use of these materials.

The objective of this experimental study is to assess the effectiveness of a barrier of oxidized regenerated cellulose to reduce adherences and intra- and peri-neural fibrosis in a model of surgical perineural induced fibrosis in rat sciatic nerve.

Materials and methods

Experimental animals

We studied 40 Sprague-Dawley male rats with a weight of 300-350 g. The study complied with current European legislation on research ethics and was approved by the ethical commission for animal research of our institution.

Procedure

Animals were anesthetized with ether inhalation followed by intraperitoneal injection of a solution of ketamine (1 ml), diazepam (0.8 ml) and atropine (0.2 ml). Preoperative anti-infectious prophylaxis was conducted by administering 30 mg/kg of intramuscular cefuroxime.

Both sciatic nerves were exposed between vastus lateralis and biceps femoris by longitudinal posterolateral incision. The sciatic nerve was dissected from the surrounding tissue with a surgical microscope, and the tibial and peroneal fascicles were proximally separated along a length of 1 cm. The perineurium of these two fascicles in the previously dissected segment was resected using microsurgery instruments, taking care to minimize nerve fascicle lesions (Fig. 1A).

In order to stimulate a local fibrogenic response around the nerve, bipolar forceps were used to cauterize the muscle bed (femoral biceps) where the sciatic nerve would rest, while the sciatic nerve itself was kept isolated. After this tissue insult, the nerve was covered with a 1.5x5 mm sheet of oxidized regenerated cellulose (Divide®; Johnson & Jonson, Raritan, NJ, USA) (Fig. 1B). This anti-adhesion mesh barrier changes to a gel consistency within a few hours of its application.

The wound was closed with silk suture in skin. The left rear limb served as internal control, applying no anti-adherent barrier before wound closure.

Twenty rats were killed at 3 weeks and the remaining twenty at 6 weeks post-surgery by overdose of sodium pentobarbital after anesthesia with ether. The nerves and surrounding soft tissues were then extracted *en bloc*.

Histopathological and morphometric study

Tissues were fixed in 4% buffered formalin and embedded in paraffin. For histopathological study, longitudinal sections were cut from the nerve-muscle complex of rat rear limb. After identifying the sciatic nerve, 4-μm sections were cut, deparaffinized, and histologically stained with hematoxylin-eosin, PAS, Masson trichromic stain or Syrius red.

Connective tissue was quantified by image analysis, assessing 20 images per sample (10 including perineurium and 10 only endoneurium) by means of a Sony camera connected to a BH-2 microscope (Olympus Optical Company, Ltd., Tokyo, Japan) with an MTV-3 adapter, using a 3.3X intermediate lens and 20X objective. Images were processed and quantified by applying Fibrosis HR® (ImaGesp, Barcelona, Spain) (Masseroli et al., 1998).

Statistical analysis

SPSS for Windows version 15.0 (SPSS Inc., Chicago, IL) was used for the statistical analyses. The

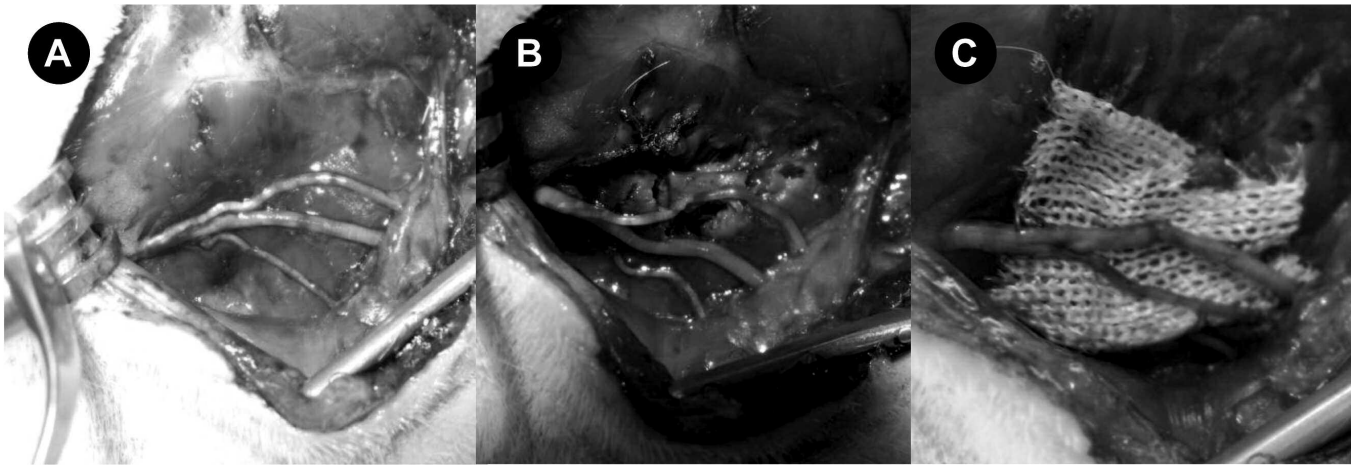

Fig. 1. A. Exposure of rat sciatic nerve. Perineurium removal and isolation of main fascicles. **B.** Thermal lesion in muscle bed with bipolar forceps. **C.** Placement of oxidized regenerated cellulose sheet between nerve and thermally-injured muscle bed.

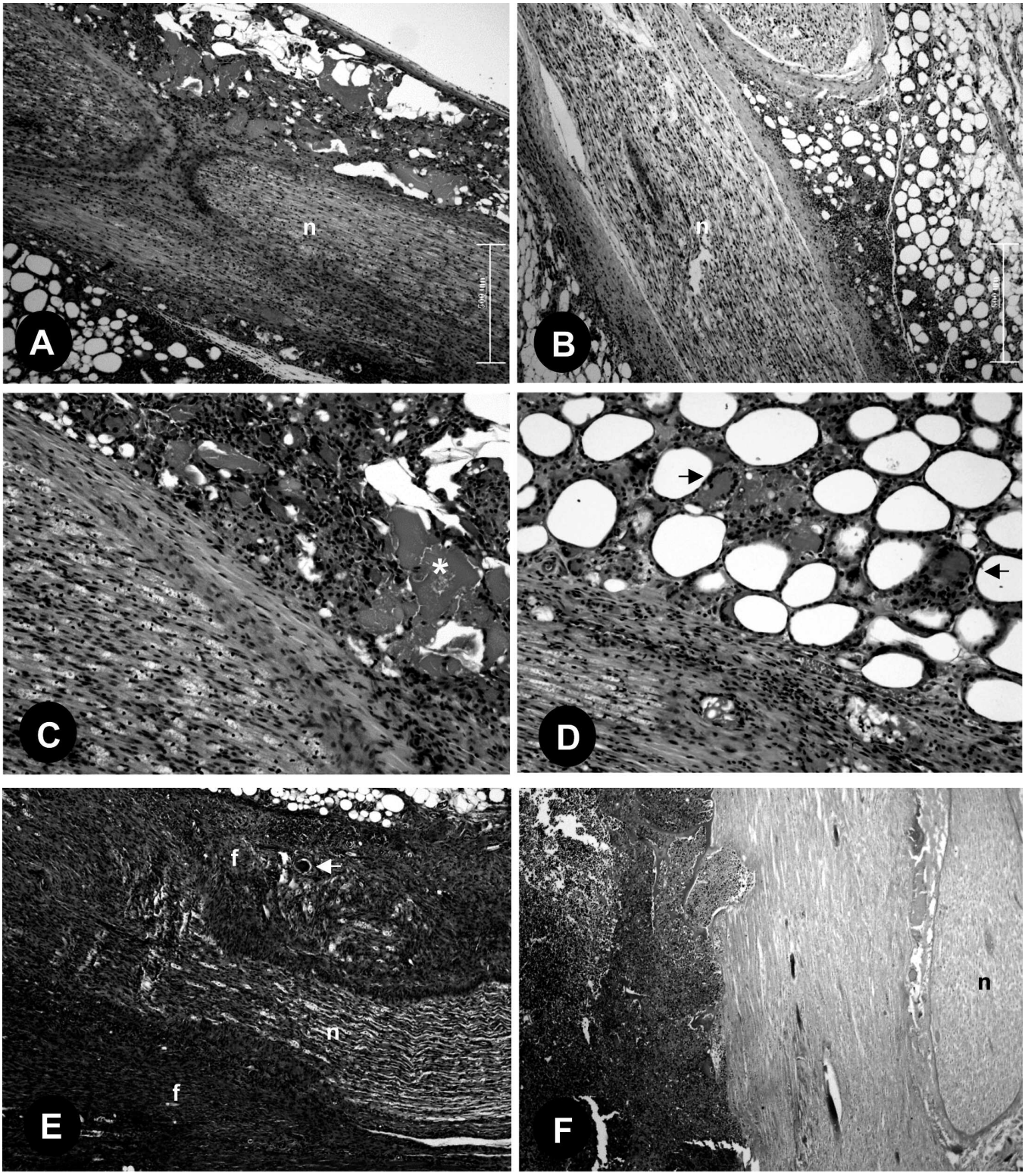

Fig. 2. Sciatic nerve (n) at 3 weeks from lesion induction with accumulations of oxidized regenerated cellulose **(A)** and extensive postoperative fat necrosis **(B)** (hematoxylin-eosin, x40). Sciatic nerve at 6 weeks from lesion induction with persistence of oxidized regenerated cellulose (*) **(C)**, chronic granulomatous reaction with presence of giant cells (arrows) **(D)** (hematoxylin-eosin, x100), intense induction of perineural fibrosis (f) **(E)**, and bacterial contamination with abscess formation **(F)** (hematoxylin-eosin, x40).

normal distribution of variables was assessed by the Kolmogorov-Smirnov test. After descriptive analysis, statistical significance was evaluated by means of the Student's t-test. Confidence intervals were 99% ($p<0.001$) and 95% ($p<0.05$).

Results

Six rats were discarded (and replaced by new animals) due to: unexplained death at 48 h post-surgery (n=1); self-mutilation of intervened limbs (n=1); suture dehiscence (n=1); and development of neurotrophic (n=3) ulcers. No signs of infection were observed during the postoperative study period.

Histopathological results

Seven (33%) of the animals treated with oxidized regenerated cellulose showed more pronounced development of foreign body granulomas surrounding nerve fibers (Fig. 2A-C). The treated group showed a more intense fat necrosis, with numerous foamy histiocytes in the perineural adipose tissue bed and, in some cases, within the nerve structure itself. A clinically undetected perineural abscess was observed in one treated rat (Fig. 2F). The semi-quantitative study found no difference between treated and control animals in the development of connective tissue in the lesioned area.

Histopathological findings in animals killed at 6

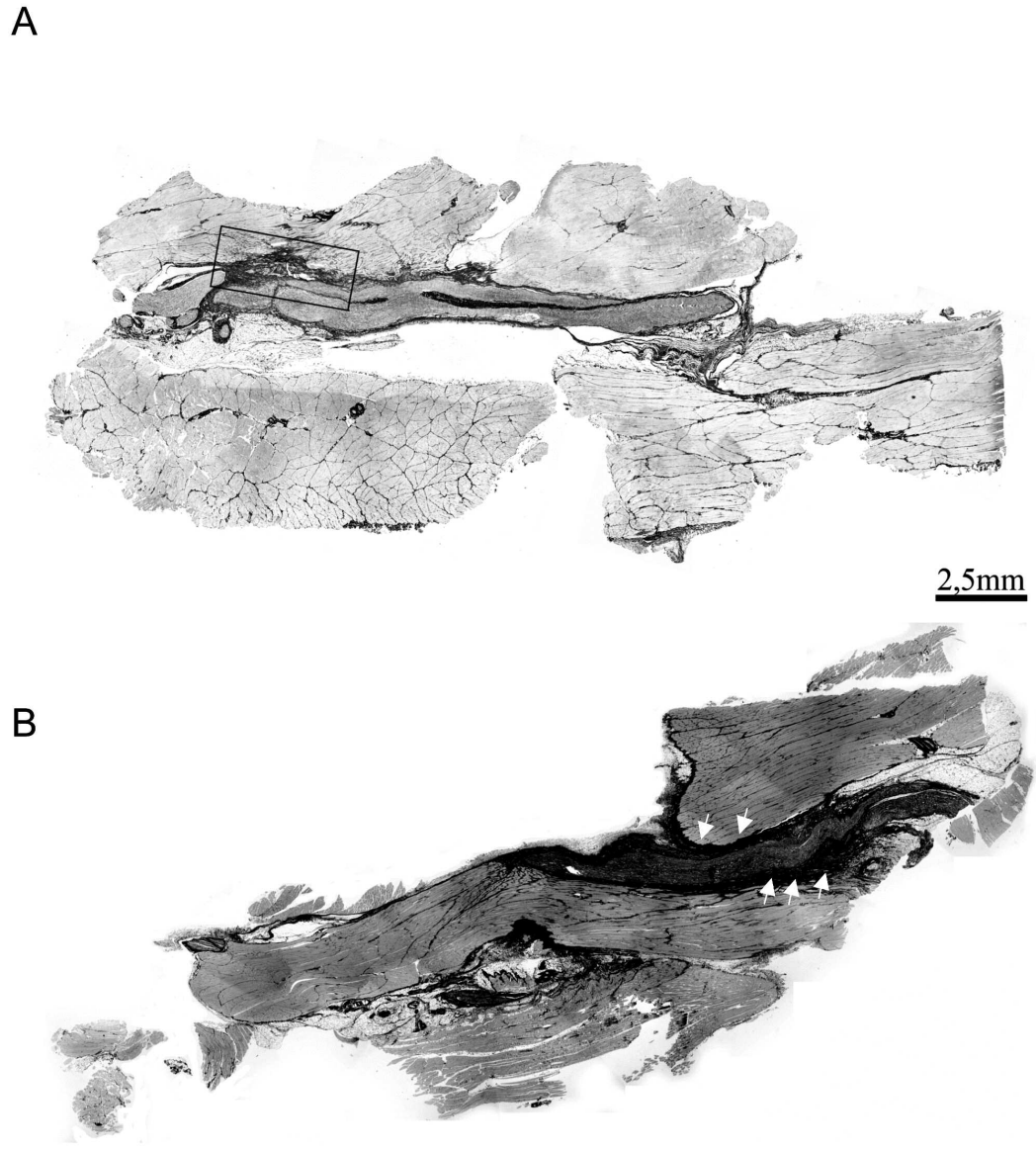

Fig. 3. Panoramic image of oxidized regenerated cellulose -treated sciatic nerve and striated muscle at 3 **(A)** and 6 **(B)** weeks post-surgery, showing the increase in perineural fibrosis over time (arrows) (Syrius red staining).

weeks revealed a lower degree of nerve and soft tissue injury in control animals; 50% of them had no nerve fiber alterations or lymphoplasmocytic chronic inflammatory infiltrate, and only a few histiocytes were observed. Control muscle tissue showed solely morphological features of thermal injury, whereas the treated muscle still showed fat necrosis, granulomatous reaction with giant cells (Fig. 2D), and histiocyte cells around the epineurium and within the sciatic nerve. In the semi-quantitative assessment of vascular connective tissue at 6 weeks, no differences were found between controls and treated animals (Fig. 2E).

Morphometric results

Table 1 shows the mean (and standard deviation) percentages and areas (in square microns) of intraneural connective tissue, including the perineurium, at 3 and 6 weeks. Morphometric study confirmed an increase in the percentage and extent of connective tissue in sciatic

Table 1. Connective tissue automatically quantified by image analysis with the Fibrosis HR® program in Syrius red-stained sections of control and oxidized regenerated cellulose-treated sciatic nerve from animals killed at 3 and 6 weeks.

Rats (n=20/group)	Intraneural connective Tissue (%)	Perineurium + connective tissue (%)	Connective tissue (μm^2)	Perineurium + connective tissue (μm^2)	P-Value
Control 3-wks	6.8±2.7	8.9±3.1	12036.3±4848.6	15569.2±5454.7	
Treated 3-wks	6.5±3.2	9.9±3.2	11489.4±5600.5	17319.1±5608.7	NS
Control 6-wks	10.4±3.8	12.5±4.4	18214.2±6740.1	21878.8±7697.1	
Treated 6-wks	11.8±1.8	15.7±3.4	20733.2±3151.5	27487.8±6010.6	NS

Values are expressed as mean ± Standard Deviation. P- values, Student t test.

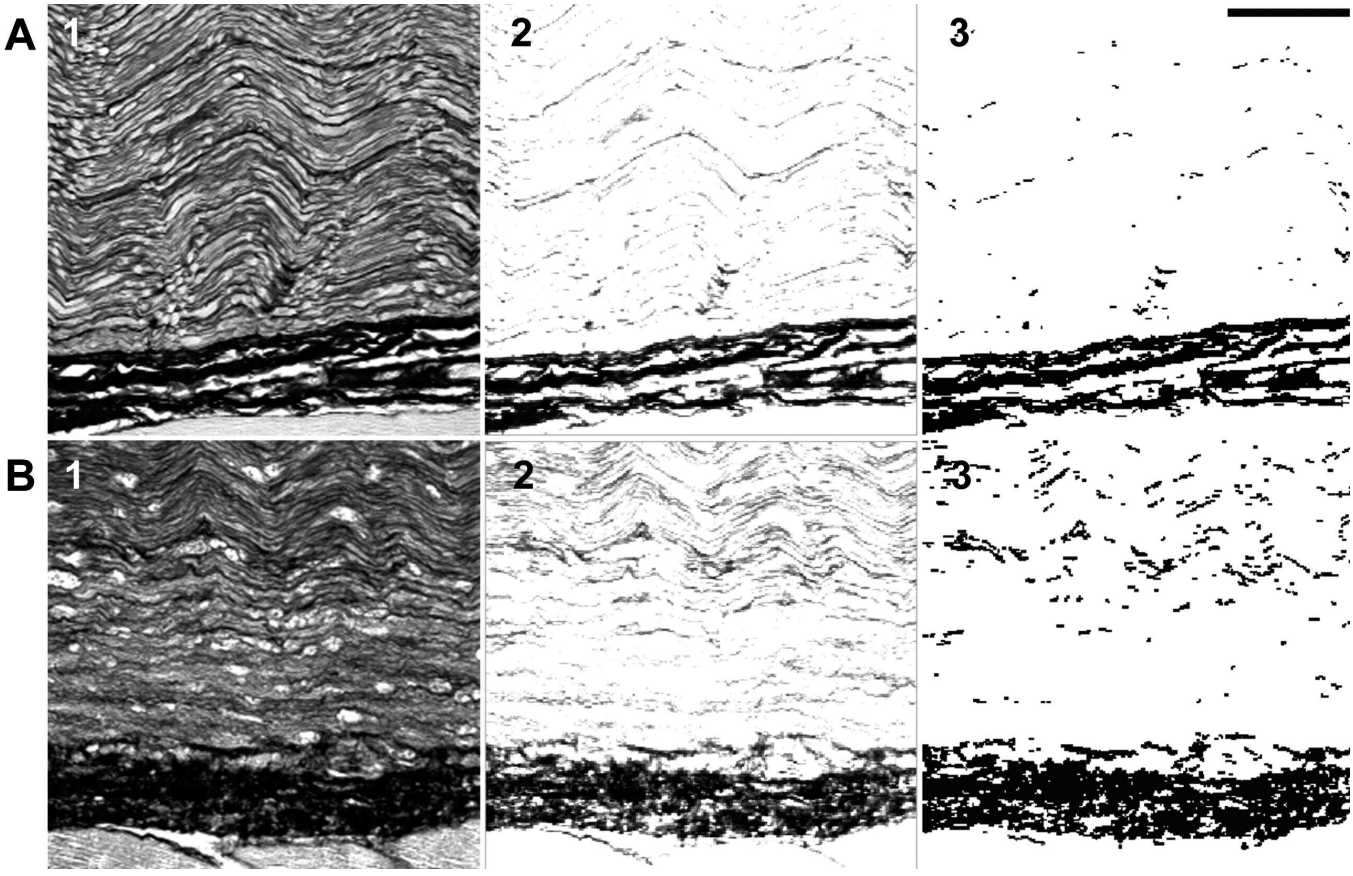

Fig. 4. Automatic image analysis processing of connective tissue. **1)** Microphotograph of rat sciatic nerve perineurium section with Syrius red-stained. **2)** Normalized digital image. **3)** Automatic segmentation of connective tissue area through Fibrosis HR® program. **A.** Control group: Connective tissue percentage 11.09%. **B.** Oxidase regenerated cellulosa-treated sciatic nerve group: Connective tissue percentage 15.39%. Bar: 100 μm.

nerve increases over time (Fig. 3) but no significant differences were found between the control and oxidized regenerated cellulose treated animals at either time point.

Discussion

Postoperative perineural fibrosis is an unresolved and frequently underestimated surgical problem (Stütz et al., 2006). Various substances such as Adcon, hyaluronic acid and derivatives, mitomycin C or polytetrafluoroethylene gels and membranes have been proposed to exert anti-adherent effects (Petersen et al., 1996; Oncel et al., 2004; Dam-Hieu et al., 2005; Ilbay et al., 2005; Massie et al., 2005; Ivanic et al., 2006), but the evidence has been non-conclusive.

Their application in human clinical trials (Petersen et al., 1996; Loick et al., 1997; McCall et al., 2001; Ganzer et al., 2003; Gerszten et al., 2003; Golash et al., 2003; Ivanic et al., 2006) has yielded controversial results of doubtful interpretation, since fibrosis is a histopathological, non-clinical entity that is not directly related to patient symptomatology and is influenced by numerous factors. Reports that these substances may inhibit conjunctive tissue formation have aroused suspicions of a possible negative influence on the healing of injuries and surgical wounds (Golash et al., 2003; Zou et al., 2004).

It is not feasible to take the requisite tissue samples from human subjects for pathological analysis, and histological assessment is limited to experimental animal models, especially rat and rabbit (Ikeda et al., 2002; Clark et al., 2003; Isla et al., 2003; Oncel et al., 2004; Dam-Hieu et al., 2005; Ilbay et al., 2005; Massie et al., 2005; Ohsumi et al., 2005; Merle et al., 2008; Temiz et al., 2008). Fibrosis analysis methodology is also highly variable. Studies have used highly subjective semi-quantitative scales (Petersen et al., 1996; Isla et al., 2003), cell counts (Ilbay et al., 2005), biomechanical tests of adherence rupture force (Petersen et al., 1996; Oncel et al., 2004; Ohsumi et al., 2005) or hydroxyproline quantification as an indirect measure (Massie et al., 2005).

Image digital analysis has been applied to the morphometric analysis of normal and pathological tissue (Gil et al., 1986; Wootton et al., 1995), allowing objective and reproducible quantifications to be obtained (Masseroli et al., 1998; Caballero et al., 2001; Pajares-López and Hernández-Cortés, 2005). The use of the Fibrosis HR® program is therefore strength of the present study. The experimental model developed by Oshumi (Ohsumi et al., 2005) was selected for its technical simplicity, controlling for any interanimal variability in wound-healing capacity by using the nerve in the other hind limb of each animal as control.

Anti-fibrotic agents currently available for humans include bovine pericardium, Silastic®, collagenous Vycril®, Gore-Tex®, Adcon-T® and oxidized regenerated cellulose such as Interceed® and Divide® (Ikeda et al., 2002; Merle et al., 2008).

Few studies have been published on the use of oxidized regenerated cellulose to prevent perineural fibrosis. Ikeda et al. (2002) found this material to be effective in preventing the formation of histologically assessed perineural adherences in an experimental model of fibrosis in rabbit sciatic nerve. Loick et al. (1997) published good clinical outcomes from its use in nine patients undergoing carpal tunnel release, epineurolysis and flexor synovectomy, although histopathological studies were evidently not possible.

Termiz et al. (2008) recently demonstrated adherence reduction in a model of tendon injury in rabbits and found no interference with wound-healing, assessing adherences on a semi-quantitative scale. Merle et al. (2008) presented a mixed rabbit experimental and clinical study on the use of oxidized regenerated cellulose in tendon surgery, also with good results, assessing adherences in the animal model from the degree of rabbit limb mobility and measuring the histological response based on descriptive microscopic criteria.

The percentage of intraneural and perineural connective tissue increased with longer postoperative follow-up; No significant difference in intraneural or perineural fibrosis was found between control and treated nerves at 3 or 6 weeks, indicating that this pharmaceutical has no antifibrogenic effect. Furthermore, inflammatory phenomena and foreign body granulomatous reactions were more frequently detected in oxidized regenerated cellulose -treated than in untreated nerves and remains of the material were still observed at 6 weeks after its application.

In conclusion, these histopathological and morphometric results do not support the antifibrogenic effect of locally applied oxidized regenerated cellulose in this experimental model, and this material induced a chronic granulomatous inflammatory response that may interfere with the healing process.

Disclaimers

The authors have no direct or indirect financial interests in the products listed in the study. F. O'Valle is one of the intellectual authors of the Fibrosis HR® program.

Acknowledgements. The authors would especially like to acknowledge the contribution of Serafín Vélez García and Carmen Ruiz Guzman, technicians at the Surgical Department of Granada University. They are also grateful to Richard Davies for assistance with the English version. This investigation was supported in part by Research Group #CTS-138 (Junta de Andalucía, Spain).

References

Caballero T., Pérez-Milena A., Masseroli M., O'Valle F., del Moral R.G. and Sánchez Salgado G. (2001). Liver fibrosis assessment with

semiquantitative indexes and image analysis quantification in sustained-responder and no responder interferon-treated patients with chronic hepatitis C. J. Hepatol. 34, 740-747.

Clark B.D., Barr A.E., Safadi F.F., Beitman L., AL-Shatti T., Amin M., Gaughan J.P. and Barbe M.F. (2003). Median Nerve trauma in a rat model of work-related musculoskeletal disorder. J. Neurotrauma 20, 681-695.

Dahlin L.B., Lekholm C., Kardum P. and Holmberg J. (2002). Coverage of the median nerve with free and pedicled flaps for the treatment of recurrent severe carpal tunnel syndrome. Scand. J. Plast. Reconstr. Surg. Hand. Surg. 36, 172-176.

Dam-Hieu P., Lacroix C., Said G., Devanz P., Liu S. and Tadie M. (2005). Reduction of postoperative perineural adhesions by Hyaloglide gel: an experimental study in the rat sciatic nerve. Neurosurgery 56, 425-433.

Ganzer D., Giese K., Völker L., Pietzner U., Follak N. and Merk H. (2003). Two-year results after lumbar microdiscectomy with and without prophylaxis of a peridural fibrosis using Adcon-L. Arch. Orthop. Trauma Surg. 123, 17-21.

Gerszten P.C., Moossy J.J., Flickinger J.C. and Welch W.C. (2003). Low-dose radiotherapy for the inhibition of peridural fibrosis after reexploratory nerve root decompression for postlaminectomy syndrome. J. Neurosurg. 99 (3 Suppl), 271-277.

Gil J., Marchevsky A.M. and Silage D.A. (1986). Applications of computerized morphometry in pathology. Lab. Invest. 54, 222-227.

Goitz R.J. and Steichen J.B. (2005). Microvascular omental transfer for the treatment of severe recurrent median neuritis of the wrist: a long-term follow-up. Plast. Reconstr. Surg. 115, 163-171.

Golash A., Kay A., Warner J.G., Peck F., Watson J.S. and Lees V.C. (2003). Efficacy of ADCON-T/N after primary flexor tendon repair in Zone II: a controlled clinical trial. J. Hand Surg. 28, 113-115.

Guillemot E., Le Nen D., Colin D., Stindel E., Hu W. and L'Heveder G. (1999). Fibrose Périneurale du median au poignet traitment par neurolyse et greffon dermo-hypodermique. Chir. Main. 18, 279-289.

Ikeda K., Yamamuchi D. and Tomita K. (2002). Preliminary study for prevention of neural adhesion using an absorbable oxidised regenerated cellulose sheet. Hand Surg. 7, 11-14.

Ilbay K., Etus V., Yildiz K., Ilbay G. and Ceylan S. (2005). Topical application of mitomycin C prevents epineural scar formation in rats. Neurosurg. Rev. 28, 148-153.

Isla A., Martinez J.R., Perez-Lopez C., Pérez Conde C., Morales C. and Budke M. (2003). A reservable antiadhesion barrier gel reduces the perineural adhesions in rats after anastomosis. J. Neurosurg. Sci. 47, 195-199.

Ivanic G.M., Pink P.T., Schneider F., Stuecker M., Homann N.C. and Preidler K.W. (2006). Prevention of epidural scarring after microdiscectomy: a randomized clinical trial comparing gel and expanded polytetrafluoroethylene membrane. Eur. Spine J. 15, 1360-1366.

Loick J., Joosten U. and Lucke R. (1997). Implantation of oxidised regenerated cellulose for prevention of recurrence in surgical therapy of carpal tunnel syndrome. Handchir. Mikrochir. Plastchir. 29, 209-213.

Masseroli M., O'Valle F., Andújar M., Ramírez C., Gómez-Morales M., de Dios Luna J., Aguilar M., Aguilar D., Rodríguez-Puyol M. and Del Moral R.G. (1998). Design and validation of a new image analysis method for automatic quantification of interstitial fibrosis and glomerular morphometry. Lab. Invest. 78, 511-522.

Massie J.B., Schimizzi A.L., Huang B., Kim C.W., Garfin S.R. and Akeson W.H. (2005). Topical high molecular weight hyaluronan reduces radicular pain post laminectomy in a rat model. Spine J. 5, 494-502.

McCall T.D., Grant G.A., Britz G.W., Goodkin R. and Kilot M. (2001). Treatment of recurrent peripheral nerve entrapment problems. Role of scar formation and its possible treatment. Neurosurg. Clin. 12, 329-339.

Merle M., Lallemand B., Lim A. and Gantois G. (2008). Experimental and clinical evaluation of an absorbable biomaterial inducing an anti-adhesive barrier (Divide). Eur. J. Orthop. Surg. Traumatol. 18, 255-263.

Millesi H., Rath T., Reishner R. and Zoch G. (1993). Microsurgical neurolysis: Its anatomical and physiological basis and its classification. Microsurgery 14, 430-442.

Ohsumi H., Hirata H., Nagakura T., Tsujii M., Sugimoto T., Miyamoto K., Horiuchi T., Nagao M., Nakashima T. and Uchida A. (2005). Enhancement of perineural repair and inhibition of nerve adhesion by viscous injectable pure alginate sol. Plast. Reconstr. Surg. 116, 823-830.

Oncel M., Remzi F.H., Senagore A.J., Connor J.T. and Fazio V.W. (2004). Application of Adcon-P or Seprafilm in consecutive laparotomies using a murine model. Am. J. Surg. 187, 304-308.

Pajares-López M. and Hernández-Cortés P. (2005). Aplicación de periostio en un modelo experimental en conejo. Rev. Ortop. Traumatol. 49, 377-389.

Petersen J., Russell L., Andrus K., MacKinnon M., Silver J. and Kliot M. (1996). Reduction of extraneural scarring by ADCON-T/N after surgical intervention. Neurosurgery 38, 976-983.

Rose E.H., Norris M.S., Kowalski T.A., Lucas A. and Flegler E.J. (1991). Palmaris brevis turnover flap as an adjunct to internal neurolysis of the chronically scarred median nerve in recurrent carpal tunnel syndrome. J. Hand Surg. 16, 191-201.

Steyers C.M. (2002). Recurrent carpal tunnel syndrome. Hand Clin. 18, 339-346.

Stütz N., Gohritz A., van Schoonhoven J. and Lanz U. (2006). Revision surgery after carpal tunnel release--analysis of the pathology in 200 cases during a 2 year period. J. Hand Surg. 31, 68-71.

Temiz A., Ozturk C., Bakunov A., Kara K. and Kaleli T. (2008). A new material for prevention of peritendinous fibrotic adhesions after tendon repair: oxidised regenerated cellulose (Interceed), an absorbable adhesion barrier. Int. Orthop. 32, 389-394.

Tham S.K., Ireland D.C., Riccio M. and Morrison W.A. (1996). Reverse radial artery fascial flap: a treatment for the chronically scarred median nerve in recurrent carpal tunnel syndrome. J. Hand Surg. 21, 849-854.

Wootton R., Springall D.R. and Polak J.M. (1995). In: Image analysis histology. Conventional and confocal microscopy. 1st ed. Wootton R., Springall D.R. and Polak J.M. (eds). Cambridge University Press. Cambridge, UK. Pp 1.425.

Zou X., Li H., Egund N., Lind M. and Bünger C. (2004). Inhibition of spinal fusion by use of a tissue ingrowth inhibitor. Eur. Spine J. 13, 157-163.

Accepted January 4, 2010