RAT SCIATIC NERVE REGENERATION USING A COLLAGEN BIOIMPLANT

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SUMMARY :

A collagen mafrix (COL) gel was used to fill a 3 mm gap resulting from transection of rat sciatic nerve. The nerve was initially stabilized with a split polyethylene tubing in order to remove elastic tension at the nerve repair site. The contralateral sciatic nerve was identically prepared but the gap was left empty. Animal survival time was 9 to 12 weeks. One day prior to killing, somatosensory evoked potentials and injections of the tracer, horseradish peroxidase on distal potentials and injections of the tracer, horseradish peroxidase on distal nerves were done. Morphological analysis of the nerve preparations included light microscopy, silver staining for axons and histochemical fluorescence of catecholamines. Horseradish peroxidase granules were observed proximal to the regenerate in both COL and controls. Light and electron misroscopic results indicate that COL-treated animals had significantly improved axonal outgrowth across and distal to the bridge matrix compared to the untreated group. The microscopic findings were supported by the somatosensory evoked potentials activity recorded from COL treated animals at the end of the observation period.

KEY WORDS :

Axon regeneration, peripheral nerve, collagen matrix, nerve repair, bioimplant.

INTRODUCTION

Adequate outgrowth of axons into the transected distal nerve stump is fundamental to restoring function in the denervated target tissue. Surgical repair of the cut nerve by suture has been the classical approach since Phillipeaux and Vulpian (1870) introduced this technique in 1870. Within the past 25 years, improvements in peripheral nerve repair have been due largely to microsurgical innovations involving suture material, fine tools and better knowledge of nerve tissue physical and hypsiological mechanics (Bischoff, 1975, Millesi, 1981). Despite these refinements, peripheral nerve repair often does not reverse the loss of sersory-motor function observed, after nerve damage (Bischoff, 1975, de Medinaceli, 1983). When a nerve is cut and elastic retraction creates a gap of several mm, re-anastomosing the stumps may introduce additional damage which can retard or impede axonal regeneration (Miyamoto, 1979). Moreover, physical injury can result from stretching the stumps and compromising the blood supply and from tension at the line of coaptation which in turn can lead to scar and edema formation at the stump junctions (Lundborg, 1973, Orf, 1979, Starkweather, 1978). correct this, autologous peripheral nerve grafts have been inserted between the cut stumps to from a bridge for regenerating axons.

Unfortunately, this procedure also presents a number of problems including the presence of 2 lines of suture coaptation, difficulty in matching the right diameter nerve graft for implantation and sacrificing a healthy nerve to replace the damaged one. This has led to the use of permeable or silicone entubulation which stabilizes the nerve preparation and presents the possibility of filling the gap with a suitable graft bridge (de la Torre, 1988, Lundborg, 1980, 1982, Rosen, 1983, Seckel, 1984, Uzman, 1983).

Based on prior successful results using this material to reconnect mammalian spinal cord (de la Torre, 1982), we developed a peripheral nerve repair technique using a collagen matrix (COL) to bridge the gap area. Preliminary evidence from our laboratory using COL on crushed (de la Torre, 1988) or transected (Goyal, 1986) rat sciatic nerve further encouraged detailed exploration of this surgical-chemical nerve preparation.

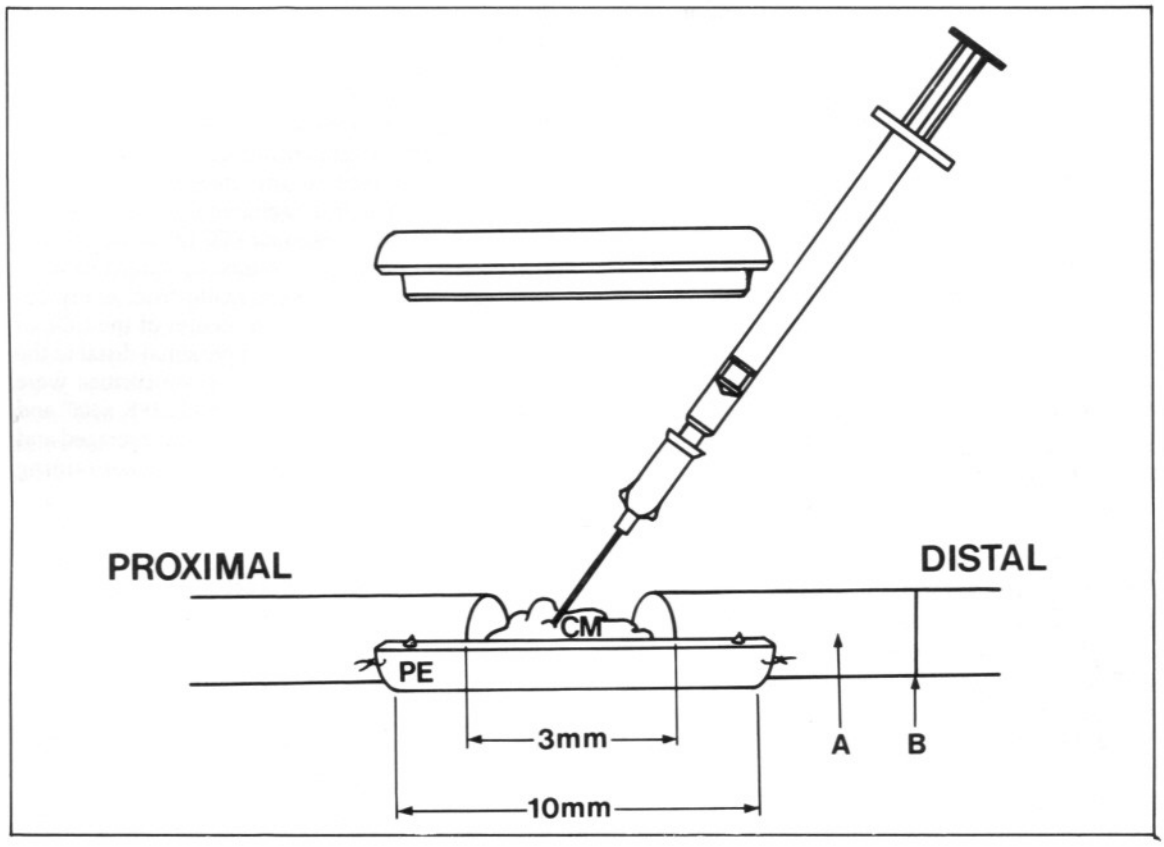

Fig.1 : Sketch of the surgical preparation. The proximal-distal sciatic nerve stumps are anchored with a 9-0 stitch to the lower half of the polyethylene tubing (PE). The gap is filled with sterile collagen matrix (COL) using a tuberculin syringe. The upper part of the PE tubing (above nerve) is placed on top of nerve preparation and held in place with a touch of glue. A - site for horseradish peroxidase injection. B - site of somatosensory evoked potentials stimulating electrodes.

COL has certain advantages over tissue grafts: it is biocompatible, bioresorbable, and has the ability to support neuritic and vascular elements migrating into the bioimplant from the host tissue (de Medinaceli, 1983, de la Torre, 1988, de la Torre, 1982). In addition, because of its fluid nature at 4° C, COl gels in excellent continuity with the host tissue (de la Torre, 1982, Knapp, 1977). The present study was designed to examine whether this COL gel bioimplant can successfully provide a bridge to axonal outgrowth after nerve transection.

METHODS

Sixteen male Long-Evans hooded rats 350-400 grams were anesthetized using 21 mg/Kg sodium pentobarbital i.p. and 60 mg/Kg ketamine i.m. The sciatic nevre was exposed bilaterally at midthigh from the sciatic notch to its trifurcation with the tibial, peroneal and sural branches. A 1 cm long polyethylene tubing (PE-240) was split in half longitudinally and placed under each nerve. Two 9-0 epineurial stitches were used to anchor the nerve to each conner of the tubing, well-away from the center of the preparation. The nerve was irrigated for 10 minutes with ice-cold 15 % polyvinyl alcohol containing 4 mM chlorpromazine (PVA/CPZ) solution according to the technique described by de Medinaceli et al (1983).

The nerve was transected mi-point in the tubing using an ultrafineedged Gillette blade mounted on a blade-breaker. A consistent 3mm gap resulted after elastic retraction of the nerve. Retraction tension on the nerve preparation was buffered by the epineurial tissue anchored to the PE tubing. The nerve stumps were bathed with the ice-cold polyvinyl alcohol/chlorpromazine solution for 15 minutes in order to harden the tissue and reduce chemical tissue damage following transection (de Medinaceli, 1983). Ice-cold saline irrigation followed, and the area was dried with cotton wicks. One of the nerves was chosen to receive the COL (Group COL); the gap was left empty in the contralateral nerve (Group GAP). Rats were then randomly separated into either COL or GAP group. About 0.3 ml of collagen gel was dispensed sterile until the gap between the nerve stumps was filled. The other split-half of the PE-240 tubing was placed

(*) Collagen type I. 35 mg/ml-Collagen Corporation, Palo Alto, CA.

on top of each nerve preparation and held in place with a spot of Krazy Glue (Fig.1).

Animals were allowed to recover and kept for 9 or 12 weeks observation in the same light-temperature controlled room. Food and water were given ad libitum.

PHYSICAL AND NEUROELECTRIC MOTOR-SENSORY TESTING

A routine examination was performed daily to prevent foot pad ulcers of deafferented lower limbs. Picric acid 5 % was poriodically sprayed over the lower limb area to discourage automutilation of toes. Neurological evaluation included motor examination of toe spreading and grasp and sensory responses to toe pinch. Rats undergoing exposure of the distal sciatic nerve for horseradish peroxidase retrograde labelling injections also had the nerves pinched at 2 mm intervals in order to locate the advancing tips of outgrowing sensory axons (Young, 1940).

The day prior to killing, animals were anesthetized as if for surgery and the sciatic nerves were exposed. Teflon-coated stimulating electrodes were hooked lightly around the nerve 3 mm distal to the transection site. Brass electrodes were screwed into on the skull for recording somatosensory evoked potentials as we have previously described in the rat (de la Torre, 1980). Square wave electrical pulses 0.1 msec duration and 0.1 msec delay were delivered every 0.5 sec. The voltage was adjusted to elicit a slight paw twitch. Signals were averaged on a Dagan 4800 computer and 254 responses were recorded using an analysis time of 250 msec. Somatosensory evoked potentials measurements were averaged from 4 trials/nerve (Table 1).

MICROSCOPIC EXAMINATION

Eight rats were anesthetized the day before killing to expose the sciatic nerves bilaterally. Using a microsyringe, 1 ul HRP (type VI, 30 % Sigma) was injected into the nerve 6 mm distal to the transection site. The rest of the animals were either perfused via the left cardiac ventricle or the sciatic nerve was removed fresh for catecholamine histofluorescence using de la Torre's SPG method (de la Torre, 1980). Perfused rats received 500 ml heparinized (10 units/ml) saline followed by 500 ml of 2 % paraformaldehyde, 1.25 % glutaraldehyde in 0.1 M phosphate buffer. After perfusion, the nerves were removed and post-fixed in 4 % paraformaldehyde and 2 % glutaraldehyde in saline for 7 days. After fixation, nerves were divided into 2 mm longitudinal blocks and embedded in Epon-araldite. Transverse 1 um thick sections were taken from each block and stained with toluidine blue for light microscopic examination. Ultra-thin sections from the center of the lesion and 5 mm distal and proximal to the center were stained and examined ultrastructurally using a Phillips 301 electron microscope. Light microscopic counts of myelinated and unmyelinated axons in the center and distal nerve region were made with the aid of an eyepiece grid encompassing an area of 625 square um^2. Unfixed 16 um cryostat sections were processed for Nissl and Palmgren silver axon stains. Serial sections were taken for SPG histofluorescence analysis. Eyepiece grid counts of catecholamine-containing varicosities were made from every 6th histofluorescent section at the center of the COL or GAP region and at 3 and 5 mm proximal-distal to the center. Catecholamine-containing varicosities were counted using a high power dry field (HPF, x300) and representative values were summated, averaged and expressed as catecholamine-containing varicosities/HPF.

TABLE 1

Group	Total number myelinated axons	catcholamine varicosities/HPF	average no. SEP peaks
GAP	3008 (+853)	70 (+12)	3
COL	4612 (+984)	220 (+32)	5

Morphologic and neuroelectric values of distal rat sciatic nerve 12 weeks after transection in GAP and COL (collagen bridge) groups.

Key :

Myelinated axons-average number of total myelinated axons 5 mm distal to transection; student's t test, p 0.02.

Catecholamine varicosities/HPF-catecholamine-containing varicosities/high power (dry) field + S.E.M.; student's t test p 0.01.

SEP - somatosensory evoked potentials; N = average number of peaks within 80 msec time base taken from 4 trials/distal nerve preparation.

STATISTICAL ANALYSIS

Data was analyzed using analysis of variance (ANOVA) followed by Dunnett's test. Pathological comparisons between groups were determined using Spearman's rank correlation test. All values were considered significant at p 0.05 and are presented a + standard error of the mean.

RESULTS
GROSS EXAMINATION

The results were similar after 9 or 12 weeks survival time. Twelve weeks after nerve transection, toe spread was present in 3/8 in GAP group and 7/8 in COL group. Light pinching of the exposed distal nevre (up to its trifurcation) prior to somatosensory evoked potentials recordings elicited a withdrawal reflex following ketamine anesthesia. This reaction was present is both COL and GAP groups 12 weeks after transection and signalled sensory fiber outgrowth (Goyal, 1986).

A mild walking deficit was present bilaterally in both groups of animals throughout the observation period. Left or right toe pinching at 12 weeks showed brisk withdrawal of limb in all animals.

Exposure of the sciatic nerve at the end of the 12 week observation period revealed bridging of the nerve stumps in both groups. The GAP group han a "hour-glass" shape in the regenerated part of the nerve at its mid-point suggesting that nerve tissue growth proceeds from both proximal and distal stump towards the center of the gap. The average diameter at the center of the regenerate in the GAP group was appreciably smaller than its proximal-distal regions, averaging 250-300 um less than the COL group regenerate. In the COL group, the diameter, at the center of the bridge implant was uniform across the entire bridge matrix and its diameter at mid-center was identical to its proximal-distal nerve.

SOMATOSENSORY EVOKED POTENTIALS

At the end of the observation period, somatosensory evoked potentials were monitored in the cerebral cortex after nerve stimulation below the transected site. Using an 80 msec time base analysis, abnormal peak-to-peak latencies were observed in al GAP treated animals (Fig. 2a). In COL treated rats, peak-to-peak latencies were indistinguishable from normal evoked potentials and were characterized by 5 consistent peaks within the same time base analysis (Fig. 2b). Both groups had normal peak-to-peak latencies waveforms when evoked potentials were taken above the transection site. All neuroelectric activity below the lesion site ceased when the nerve was re-transected proximally.

MICROSCOPIC EXAMINATION

Light microscopic examination of the GAP and COL groups using toluidine blue showed a central reduction of thickness in the GAP nerve regenerate but not in the latter group. GAP nerves contained well-organized myelinated axons extending from proximal to distal stump. Most of these axons were seen to enter the distal nerve but a few bundles of misdirected fibers were observed outside the epineurial sheath.

In COL nerves, the center of the gap was identified in toluidine blue sections by the presence of several microscopic remnants of unresorbed collagen matrix. Axons coursing through the COL did not always follow a straight path towards the distal stump but rather a sinewy trail using the space between the collagen fibers for support. There appeared fewer misdirected axons outside the epineurial sheath in COL treated sections than in similer GAP preparations.

Electron microscopic examination of the proximal stump in GAP and COL confirmed the presence of well-myelinated axons extending into the gap or COL regenerate. GAP regenerate contained multiple fascicular units composed of thinly myelinated and unmylenitade fibers. A moderate number of macrophages were seen in the regenerated region. In addition, distal GAP axons revealed large numbers of thinly myelinated and unmyelinated fibers which did not appear to form "subunit" fascicles.

The regenerated region in the COL group contained a few macrophages and numerous myelinated fibers and mesenchymal cells. Examination of distal to the COL bridge, eyepiece grid counts revealed 53 % more myelinated axonal fibers within fascicular bundles than in the GAP group (Fig. 3; Table 1). This difference was considered significant (p 0.02).

Histofluorescent examination of intra-axonal cetecholamine-containing varicosities were quantitated by high power dry field (HPF) in longitudinal sections of peripheral nerve. Proximal distribution of catecholamine axons was similar in both GAP and COL nerves. The regenerate of GAP showed an average of 70 varicosities/HDF which were observed extending to the distal stump in diminishing numbers. Values in COL regenerate averaged 220 varicosities/HPF (Fig. 4; Table 1). These differences were statistically significant at p 0.01 using ANOVA.

Three mm distal to the transection site, catecholamine density in the COL group showed a mean three-fold higher density of intra-axonal varicosities when compared to the GAP group (Fig. 5). This ratio increased slightly 5 mm distal to the transection site.

Distally-injected horseradish peroxidase granules were found localized in dorsal and ventral root ganglion neurons as well as intra-axonally in both GAP and COL nerves proximal to the transection site. Quantitation of labelled dorsal root ganglionic neurons was not done due to the inconsistent uptake of the tracer by nerve fibers.

DISCUSSION

COL is a highly purified, cell-free, type I collagen that contains 35 mg/ml of the active material. COL remains in al liquid state at 0-4° C. At body temperature, the molecular structure of COL undergoes polymerization (hardening) and forms an opaque gel within 1-2 hours (de la Torre, 1982, Knapp, 1977). The COL fibers' loosely arranged helical structure is in direct contrast to the densely packed, parallel-oriented collagen fibers seen after tissue scarring or inflammatory responses. COL is supplied sterile and can be easily dispensed with a syringe.

Circumferential tubular implans have been previously used as substitutes for nerve grafts (Lund-

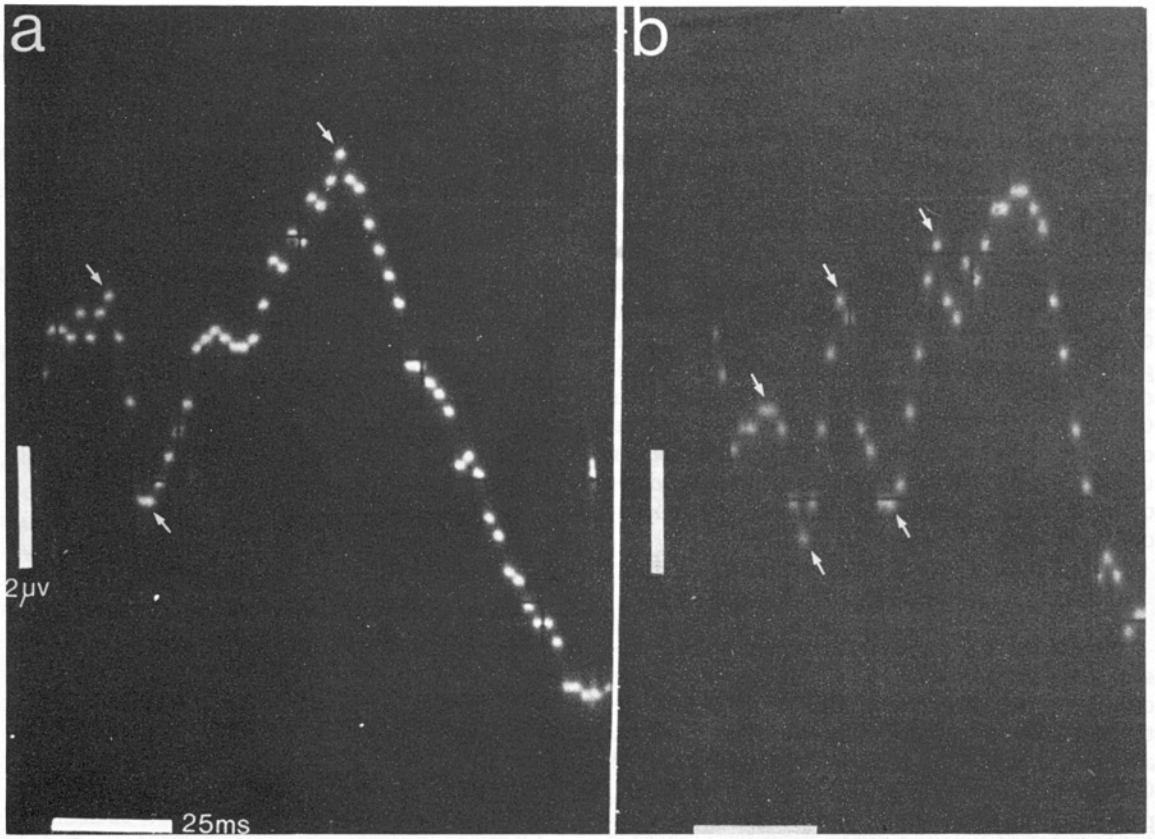

Fig.2 : Oscilloscopic picture of typical somatosensory evoked potentials taken 12 weeks after stimulation of sciatic nerve distal to the transection site. a) GAP nerve evoked potentials shows absence or delay of peak activity (arrows). b) COL experimental nerve shows normal waveform pattern (arrows). Time base 250 msec., 254 responses.

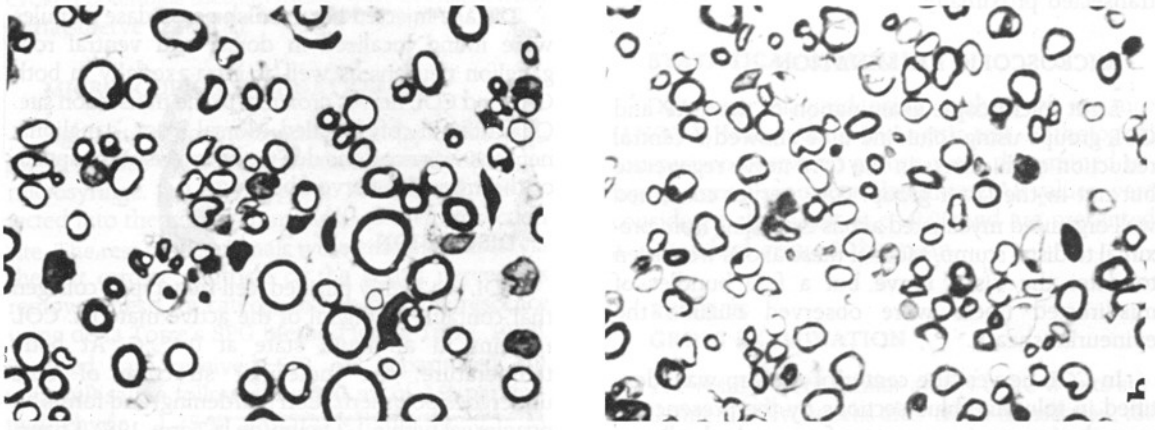

Fig.3 : Epon-embeded 1 um light microscopic sections of GAP (a) and COL (b) sciatic nerve 3 mm distal to transection site. A 53 % increase in total number of axonal fibers is seen in COL as compared to GAP treated group (p 0.01).

borg, 1982, Rosen, 1983, Seckel, 1984, Weiss, 1943, Williams, 1983, Fawcett, 1986). Placement of cut or damaged nerve within a stiff tubing has the advantages of stabilizing the preparation from external tissue contact and may provide a guide to redirect the elongating axons across the nerve tips. We

modified the standard entubulation technique by using a split tube (Fig. 1). This enabled us to anchor the nerve at an area distant from the site of transection in order to prevent tension at the point of nerve-bioimplant line of coaptation. Manipulation of the cut nerve ends was therefore avoided, a problem associated with previous suturing methods. Moreover, our technique offers the advantages of entubulation (Lundborg, 1982, Fawcett, 1986, Nieto-Sampedro, 1983), without the associated mechanical handling of the nerve tips which can lead to neuromas or scar formation often seen after nerve repair (Millesi, 1981). Pilot experiments in our laboratory have indicated that the collagen material used in this study appears superior to the commercial collagen product Vitrogen with respect to support of axonal regeneration. Our results indicate that a critical obstacle to axon regeneration. Our result indicate that a critical obstacle to axon regeneration is removed with our procedure since the longitudinal stress tension at the site of repair is minimized (Miyamoto, 1979, Orf, 1978). In addition, the use of ice-cold polyvinyl alcohol/chlorpromazine treatment appears to eliminate undesirable axoplasmic extrusion of the nerve stumps which occurs after the nerve transection (de Medinaceli, 1983, de la Torre, 1988). Polyvinyl alcohol may reduce the loss of nitrogenous substances following transection of the nerve while hardening the nerve tissue so that compression damage is minimized (de la Torre, 1988). Chlorpromazine has a spectrum of chemical activity but may be beneficial in this nerve preparation because it can prevent increased calcium permeablitiy into the axonal cytoskeleton (Chien, 1978), a phenomenon that may trigger Wallerian degeneration (Schlaepfer, 1974, Anden, 1964). Pilot experiments had shown that when ice-cold saline was used, axoplasmic extrusion still resulted following nerve transection, a finding that further supports a chemical basis for polyvinyl alcohol/chlorpromazine rather than a local cooling effect on the nerve tissue. Finally, the physical reunion of the cut nerve stumps appeared to be accelerated after 12 weeks when COL was placed in the gap region. The reason for achieving superior repair with COL than with GAP remains unclear. Williams and Varon (1983) have suggested that axonal outgrowth after nerve transection is dependent on a "fibrin" mesh secreted between the nerve tips which allows Schwann cell and vascular proliferation to physically link the two stumps. It is conceivable that COL may eliminate the need for a fibrin mesh so that cell and vascular migration into the gel occurs more quickly than when the gap is left empty.

The presence of catecholaminergic axons distal to the nerve transection indicates axonal outgrowth occurred in both groups of animals after 12 weeks since catecholamines originate above the nerve in postganglionic neurons (Adnen, 1964). However,

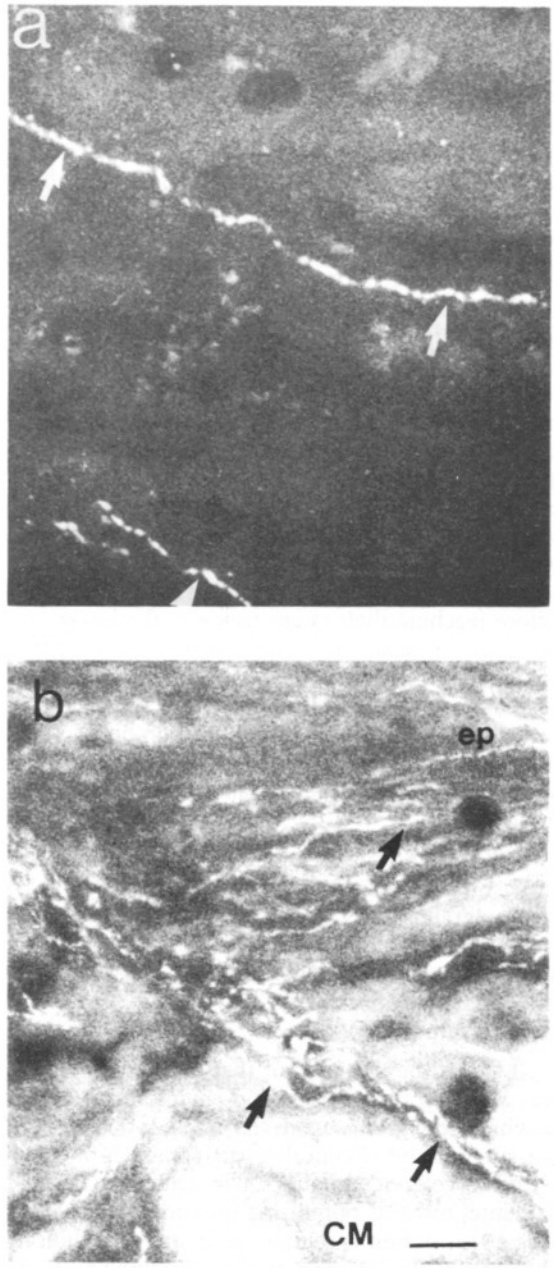

Fig.4 : Center of regenerate. a) Few catecholamine-containing varicosities (arrows) along the epineurium in GAP group. b) Moderate catecholaminergic axon density in COL group (arrows) crossing collagen matrix (CM) and epineurium (e.p.). Longitudinal section. SPG histofluorescence method: x 300. Bar = 50 um.

histofluorescence and microscopic analysis of GAP and COL nerve regenerate showed that axonal outgrowth and catecholamine-containing varicosities were more abundant in COL that GAP after nerve transection (Table I). This may be the reason why the

diameter of the COL regenerate was not diminished in relation to its proximal- distal stumps; unlike the GAP treatment which was hour-glass shaped and visibly thinner at its junction with the host tissue. On the other hand, filling the nerve stump gap with any implant does not guarantee optimal axonal outgrowth. For example, we observed in a previous study that filling a transected sciatic nerve gap with peripheral nerve tissue or tissue was inferior to filling the gap with COL (de la Torre, 1985).

The morphological data is also supported by the relative neuroelectrical responses seen with GAP and COL treated nerves when evoked potentials were recorded using the distal nerve stump 12 weeks after transection. The finding that waveforms are present and better organized in COL than GAP nerves, suggests an improvement in the axonal density, organization, or rate of outgrowth using the former treatment. We believe that motor function of the lower limbs was not affected by either treatment because the regenerated axons require longer than 12 weeks before reaching their target tissue in the lower leg musculature (de la Torre, 1988, Restrepo, 1983)... Both groups appeared to react to the distal nerve pinch test, suggesting that axonal sersory fibers regenerate regardless of treatment although the morphologic and neuroelectric findings indicate that superior axon density outgrowth was achieved using a COL bioimplant bridge.

The results of horseradish peroxidase labelling suggest that both GAP and COL groups are capable of regenerating through a 3 mm gap within a 9-12 week period. Unfortunately, the high variability of labelled dorsal root ganglion cells was unsatisfactory for quantitative comparisons between the experimental and control group.

It should be pointed out that GAP nerve repair was aided by the physical and chemical treatment to which both groups were subjected. If this is true, our surgical and chemical repair approach of transected nerve even without COL bridging may be fundamental towards optimizing proximaldistal nerve repair This notion is reinforced by a recent clinical observation on a young man who sustained a 14 cm gap created after median nerve injury. Nerve stump intergap distance appears to be growing successfully after 18 months using the same surgical approach as the GAP preparation described here (Karaca, personel communication).

The main advantages of COL over solid tissue bridges, or re-anastomosis or leaving the nerve gap empty are threefold. First, COL is biocompatible with the nerve tissue. None of our preparations has resulted in a cellular reaction to this implant. There is therefore no danger of potential collagen scarification or fibrosis between COL and host tissue which often occurs even with autografts. We speculate, that

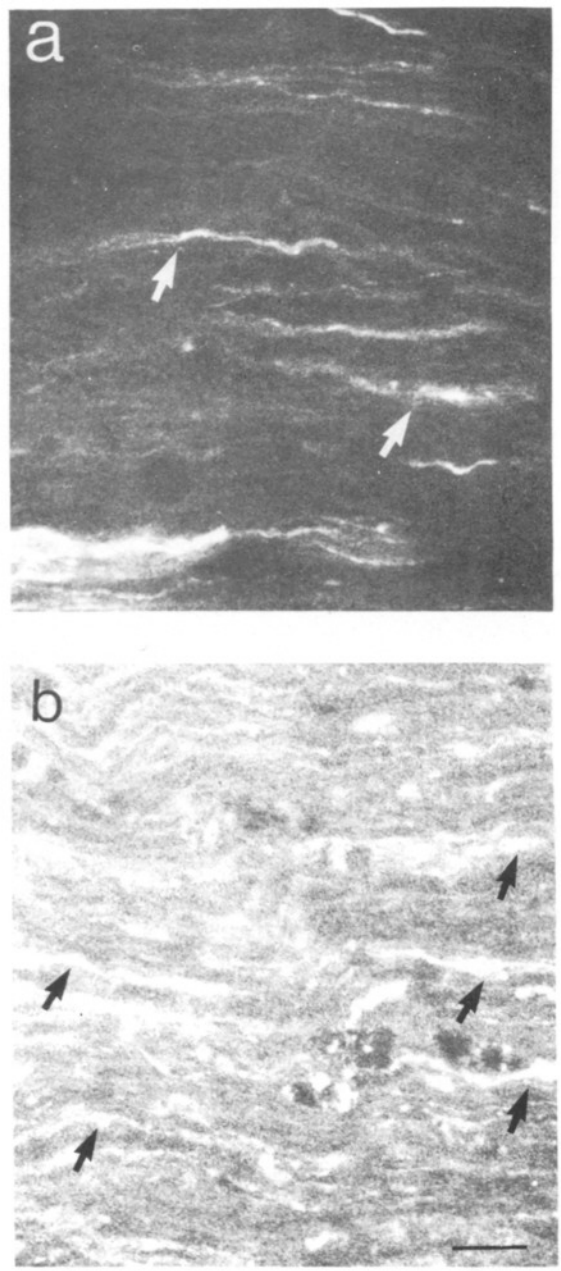

Fig.5 : 5 mm distal stump. a) catecholamine-containing varicosities (arrows) in GAP group. b) increased density of varicosities (arrows) in COL group. Longitudinal section. SPG histofluorescence method; x 300. Bar = 50 um.

using COL on the nerve gap may provide a chemical signal to arrest fibrogenesis despite the fact that COL and scar-generated collagen fibers have totally opposite halical structures. If our hypothesis is correct, tissue collagen scar formation would be reduced or absent. This possibility is further supported by the near absence of fibroblasts or collagen scar formation

in injured and transected rat spinal cord (de la Torre, 1982) and in the present model when COL was used. Second, COL is bioresorbable. Cellular, vascular and neural proliferation eventually migrate into the implanted COL bridge so the bioimplant is transformed into a local extension of host tissue elements. Third, COL is capable of supporting optimal axonal regeneration by its ability to form tight contact with the host tissue in a manner hpysically achieved by solid tissue grafts (de la Torre, 1985). COL may also be able to retain within its structure local putative or circulating neurotrophic factors during the period of neural reconstruction.

The present findings suggest that axonal regeneration after sciatic nerve transection results after surgical-chemical treatment of the nerve stumps and that marked improvement in the axon density, neurotransmitter content, and neuroelectric activity can be further achieved by bridging the nerve gap with COL. Such an approach, may have important clinical application in the area of peripheral nerve injury expecially where a significant gap space is created.

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