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Reconstruction of the Facial Nerve in Pigs with Facial Nerve Allografts Wrapped in a Fibrin Scaffold Containing Fibroblasts Transduced with Adenovirus Encoding VEGF 156

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Abstract

Nerve allografts, which are nerves transplanted between genetically non-identical individuals of the same species, have been studied in experimental and clinical works and seem to be an effective method for nerve reconstruction. We aim to evaluate the effects of VEGF-secreting scaffold holding fibroblasts transduced with an adenovirus on the survival of facial nerve allografts in a model developed in pigs. We operated on 10 white large pigs using one of the following surgical protocols: protocol I, 5 subjects receiving nerve allografts wrapped with a fibrin scaffold holding fibroblasts; and protocol II, 5 subjects with nerve allografts wrapped in a scaffold containing fibroblasts transduced with adenovirus encoding VEGF 156. Statistically significant differences were found by the Mann–Whitney test between protocols I and II for the number of neurofilaments, the number of vessels per field, the number of nerve bundles, and the percentage of neural tissue, with the scores higher for protocol II subjects (p= 0.008 for the above variables). There were no differences in the diameter of the nerve between the protocols. When Student's t was used, the number of neurofilaments and the percentage of neural tissue were significantly different (p= 0.002). This experiment showed that in pigs receiving a 4-cm facial nerve allograft, conditioning with adenovirus-mediated VEGF-secreting heterologous fibroblasts embedded in a fibrin scaffold, improved axonal passage throughout the nerve allograft and helped to maintain the continuity and structure of the new facial nerve.

Introduction

Gene therapy is an emerging prospect in tissue engineering of damaged organs, including those of the neuromuscular system [1,2] and in the treatment of traumatic nerve injuries. Following nerve damage, axon continuity is interrupted and the distal axonal tract begins to degenerate. As a consequence, the anatomical and functional connections between motor neurons and skeletal muscle fibers are lost, inducing progressive muscle atrophy. If nerve continuity is surgically repaired, the proximal stumps of damaged axons can regenerate within the distal nerve stumps, reach the denervated muscle, and restore functional connection with it [3]. Gene therapy based on adeno-associated viruses appears to be a rational approach to reach this goal as they have already been used to efficiently deliver therapeutic genes to nerves and to induce neural protection and repair in several experimental models [4-8]. By means of adenoviruses, we can deliver various growing factors for promoting axon regeneration, in particular vascular endothelial growth factor VEGF [9-11].

Nerve allografts, which are nerves transplanted between genetically non-identical individuals of the same species, have been studied in experimental and clinical works [12,13] and appear to be an effective method for nerve reconstruction, as area cellular nerve allografts [14,15]. We aimed to evaluate the effects of VEGF-secreting scaffold holding fibroblasts transduced with an adenovirus on the survival of facial nerve allografts in a model developed in pigs. We operated on 10 white large pigs using either of the following 2 surgical protocols: 5 subjects receiving nerve allografts wrapped with a fibrin scaffold holding fibroblasts, and 5 subjects with nerve allografts wrapped in a scaffold containing fibroblasts transduced with adenovirus encoding VEGF156. The aim of the study was to determine whether gene therapy improves nerve regeneration through allografts placed in the facial nerves of pigs.

Materials and Methods

Animals

Ten white large male pigs weighing between 20 and 25 kg were used. The European Communities Council Directive (86/609/EEC) guidelines for the care and use of laboratory animals were followed. The animals were distributed at random into 2 groups of 5 animals each and subjected to the following experimental protocols:

• Protocol I: Five pigs received a 4cm facial nerve allograft conditioned with a fibrin scaffold holding heterologous fibroblasts.
• Protocol II: Five pigs received a 4cm facial nerve allograft conditioned with adenovirus-mediated VEGF-secreting heterologous fibroblasts embedded in a fibrin scaffold.

Anesthesia and Post-Treatment Follow-Up

The animals received general anesthesia and endotracheal intubation. Intramuscular injections of 2 ml of a solution of 5 mg xylazine (Rompun[c]) and 50 mg ketamine (Ketolar[c]) plus 0.001 mg/kg of fentanyl (Fentanest[c]) at intervals of 30min were given. Following surgery, the pigs were administered a daily dose of antibiotics subcutaneously: benzyl-penicillin procaine, 200,000 IU; dihydro-streptomycin sulfate, 250 mg in doses of 1 ml/10 kg, and 0.01 mg/kg of buprenorphine (Buprex[c]) and ketorolac, 1mg/kg/day, for analgesia. To avoid rejection reactions to the transplanted allografts and cells, the subjects were treated daily with 4 mg/kg/day of intravenous cyclosporine A (Sandimmune[c]), Azatioprine (Imurel[c]), 1 mg/kg/day, and methyl prednisolone, 4 mg/kg/day, from the day of surgery until sacrifice.

Surgical Protocol

Following standard protocols of asepsis and antisepsis, animals were operated on in groups of two. Two surgical beds were placed in the experimental operation theater. Two surgical groups initiated the operations at the same time, maintaining full synchronization of surgical steps. Initially, a vertical incision was made, following the vertical ramus of the mandible. The incision was extended to the antero-lateral border of the neck. The homolateral deep jugular vein was dissected, and a central venous catheter was introduced. The catheter was extracted at the posterior side of the neck, to avoid it being bitten during the postoperative period. On the face, the initial incision was extended under the horizontal ramus of the mandible, and a musculocutaneous flap was undermined, which includes a part of the parotid gland. The main trunk of the facial nerve was identified (Figure 1) and tested with a neurostimulator. Four centimeters of the central part of the facial nerve were removed. The proximal and distal stumps of the nerve were identified and marked with a 6/0 monofilament suture.

The 4 cm segments of the facial nerve were exchanged between the pigs and sutured end-to-end using a 10/0 monofilament suture, under magnification loupes. In one pig, the nerve was wrapped with the fibrin scaffold holding fibroblasts transduced with adenovirus, but in the other pig, the fibrin scaffold was seeded with non-transduced fibroblasts. A silicone sheet helped to place the scaffold around the nerve. It also helped to avoid the invasion of the operated nerve by fibrous tissue. The myocutaneous flap was closed and a drain was placed under the flap.

The animals were sacrificed 12 weeks after the initial surgery by an overdose of intravenous sodium pentobarbital, but general anesthesia was performed in advance, to obtain in vivo the surgical specimens to be analyzed. Using the same surgical approach, the main trunk of the facial nerve was carefully removed and placed in 10% buffered formalin.

The specimen consisted of 3 parts: proximal stump, allograft, and distal stump. Each specimen was sectioned in two parts: proximal, with the proximal stump and proximal part of the allograft; and distal, with the distal part of the allograft and the distal stump (Figure 2), and processed for paraffin embedding.

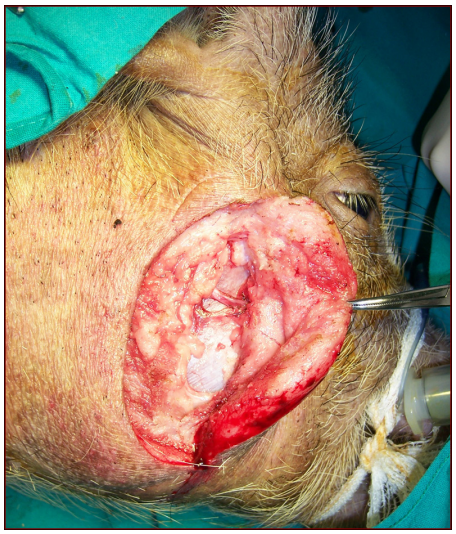


Figure 1. Exposure of the main trunk of the right facial nerve in one of the pigs.

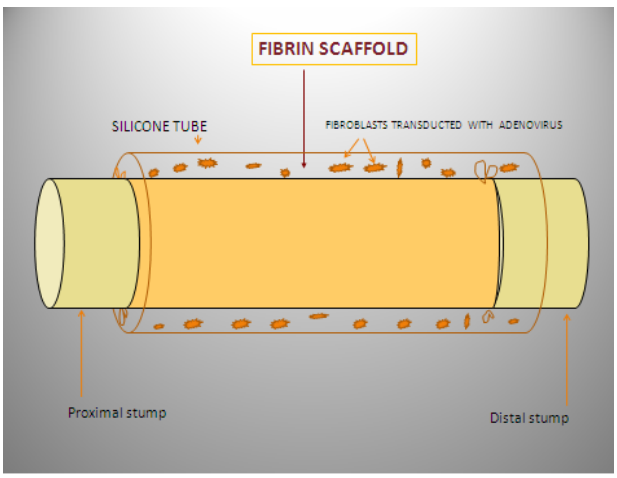


Figure 2. Representation scheme of the facial allograft, wrapped by a fibrin scaffold holding fibroblasts. In group II the fibroblasts were transducted with adenoviruses encoding VEGF-156.

Fibrin Gel Preparation

Fibroblast cells were harvested from a skin biopsy obtained from one of the pigs in the study, according to a method previously described [16]. Cells were cultured in Iscove's modified Dulbecco's medium: Ham's F12 (1:1) containing 10% FCS supplemented with glutamax I, 100 IU/ml penicillin G (sodium salt), 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Cells of three or fewer passages were used. In vitro gene transfer into cells was performed with confluent cultures by incubation with the adenoviral vector in low-serum medium for 3 h at 37ºC. After washing twice with PBS, cells were incubated in growth medium for 24 h and then treated with trypsin/EDTA to obtain individual cells. Fibrin gels containing transduced fibroblast cells were prepared using the protocol described for fibrin-fibroblast gels [9] with modifications. Fibrinogen from pig blood plasma cryoprecipitates was used as the fibrin source. Cryoprecipitates were obtained according to the standards of the American Association of Blood Banks [17]. To produce a fibrin gel, 3ml of fibrinogen solution was added to 12 ml of DMEM/10% FCS containing 5×10^5 transduced endothelial cells. Immediately afterwards, 1ml Cl_2Ca (0.025 mM, Sigma) with 11 IU of bovine thrombin (Sigma) was added. Finally, the mixture was seeded in a 75 cm^2 culture flask and allowed to solidify at 37°C. The gel was covered with culture medium and used 24 h later.

Macroscopic and Light Microscopic Assessment

Motor assessment of facial motion was evaluated, as well as complications associated with surgery, such as local infections, seromas or hematomas. A pathologist assessed the hematoxylin-eosin- and Masson-stained slides and the sections subjected to immunohistochemical labeling of neurofilament (mouse anti-human neurofilament 2F11. CodeIR607/IS607.DAKO[c]) and anti-VEGF (VEGF: SC-7269. Santa Cruz Biotechnology Inc[c]., Santa Cruz, CA, USA).

The architecture of the facial nerve allograft was evaluated with respect to the distribution of axons, the diameter of the nerve, areas of neovascularization, total number of blood vessels labeled with the anti-VEGF antibody (microscopy field, 40×), percent of neural tissue, and inflammatory cell infiltrations.

Neurofilaments belong to the family of intermediate filaments and are structural elements of the neuronal cytoskeleton in interconnection with actin microfilaments, microtubules, and other intermediate filaments. In neurofilament-labeled sections, we counted the nerve fibers in cross-section slides at three different points of the allograft. We ignored any areas with inflammatory reactions and considered only areas that showed no alteration in the nerve anatomy.

Statistical Analysis

The descriptive statistical analysis comprised the calculation of means, median values, and standard deviations of the number of positive neurofilaments per cross-section in the allograft, percent of neural tissue, and number of positive anti-VEGF vessels (single light microscopy field, 40×).

The statistical analysis employed the nonparametric Mann–Whitney test and also Student's t. SPSS software was used for calculation.

Conclusions

This experiment showed that in pigs receiving a 4-cm facial nerve allograft, conditioning with adenovirus-mediated VEGF-secreting heterologous fibroblasts embedded in a fibrin scaffold improved axonal passage throughout the nerve allograft and helped to maintain the continuity and structure of the new facial nerve.

Results

Facial motion function was slightly altered in all animals, with reduced range of motion of the snout on the operated side. They presented no alteration of eyelid motion. All were able to eat normally. No infection appeared during the postoperative period, but one animal of protocol II presented a severe inflammation of the operated cheek resulting from a local hematoma, which was drained.

Observation of the hematoxylin–eosin and Masson-stained longitudinal and cross sections revealed that the diameter of the allograft in the protocol that employed genetically manipulated cells was greater than that of the allografts with non-genetically modified cells.

Study of the architecture of the allograft revealed that the distribution of axons was longitudinal in both protocols and denser in protocol II subjects. In these cases some normal nerve bundles were present at the allograft and at the proximal and distal stumps. The axons were surrounded by hypertrophied Schwann cells, especially at the allograft. In the allografts of protocol I, we observed more nerve distortion and vacuola produced after Schwann cell destruction. This finding was also evident in some parts of the allografts in protocol II, but less frequently than in protocol I.

The group treated according to protocol II exhibited greater capillary density than the other group, as revealed by Masson staining and anti-VEGF (Figure 3). Vessels in subjects treated with transduced cells were markedly dilated, compared to those of subjects treated with non-genetically modified cells.

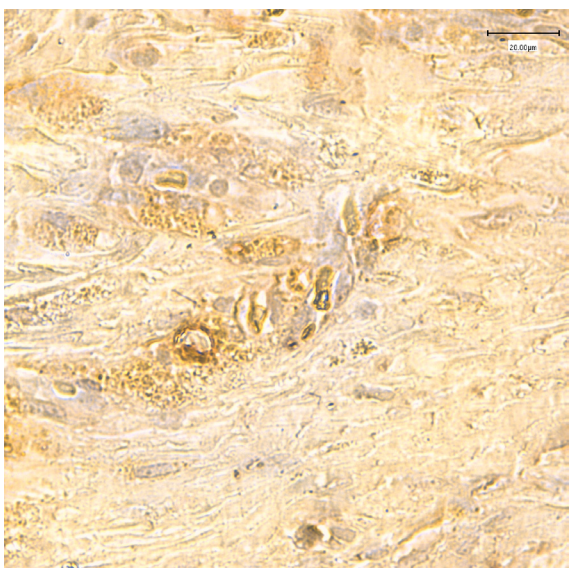
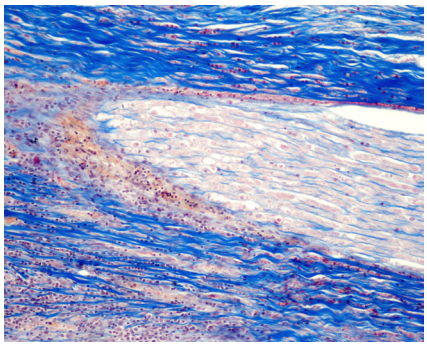


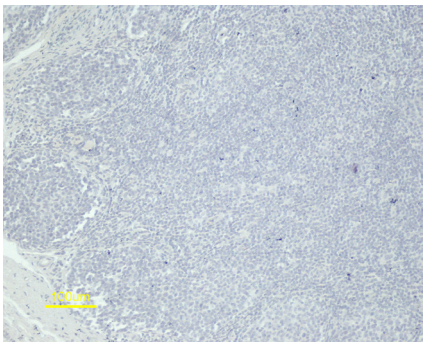
Figure 3. Positive anti-VEGF immuno staining in a new vessel of the nerve allograft in a pig from group II.

Inflammatory response to the nerve allograft was presented in all cases. We classified the degree of rejection in 3 types: severe, moderate and low. Subjects treated with genetically modified cells presented moderate infiltration along the allograft, alternating with regions of low infiltration; those treated with non-genetically modified cells presented moderate to severe degrees (Figure 4 a,b,c). However, in cases that exhibited necrotic areas, inflammatory foci replaced the stroma derived from the gel.

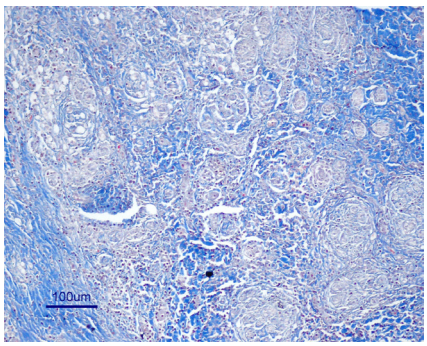
Figure 4 a,b,c



a. Axonal sprouting in an allograft in group II. Masson staining (x10).



b. Group I: Severe inflammatory response to nerve allograft. Massson staining (x10).



c. Group II: Moderate inflammatory response to the nerve allograft which exhibited inflammatory foci replacing the stroma derived from the gel. Massson staining (x10).

Percent of neural tissue was measured in all specimens, in both longitudinal and cross-sectional slides. We described values attributed to the incorporated nerve allograft (Table I).

Results of anti-neurofilament stain in both protocols were recorded. In some cases, the structure of the fascicles was distorted, but there was a normal account of neural structures under neurofilament staining (Figure 5 a,b,c). These results are presented in (Table II).

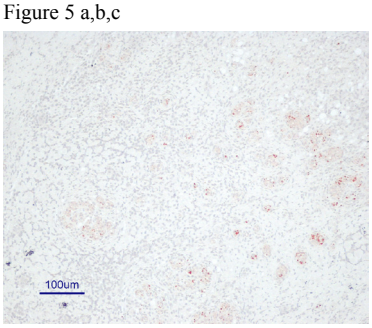
Median/Percentile 25-75	DIAMETER	NUMBER OF BUNDLES	NEUROFILAMENT	CAPILLARY DENSITY	PERCENT NEURAL TISSUE	DEGREE INFLAMMATION
Group I	30/25-38	1/1-2	89/74-95	0/0-0	20/5-25	3/2-3
Group II	60/33-100	3/3-5	139/119-155	2/2-3	55/38-58	2/2-3
Mann-Whitney U (p values)	p=0.095	p=0.008	p=0.008	p=0.008	p=0.008	p=0.22

Table 1: Median, percentile 25th and 75th and p values after Mann-Whitney test corresponding to nerve allograft diameter, number of bundles, neurofilament, capillary density, percent of neural tissue and degree of inflammation.

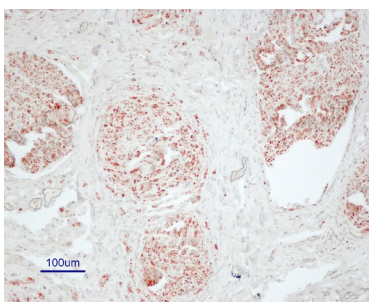
Anti-neurofilament Immuno-staining	Case I	Case II	Case III	Case IV	Case V
Protocol I	89	100	77	90	70
Protocol II	139	169	105	131	140

Table 2: Anti-neurofilament immuno-staining. Number of positive structures in cross-sectional slides (x40).

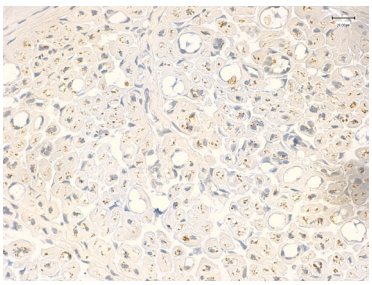
Figure 5 a,b,c



a. Cross section in an allograft of group I. Neurofilament immunostaining (x10).



b. Cross section in an allograft of group II.Neurofilalment immunostaining (x10).



c. Detailed cross section in an allograft of group II. Neurofilament immunostaining (x40).

Statistically significant differences were observed by the Mann-Whitney test between protocols I and II for the number of neurofilaments, the number of vessels per field, the number of nerve bundles, and the percentage of neural tissue, with the scores higher for protocol II subjects ($p= 0.008$ for the above variables). There were no differences in the diameter of the nerve between the protocols.

When Student's t was used, the number of neurofilaments and the percentage of neural tissue were significantly different ($p= 0.002$).

Discussion

The peripheral nervous system is composed of the nerves extending from the brain and the spinal cord to all points of the body. Peripheral neurons consist of a cell body and a long axon, which may reach 1 m in length. Short segments of the axon are wrapped with an insulating myelin sheath formed by Schwann cells, which also plays several important roles in the axon-regeneration process. Nerves can be damaged by trauma caused by compression or cuts as a result of accidents, or by diseases such as sclerosis, diabetes, and polio. Mature neurons do not replicate, but axon extension across gaps caused by injury can occur, reconnecting the axon with the distal stump and eventually reestablishing functional contact.

When a nerve is cut or crushed and nerve function is lost, the portion of the nerve distal to the injury dies and degenerates, but the proximal segment may be able to regenerate and re-establish nerve function. When the axon is disconnected from the cell body by injury, its distal segment gradually degenerates and eventually disappears, a feature known as Wallerian degeneration [18].

A crush often leaves a continuous tubule structure through which the axon can grow, but a cut creates a gap across which the growth cone of the regenerating axon must navigate. To improve recovery, severed nerves can be surgically sutured end to end over small gaps. However, large gaps must be repaired with a graft inserted between the proximal and distal nerve stumps as a guide for regenerating axons. For large garps, (more than 3 cm in humans), nerve segments can be taken from the patient (autograft) or from a donor (allograft) for suturing the stumps without tension. Disadvantages of the nerve autograft include a second surgical procedure, limited availability, permanent denervation of the donor site, and mismatch between nerve and graft dimensions [13]. Allografts have also been used, and data suggest that processed nerve allografts offer a safe and effective method of reconstructing peripheral nerve gaps from 5 to 50 mm in length. These outcomes compare favorably with those reported in the literature for nerve autograft and exceed those reported for tube conduits [19,20].

Avoiding the problem of availability and immune rejection, a promising alternative for extending the length over which nerves can successfully regenerate is the nerve guidance channel. It can be artificial or natural, biodegradable or non-biodegradable [21-24]. Guidance channels bridge the gap between the nerve stumps and direct and support nerve regeneration, reducing cellular invasion and scarring of the nerve and providing directional guidance to prevent neuroma formation or excessive branching. The conduit may be implanted empty or may be filled with growth factors, cells or fibers. We used an artificial channel to protect the scaffold that was wrapping the allograft and the proximal and distal stumps in a way similar to that reported by other authors [25,26]. It prevents fibrous tissue from invading the scaffold and nerve allograft and it is stable as long as the regenerating nerve fibers are not mature. Finally the scaffold will be integrated around the nerve allograft rather than being removed, to avoid the risk of injuring the repaired nerve.

Scaffolds made up of both natural and synthetic materials are another important alternative in nerve tissue engineering, and those made of fibrin glue do not impede nerve regeneration or functional recovery after surgical repair of a segmental nerve defect, but provide structural support for axonal growth and migration [27-29].

Scaffolds may provide bioactive factors that further enhance nerve migration; interestingly, controlled release of VEGF may be achieved over more than 10 days by means of VEGF-loaded polilactic-glicolic acid microspheres [30]. This sustained release is one of the main properties of our fibrin scaffold, given that it is an effective method of maintaining the viability of the fibroblasts encoding VEGF, as previously reported [31,32]. The time-release delivery of growth factors in engineered gel promotes the migration and promotion of embedded cells (in our case, aimed at neural tissue regeneration) and stimulates Schwann cell production and angiogenesis in the allograft.

VEGF was selected because angiogenesis has long been recognized as a necessary step in tissue repair. VEGF promotes angiogenesis by increasing the permeability, growth, and migration of endothelial cells. It also plays an important role in neuronal function and integrity [33]. It enhances vascularization of the nerve graft and Schwann cell migration, finally producing abundant sprouting of nerve axons along the graft [34]. Haninec [8] demonstrated that increasing VEGF protein in nerve stumps resulted in a higher quality of axon regeneration and functional reinnervation after both end-to-end and end-to-side neurorrhaphy. Moreover, Sondell et al. [9] reported that VEGF stimulated Schwann cell invasion and neovascularization after sciatic nerve transection.

This effect has already been described in acellular nerve grafts. In one study, when grafts were loaded with betaNGF and VEGF, the number and mean diameter of axons and neovascularization in the regenerated nerves increased at 1 month [35]. Also in nerve allografts, therapy with VEGF appears promising [14]. VEGF enhanced vascularization and, indirectly, axonal regeneration in a silicone nerve regeneration chamber [36]. It has been described that VEGF and its receptors are expressed in non-vascularized human nerve grafts for facial reanimation [37].

Nerve allografts or homografts are nerves transplanted among genetically non-identical individuals of the same species. Nerve allografts elicit an immune response, but one lower than that of skin, muscle and tendons. Schwann cells express the highest antigenicity among all the components forming the peripheral nerve [38]. Mackinnon et al. have reported several studies with cadaver nerve allografts to restore nerve continuity in clinical cases. These allografts act like viable conduits, their function is provided by the regenerating autologous nerves, and the regeneration is supported by the allogenic cells. It appears that in the early post-transplant period, revascularization is important to maintain Schwann cell viability and produce minimal fibrosis [39]. Xenogenicacellular nerve grafts may also represent an alternative for the reconstruction of defects affecting the facial nerve [40].

Once the allograft is revascularized, it is invaded by macrophages and lymphocytes, producing demyelination around the venules. Schwann cells are the target of the rejection response. Their necrosis is promoted by lymphatic infiltration of the allograft and destruction of the perineurium. If anti-rejection measures are not taken, the nerve becomes fibrotic and non-functional. To approximate clinical nerve allograft reconstruction, a series of long-segment (8-cm) nerve allografts were performed in the ovine model. Animals received nerve allografts under one of the following conditions: fresh nerve autograft, fresh nerve allograft, cold-preserved nerve autograft, and cold-preserved nerve allograft. Excellent regeneration occurred across the nerve autograft, but the long nerve allograft did not support axonal elongation. Similarly, cold nerve preservation did not enhance regeneration [41]. Although several methods have been developed to avoid nerve rejection, including cryopreservation, lyophilization, irradiation or storage of nerves in Wisconsin solution, the best results are obtained with pharmacological immunosuppression of the recipient. However, it is possible to prolong xenograft survival with modified cultured fibroblasts [42].

The toxicity associated with immunosuppression required for graft acceptance must be compared with the relative benefits of reinnervation. Otherwise, the risk of suppression remains a barrier to the advance of tissue transplantation. However, immunosuppressant drugs can enhance the outcomes in nerve allografts. In sheep receiving cyclosporine A, allografts models showed axon regeneration, while non-immunosuppressed allografts were clearly rejected. Such rejection has also been demonstrated in ovine models [43,44]. Azathioprine has also shown good results in nerve allotransplantation [45].

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