Original Article

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The Effect of Low-Dose Tacrolimus on Recurrent Laryngeal Nerve Regeneration in Pigs: A Pilot Study

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Abstract

Objective: The functional neurotrophic effects of systemic tacrolimus in a sub-immunosuppressive regimen in the minipig model of laryngeal reinnervation were investigated.

Methods: Right recurrent laryngeal nerve transection and phrenic-abductor branch of recurrent laryngeal nerve anastomosis were performed in two minipigs that were administered low-dose oral tacrolimus (0.125 mg/kg) for one month. Vocal cord abduction at four months was rated by two blinded expert assessors on a four-point Likert scale from poor (1) to complete (4), and compared with five fully matched historical control animals that received the same surgery but no other interventions.

Results: Right vocal cord abduction was more complete in tacrolimus-treated animals than controls (mean abduction score 2.3 vs. 1.5, p=0.019).

Conclusion: Tacrolimus may have an important role in laryngeal reinnervation following allograft transplantation, and low-dose regimens may have applications in cranial and peripheral nerve injuries or in the reinnervation of tissue-engineered laryngeal constructs, but further studies are required.

Key words: *Tacrolimus, nerve regeneration, larynx, recurrent laryngeal nerve*

Introduction

Airway transplantation is an increasingly viable option for patients with irreversible airway disease including trauma, congenital abnormalities and neoplastic disease [1], and implantation of tissue-engineered tracheal allografts has recently been reported [2,3]. The first fully documented human laryngeal transplant was a qualified success in that the patient retains a stoma to breathe but has an excellent voice [4]. A second laryngeal transplant has been carried out successfully more recently, with the patient achieving a reasonable voice only four months after the procedure [5]. Tissue engineering will probably be the future treatment of choice for long segment tracheo-bronchial disease or laryngeal replacement therapy, though it will be some time before functional neuromuscular activity will be achieved in this way. However, recent advances in producing decellularized laryngeal scaffolds [6,7] and successful translation of other tissue-engineered constructs such as a bladder [8] suggests that we are moving closer to this treatment modality for irreversible laryngeal disease.

For tracheal and laryngeal allografts reported in the literature, the calcineurin-inhibitor tacrolimus (FK506) has been the principal immunosuppressant of choice [4,9,5]. Tacrolimus functions as an immunosuppressive agent by inhibiting calcineurin-mediated T cell activation via inhibition of the calcineurin-nuclear factor of the T cell activation (NFAT) pathway [10]. Tacrolimus has, however, also been found to have neurotrophic effects [11-13]; the mechanism is thought to be independent of its immunosuppressive activity. Though not fully understood, it is thought to involve binding to FK506 binding protein 52 (FKBP-52), a chaperone component of mature steroid receptor complexes, with subsequent dissociation of p23 from heat shock protein-90 (Hsp-90) in the complexes and extra-cellular signal-related kinase (ERK) activation (Figure 1) [14-16]. Animal studies demonstrate that tacrolimus enhances the rate of peripheral axonal regeneration [13], with this effect appearing to be dose-dependent [17].

The problem posed in laryngeal reinnervation is the difficulty in directing regenerating axons to their appropriate targets, given that the recurrent laryngeal nerve supplies all the intrinsic muscles of the larynx except for the cricothyroid muscle. Simple anastomosis of the transected recurrent laryngeal nerve (RLN) leads to synkinesis of the intrinsic laryngeal muscles. Vocal cord abductors (posterior cricoarytenoid (PCA) muscles) must be reinnervated selectively in a way in which vocal cord abduction can be timed with inspiration. We have developed a minipig model of functional laryngeal reinnervation using a phrenic nerve-to-abductor branch of recurrent laryngeal nerve transfer [18], and have used this to demonstrate improved reinnervation times, but not function or myosin heavy chain (MyHC) morphology, using the neurotrophic agent neurotrophin 3 [19].

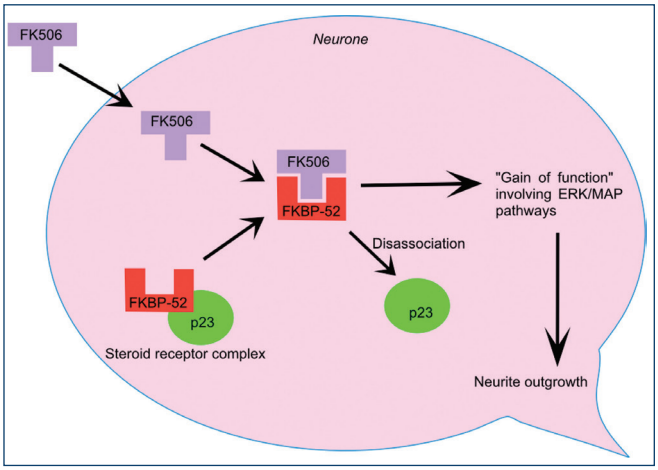

Figure 1. A diagram illustrating the proposed neurotrophic action of tacrolimus in the neurone (FK506=tacrolimus, FKBP-52=FK506 binding protein 52, ERK=extracellular signal-related kinase, MAP=mitogen-associated protein kinase).

Therapeutic immunosuppression is not desirable unless graft rejection is an issue. In the case of tissue-engineered constructs, the antigenicity of allograft tissue is often removed through a decellularization process utilizing enzymes and detergents. Reinnervation of a tissue-engineered construct would not require systemic immunosuppression, and due to the low concentrations of tacrolimus necessary for its neurotrophic effects demonstrated in vitro [17], it is possible that sub-immunosuppressive doses could be used to speed reinnervation whilst minimizing side effects and morbidity.

This pilot study aims to perform a preliminary investigation into the neurotrophic effects of systemic tacrolimus in a low-dose regimen in the minipig model of laryngeal reinnervation, with potential applications in RLN and other peripheral nervous system injuries and the reinnervation of laryngeal allografts or tissue-engineered laryngeal constructs.

Materials and Methods

Two male MHC-matched minipigs each weighing 16 kg at the time of operation underwent a phrenic-to-abductor branch of recurrent laryngeal nerve transfer using our published technique [18]. Animals were reared under conventional husbandry conditions. These experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by the local ethics committee of the University of Bristol and by the UK Home Office.

General anesthesia was induced with intravenous propofol (Propoflo; Abbott Laboratories, Queenborough, UK) to effect after sedation with azaperone (Stresnil; Janssen Animal Health, High Wycombe, UK) and ketamine (Vetelar; Pfizer Ltd, Sandwich, UK). The trachea was intubated, and anesthesia maintained with the volatile agent sevoflurane (Sevflo; Abbott Laboratories Ltd). Analgesia was provided using morphine sulphate (Martindale Pharma, Brentwood, UK) and meloxicam (Metacam; Boerhringer Ingelheim Ltd, Bracknell, UK), which was continued post-

operatively. The larynx was exposed via an anterolateral cervical incision, the strap muscles were retracted, and the posterior crico-arytenoid (PCA) muscles identified with rotation of the larynx. The right recurrent laryngeal nerve (RLN) was then divided distal to the branches to the PCA. The RLN trunk was electrically stimulated to ensure the adductors were denervated. The right RLN was then divided at the thoracic inlet. The phrenic nerve, located under the jugular venous confluence, was confirmed by electrical stimulation, causing visible diaphragmatic contractions, and then divided distally. Phrenic-recurrent entubulation neurorrhaphy was achieved using a saline-soaked 15 x 10-millimeter (mm) polyhydroxylbutyrate (PHB) mat (Astra Tech AB, Mölndahl, Sweden). A single 8/0 Prolene (Ethicon, Gargrave, UK) epineural suture was used to fix each nerve end to the PHB mat, with a 5mm separation between nerve ends under an operating microscope with 10x magnification (Leica Microsystems GmbH, Wetzlar, Germany). The mat was then rolled longitudinally to form a conduit which was closed with three further 8/0 Prolene sutures. The wound was closed in layers using 4/0 subcutaneous Vicryl (Ethicon, Gargrave, UK) and 3/0 subcuticular Dexon (Davis & Geck Wayne, NJ, USA) sutures, and dressed with a single Mepore (Mölnlycke Healthcare A B, Göteborg, Sweden) absorbent dressing that was removed after five days. After surgery the animals were allowed to recover. One animal sustained a fracture of the right hind tibia on recovery from the general anesthetic. After careful consideration a plan was made to treat the fracture conservatively with supportive dressings and a fiberglass half cast, rather than euthanize the animal.

The animals were individually fed a powdered food preparation with oral tacrolimus (Prograf; Astellas Pharma, Staines, UK) thoroughly mixed in, in a dose of 0.125 mg/kg/day. This regimen is in line with low-dose tacrolimus regimens used in previous pig transplantation studies [20,21], and was selected to minimize immunosuppressive effects. The animals were supplied this dose for one month during the initial period of nerve regeneration, with weekly recalculation of the dosage according to the weight of the animals. Trough levels of tacrolimus from whole blood were measured at one month. The animals underwent laryngeal endoscopy at four months and were then killed (using a Schedule 1 method of anesthetic overdose in accordance with UK Home Office regulations). Laryngeal endoscopy was performed under a brief general anesthetic with no neuromuscular blockage and was maintained with inhaled low-dose sevoflurane, allowing active reflexes and spontaneous breathing. The larynx was viewed with a flexible bronchoscope (Karl Stortz, Tuttlingen, Germany), with the images being recorded via the stack system media recorder (Karl Stortz, Tuttlingen, Germany) (Figure 2). Prior to recording, the endotracheal tube was removed, allowing spontaneous vocal cord movement to be captured on video.

Nerve specimens were harvested via anterior cervicotomy after the animals were killed. A nerve specimen consisting of a 1cm proximal (phrenic) nerve, and a 1cm distal (RLN) nerve were immediately harvested, pinned out for orientation and fixed in 4% paraformaldehyde (Sigma-Aldrich, Gillingham, UK). Contralateral control samples of RLN were also harvested and fixed. Immunohistochemical staining for S100 and βIII-tubulin was carried out. Briefly, the nerve specimens were embedded in Optimal Cutting Temperature compound (Invitrogen, Paisley, UK) and cut transversely into 12-μm sections using a cryostat. Sections were mounted onto slides and allowed to dry for 2 h. Sections were then washed in phosphate-buffered saline (PBS) and then blocked with goat and horse serum (both 5% v/v; Sigma-Aldrich) in the presence of 0.1% (v/v) Triton X-100 (Sigma-Aldrich) for 1 h at room

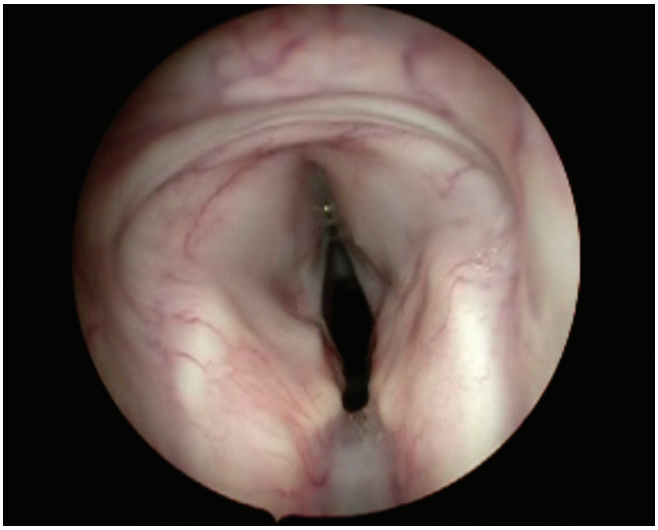

Figure 2. Endoscopic image of pig larynx taken with flexible bronchoscope.

temperature. Next, rabbit S100 polyclonal antibody (1:1000; Dako, Ely, UK) and mouse monoclonal β-III tubulin antibody (1:1000, Sigma-Aldrich) were applied for 2 h at room temperature. After rinsing in PBS, secondary goat anti-rabbit and goat anti-mouse antibodies — Alexa Fluor 568 and Alexa Fluor 488 (both 1:100; Molecular Probes, Eugene, Oregon, USA) — were applied for 1 h at room temperature in the dark. The slides were washed in PBS and then cover-slipped with anti-fading mountant — Prolong Gold containing 4'-6-Diamidino- 2-phenylindole (DAPI; Molecular Probes, Eugene, Oregon, USA). The staining specificity was tested by omission of primary antibodies. Sections were analyzed under fluorescence microscopy, with images being captured with a Nikon DXM1200 digital camera.

Laryngeal endoscopy footage was edited using Windows Movie Maker software (Microsoft, Redmond, USA); 10 x 20-second excerpts (five different excerpts for each animal) were arranged in a random sequence with 10 x 20-second excerpts of historical control videos from experiments on five MHC-matched minipigs of the same sex and age (two different excerpts for each animal). The control pigs underwent identical surgery but were not administered tacrolimus or any other treatments in a previous study performed by the same team 12 months beforehand. The control animals were raised in exactly the same conditions at the same facility. Laryngeal endoscopy at four months was performed in an identical manner. The video compilations were independently assessed by two trained experts (consultant otolaryngologists with a special interest in voice or airway pathology) who graded vocal cord movement using a scoring pro-forma which is adapted from a similar validated scoring system used successfully by Lith-Bijl et al. in 1998 [22]. Abduction was graded on a four-point Likert scale from poor (1) to good (4), and adduction was rated on a four-point Likert scale from paradoxical (1) to normal (4) (Table 1). The trained assessors were blinded to which video excerpts corresponded to controls or intervention animals.

Statistical Analysis

Statistical analysis was carried out using SPSS version 16.0 software (SPSS Inc., Chicago, USA). Mean abduction and adduction grades were calculated from the videolaryngoscopic assessment. Grading of abduction and adduction of vocal cords was compared using the Mann-Whitney U test, as the data were non-parametric. The p values reported are two-tailed. Significance was considered with a p value less than 0.05.

Results and Analysis

The pigs tolerated the low-dose tacrolimus regimen well. Both animals suffered no hypersensitivity reactions or side effects from the medication during the month of administration, and developed no immunosuppressive complications throughout the course of the study. Additionally, the surgery was tolerated well, as documented previously [23,18]. Despite the right-sided hemidiaphragm paresis that resulted from the phrenic nerve transection, there were no respiratory complications or issues apart from early post-operative paradoxical breathing in one animal, manifested only during episodes of anxiety. This settled during the first post-operative week. One animal sustained a fractured hind tibia during recovery from the initial general anesthetic. Given the circumstances and the animals' age, health status and intended interventions, it was decided after discussion with a specialist veterinary anesthetist and veterinary orthopedic surgeon with approval from the Named Veterinary Surgeon that the animal should have the fracture treated conservatively with reduction and plaster cast fixation. Due to the growth rate of the pig, the cast had to be changed every week for six weeks until radiographic evidence of stability was confirmed.

Table 1: Scoring system for vocal cord movement assessment by trained experts.

Score	Vocal cord abduction movement	Vocal cord adduction movement
1	Poor (if no effective or slight abduction occurs)	Paradoxical (one cord adducts while the other abducts)
2	Limited (if abduction on inspiration is less than half)	Immobile (no visible movement on that side)
3	Adequate (if abduction on inspiration is more than half)	Decreased (if adduction is less than normal)
4	Good (complete)	Normal (complete)

The trough FK506 levels taken at one month were 1.0 ng/ml and 1.3 ng/ml. These levels are appropriate for the low-dose regimen of FK506 used in this study and well below therapeutic immunosuppressive levels (5–15 ng/ml).

Videolaryngoscopic Analysis

There was no statistically significant difference in left vocal cord (non-operated side) abduction between the animals that received tacrolimus (mean score 2) and controls (mean score 2.5) (Mann-Whitney U test statistic 66.5, p=0.111) (Table 2). There was, however, a significant difference in right vocal cord (operated side) abduction between the tacrolimus-receiving animals (mean score 2.3) and controls (mean score 1.5), with Mann-Whitney U test statistic 49.5, p=0.019. Additionally, there was no statistical difference between left vocal cord adduction in tacrolimus-receiving pigs (mean score 2.8) and controls (mean score 3.0) (Mann-Whitney U test statistic 82.0, p=0.341). There was no statistically significant difference between right vocal cord adduction either (Mann-Whitney U test statistic 77.5, p=0.234) (Table 2; Figure 3).

Histological Analysis

Nerve specimens distal to the conduits from the operated side and the control recurrent laryngeal nerve from the non-operated side in the tacrolimus-treated pigs were compared after staining with S100 and βIII-tubulin as markers for Schwann cells and neuronal axons respectively (Figure 4 / Figure 5). These images demonstrate regeneration of axons through the PHB conduit, with clearly visible axons seen in sections distal to the conduit (Figure 4) that stain for βIII-tubulin

Table 2: Mean vocal cord movement scores for intervention (surgery and tacrolimus treatment) and controls (surgery only), and comparison with Mann-Whitney U test for significance (*).

	Intervention	Control	P value (two-tailed)
Mean left vocal cord abduction score (1–4)	2.0	2.5	0.111
Mean right vocal cord abduction score (1–4)	2.3	1.5	0.019*
Mean left vocal cord adduction score (1–4)	2.8	3.0	0.341
Mean right vocal cord adduction score (1–4)	2.95	2.7	0.234

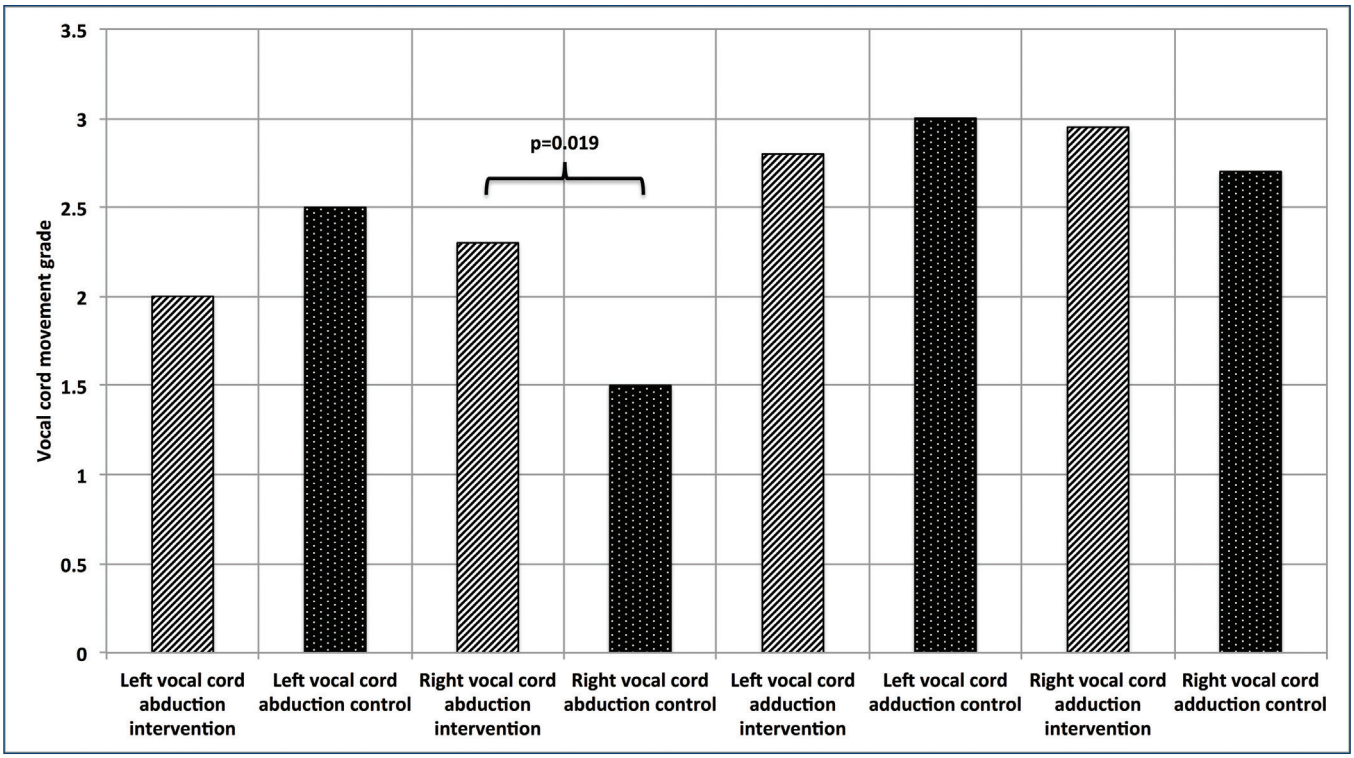

Figure 3. Mean scores for vocal cord abduction and adduction in the intervention group (tacrolimus-receiving animals, n=2) and controls (non-tacrolimus-receiving animals, n=5), with a significantly higher right vocal cord abduction score in the intervention group (*).

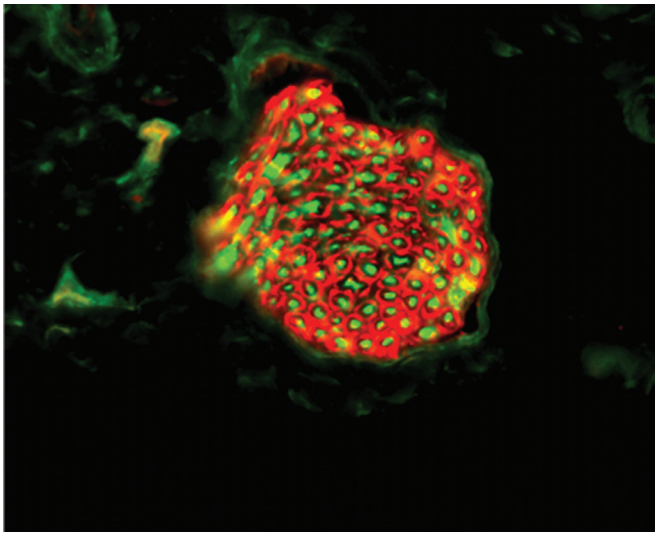

Figure 4. Control recurrent laryngeal nerve stained with S100 (red) to highlight Schwann cells, and βIII tubulin (green) to highlight axons (original magnification x160).

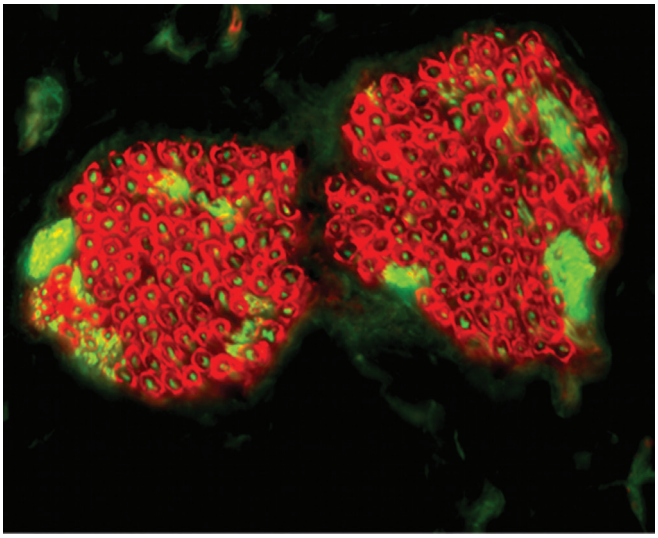

Figure 5. Right recurrent laryngeal nerves distal to anastomosis stained with S100 (red) to highlight Schwann cells, and βIII tubulin (green) to highlight axons, demonstrating qualitative evidence of axonal regeneration (original magnification x160).

(green in Figures 4 and 5); however, the axonal diameter is qualitatively smaller than that of the control recurrent laryngeal nerve.

Discussion

These preliminary results suggest that tacrolimus administered for one month in a low-dose regimen may accelerate functional reinnervation of the posterior cricoarytenoid muscle. Though a significant improvement in operated-side vocal cord abduction in tacrolimus-treated animals compared with controls was observed, the difference of 0.8 versus 0.5 on the non-operated side is small and this underpowered analysis requires repeating with a larger sample size to provide more meaningful results. Evidence of reinnervation was, however, histologically corroborated by qualitative evidence of axonal regeneration in nerve specimens distal to the anastomosis compared with the control recurrent laryngeal nerve. As expected, no differences were seen in vocal cord adduction between tacrolimus-treated pigs and controls, or between the non-operated sides of tacrolimus-treated animals and controls. Additionally, there were no adverse events observed due to either the selective laryngeal reinnervation surgery or the low-dose tacrolimus regimen.

This study is severely limited by the small sample sizes (n=2 in comparison with five controls), and the results must be confirmed with a larger-scale study using the same model of laryngeal reinnervation. Additionally, due to technical problems with the specimens, it was not possible to perform any quantitative morphological investigations on nerve and muscle specimens; only qualitative comparison can be drawn between operated and control nerves. However, this pilot study demonstrates that it is feasible to undertake experiments investigating the functional neurotrophic effects of tacrolimus in laryngeal reinnervation in pigs, and the role of low-dose tacrolimus for this application is promising. Our minipig model of functional laryngeal reinnervation is appropriate for this purpose, and the data generated in this pilot study can be used to inform sample size calculations for a larger-scale study.

Based on these preliminary results, potential future clinical applications for tacrolimus include traumatic or iatrogenic recurrent laryngeal nerve injury, enhanced reinnervation following laryngeal allograft transplantation, and reinnervation of tissue-engineered laryngeal constructs. Additionally, low-dose tacrolimus may have a role to play in the regeneration of other cranial and peripheral nerves following injury.

The results of this study are supported by Gold et al. [13] who reported that in the rat sciatic nerve crush model, a 16% increase in the regeneration rate between days 10 and 15 was observed in animals treated with systemic tacrolimus compared with saline-treated animals (4.4 mm/day compared with 3.8 mm/day). The dose dependency of the neurotrophic effects of tacrolimus was subsequently reported, with more regenerated myelinated axons seen in animals treated with 5mg/kg

tacrolimus [17]. Above this level the neurotrophic effects appeared to diminish. Though no serum trough levels were taken, this study cites unpublished work by Gold and Zeleny-Pooley which found that low (pM to nM) concentrations of tacrolimus markedly promote neuritic outgrowth in vitro from SH-SY5Y human neuroblastoma cells, whereas high (mM) concentrations of FK506 actually inhibit neuritic elongation. Subsequent in vivo studies have demonstrated accelerated nerve regeneration in rats at lower sub-immunosuppressive systemic doses of 0.5 and 1.0 mg/kg/day [24,25]. The low-dose regimen selected in this study is comparable with low-dose regimens used in previous porcine studies [26,21,20], and was chosen to avoid immunosuppressive side effects and morbidity.

The neurotrophic effects of tacrolimus in recurrent laryngeal nerve injury have not been specifically investigated before in a large animal model. However, a study by Gorphe et al. demonstrated no difference in myosin heavy chain type 1, 2a and 2b distribution in the intrinsic laryngeal muscles of tacrolimus-treated rats 45 days after non-selective vagus nerve injury and repair compared with healthy unoperated animals, suggesting effective reinnervation [27].

A recent laryngeal allograft transplant performed by Farwell and Birchall et al. in 2010 demonstrated faster reinnervation of intrinsic laryngeal muscles than expected, with the patient being able to speak with a near-normal voice by four months with functional vocal cord movement, reinnervating in approximately half the expected time [5]. The patient was on a full immunosuppressive dose of tacrolimus before the laryngeal transplantation procedure due to previous pancreatic and renal allograft transplantations, with the most likely explanation for the improved reinnervation time being the systemic tacrolimus therapy. If this is the case, tacrolimus is the ideal immunosuppressive agent to use in composite tissue transplantation.

Upstream agents with the immunophilin-binding profile of tacrolimus, but without calcineurin inhibition and immunosuppressive activity, have been shown to accelerate nerve regeneration in vitro and in vivo [28-30]. Without immunosuppressive side effects, these agents may also be applicable in laryngeal replacement therapies following malignant disease. Therapeutic immunosuppression for laryngeal allograft transplantation would considerably increase the risk of recurrent disease in these patients. Tissue engineering of laryngeal constructs is likely to be a future method of overcoming this problem, and sub-immunosuppressive doses of tacrolimus or non-immunosuppressive derivatives of tacrolimus may play an important role in reinnervation of such constructs.

A possible future direction of research includes the development of nerve guidance conduits that elute tacrolimus locally to the nerve repair zone rather than systemic administration. This could find application in treating peripheral and cranial nerve injuries outside the realm of transplantation. Tacrolimus-eluting nerve conduits could also help deliver tightly controlled optimal concentrations of tacrolimus to nerve repair zones in cranial and peripheral nerve transection injuries.

Conclusion

Low-dose tacrolimus may enhance laryngeal reinnervation in the pig model. Applications include recurrent laryngeal and other cranial or peripheral nerve injuries, reinnervation following laryngeal transplantation, and the reinnervation of tissue-engineered laryngeal constructs. More studies are required to further quantify the safety and neurotrophic effects of tacrolimus in peripheral and cranial nerve regeneration.

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Summary

- Methods of providing functional laryngeal replacement are necessary to treat irreversible laryngeal diseases, which may include laryngeal allograft transplantation and tissue-engineered laryngeal constructs.
- The immunosuppressant tacrolimus has potent neurotrophic effects at low doses, which have been demonstrated in a variety of animal models.

- Phrenic-to-abductor branch of recurrent laryngeal nerve transfer is a well-described technique of providing functional laryngeal reinnervation.
- This pilot study demonstrates that tacrolimus may enhance laryngeal reinnervation in a low-dose regimen based on functional observations in the pig model.
- Potential applications of low-dose tacrolimus include repair of cranial and peripheral nerves, laryngeal allograft transplantation, and reinnervation of tissue-engineered laryngeal constructs.

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