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PREVENTION OF POST-TRAUMATIC REINNERVATION WITH MICROTUBULE INHIBITORS

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Abstract

Objective/Hypothesis—Functional recovery after a recurrent laryngeal nerve or facial nerve injury may be impaired due to aberrant reinnervation. Previous work in a rat peripheral nerve injury model found vincristine to be a potent inhibitor of reinnervation, and it has since been used to effectively block neural regeneration in other animal models. However, vincristine's narrow therapeutic index may limit its utility; therefore, another microtubule inhibitor, paclitaxel, which has a higher therapeutic index, was tested.

Study Design—Animal (rat) study.

Methods—After controlled injury to the rat posterior tibial (PT) nerve, the gastrocnemius/soleus complex was injected with saline (control, n=14), vincristine (n=30), or paclitaxel (n=20). Injections without a crush injury were performed using saline (n=5) or paclitaxel (n=9). The functional recovery (FR) of the posterior tibial nerve was assessed using walking track analysis.

Results—At six weeks, controls had already recovered to baseline (FR=1.0) while the paclitaxel group had FR =0.724 ±0.064 and the vincristine group had FR =0.709 ±0.078. At six months the paclitaxel rats had FR = 0.798 ±0.167 and the vincristine rats had FR =0.754 ±0.240. These differences were significantly different from baseline, but the two agents were not different from each other. Paclitaxel did not affect the FR in the absence of a nerve injury.

Conclusion—Intramuscular paclitaxel and vincristine both significantly inhibit regeneration of the PT nerve after crush injury for at least 6 months. Potential clinical uses of inhibition of reinnervation are discussed.

Level of Evidence—NA

Keywords

Unilateral Vocal Fold Paralysis; neurotoxin; Paclitaxel; Vincristine; neural regeneration; larynx; laryngeal nerve

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Level of evidence: NA (animal study)

INTRODUCTION

Peripheral nerve injuries in the head and neck region are commonly seen by practicing otolaryngologists. Following axonal injury and Wallerian degeneration,{1} nerves that innervate multiple muscles (such as the facial nerve) or antagonistic muscles (such as the recurrent laryngeal nerve) may experience misdirected neural regeneration into the wrong muscle (“synkinesis”).{2,3} The random nature of this aberrant reinnervation results in varying degrees of impairment of functional recovery.{4} Muscles can become partially reinnervated and demonstrate synkinetic activity, or spontaneous reinnervation may be inadequate to generate any observable movement.{5,6} Therefore, most clinicians initially treat patients medically or employ a “wait-and-see” approach to allow the nerve to recover naturally.{5} Currently, there are no early, minimally invasive treatment options available to block potentially aberrant neural regeneration in these patients.

The notion of using a local neurotoxin to selectively block aberrant nerve regeneration was first proposed in 2001 by Paydarfar and Paniello.{7} Drugs that inhibit microtubule assembly are attractive candidates, as microtubules are essential components of nerve regeneration.{8} Vincristine had previously been shown to inhibit sciatic nerve regeneration in a rat model when administered systemically or injected directly into the nerve,{9,10} but Paydarfar and Paniello gave *intramuscular* injections and found that, following a posterior tibial nerve crush injury, vincristine in the gastrocnemius muscle resulted in prolonged impairment of functional recovery.{7} Yian et al. showed that vincristine prevented facial nerve recovery into a target muscle after a transection injury with primary neurorrhaphy, which may have led to enhanced reinnervation of adjacent muscle groups as well as a reduction in synkinetic muscle activity.{11} Studies by McRae et al.{12} in a rat larynx model, and by Paniello{13} in a canine larynx model, both confirmed that vincristine is effective in blocking neural regeneration when administered to the posterior cricoarytenoid muscle.

Vincristine is a potent mitotic inhibitor that is used in various chemotherapeutic regimens and is associated with neutropenia, peripheral neuropathy, and GI symptoms.{14} The dose-limiting toxicity of vincristine is the development of peripheral sensorimotor neuropathy. Vincristine has a narrow therapeutic index, i.e. there is small margin between the therapeutic dose and the toxic dose. In this study we propose the use of another potential neurotoxic agent, paclitaxel, which has a higher therapeutic index. Like vincristine, paclitaxel is associated with neutropenia, peripheral neuropathy, and GI symptoms; however, dose-limiting peripheral neuropathy is sensory and rarely severe at doses <200mg/m² per cycle. {15,16}

The goal of this study was to investigate alternative agents that could prevent neural regeneration similar to vincristine but with a more favorable toxicity profile. Paclitaxel was investigated using the same peripheral nerve regeneration model as previously described.{7} Walking track analysis was performed to assess and measure functional recovery. The rat normally plantarflexes its foot and walks on its toes. After a posterior tibial nerve injury, the rat is no longer able to plantarflex and bears its weight on its heels, resulting in an increased

footprint length. As the nerve recovers over time, the rat regains the ability to plantarflex, resulting in a gradually shortening print length until it returns to normal. The change in print length correlates with the degree of nerve recovery.{3,7,17}

MATERIALS & METHODS

Animal Model

The experimental protocol was approved for use by the Institutional Animal Care and Use Committee (IACUC) at Washington University in St. Louis. Adult male Sprague-Dawley rats weighing between 225–250g were used. The animals were housed in a central animal care facility with alternative 12-hour light and dark cycles and were fed rat chow and water *ad libitum*.

Experimental Design

A paclitaxel dosing trial was performed using 2 rats each for 5 dose groups ranging from 60µg to 1.2mg; doses of 0.6 and 1.2 mg were found to be effective and were utilized in subsequent experiments described below. Post-operatively rats were monitored closely for any adverse reactions.

An additional 78 rats were then divided among 6 experimental groups (Table 1). Rats in the “crush” groups underwent a crush injury to the posterior tibial nerve, which innervates the gastrocnemius/soleus muscle complex (see below). Rats in the “no crush” group had the same dissection with nerve exposure, but the nerve was not crushed.

Vincristine injections were previously shown not to cause functional impairment in the absence of a crush injury.{7} A paclitaxel-no crush control group was created to assess the effect of paclitaxel injection alone on nerve function in the absence of a crush injury. A saline without crush control group was created to show that the microsurgical technique alone does not cause a functional deficit.

Surgical Procedure

Rats were anesthetized via intraperitoneal injection of 40mg/kg ketamine and 5mg/kg xylazine. After the surgical site was shaved and prepped using iodine paint, an incision was made over the right gluteal muscle and microsurgery was performed to expose the three major fascicles of the left sciatic nerve. The posterior tibial nerve was isolated and, for “crush” groups, was crushed 5mm distal to the trifurcation point with one “click” of a small hemostat for 30 seconds. The incision was closed and rats were recovered.

Neurotoxin Injection

Table 1 lists the agent and its corresponding administered dose for each experimental group. Immediately after wound closure, all groups underwent injection of the gastrocnemius/soleus complex with 0.2mL of normal saline, vincristine, or paclitaxel. Paclitaxel solubilized in Cremophor EL (6mg/mL; APP Pharmaceuticals) and vincristine sulfate in aqueous solution (2mg/2mL; Hospira) were used.

Walking Track Analysis

Assessment of nerve recovery was obtained using a well-established walking track method. {7,18} A custom-built walking track measuring 61cm × 14.2cm × 22.8cm was used. Plain packing paper was laid at the base of the walking track along its full length. The hind feet of the rat were each painted with a different color of non-toxic Crayola paint, from the toes to the heel. The rats were then placed at one end of the walking track and allowed to ambulate to the other end, leaving footprints on the paper. The rats were conditioned to the walking track and baseline footprints were obtained just prior to undergoing any intervention. Rats were then walked weekly after intervention for 6 weeks. Selected rats were walked for 6 months (monthly walking tracks after 6 weeks).

The walking tracks were measured in random order by an investigator blinded to the labeling of each walking track. The experimental print length (EPL) was compared to the normal print length (NPL) and a print length factor (PLF) was calculated:

$$PFL = (EPL - NPL) / NPL.$$

Then, functional recovery is calculated using:

$$\text{Functional Recovery (FR)} = (1 - PLF) \times 100.$$

Statistics

Median and range were used to describe the distribution of FR for each group and at each time point. The Mann-Whitney U test was used to compare crush and non-crush groups at each time point and Kruskal-Wallis test was used to compare different drug groups at each time point. When Kruskal-Wallis test was found significant, Mann-Whitney test was employed for post hoc analysis with the alpha level adjusted for number of comparisons using Bonferroni's correction. Friedman's ANOVA was used to compare medial functional recovery across different time points. A mixed within-between subjects ANOVA model was used to compare the treatment groups across all time points. Estimated marginal means were calculated and used to understand and explain the time*drug group interaction effects. Data was analyzed using IBM-SPSS statistical package version 20.0 and all statistical tests were 2 sided and evaluated at the alpha level of 0.05, except for post hoc analysis.

RESULTS

Figure 1 illustrates the calculated functional recovery for Saline Crush(control), Saline Only (control), Taxol Crush, and Vincristine Crush over 6 weeks. There was a significant difference between the two saline control groups for the first 2 weeks ($p < 0.05$), but no difference between the two groups from weeks 3 to 6. The mean Taxol Crush results shown in the plot are the combined data for rats receiving 0.6mg ($n=10$) and 1.2mg ($n=10$), as there was no statistical difference between these two subgroups. The saline-no crush group demonstrated that the microsurgical approach did not impair neural function.

Both the paclitaxel groups and the vincristine group demonstrated significant functional impairment, confirming our hypotheses. Although the paclitaxel groups appear to have recovered slightly more functionality than vincristine at the end of 6 weeks, the difference observed was not statistically significant. There is also a significant within-between rats interaction ($p < 0.001$) demonstrating that there is a significant change from week to week in the calculated functional recovery, and that this change is significantly different among different neurotoxic agent groups. As shown in Figure 1, and as proven by post hoc pairwise comparisons, saline was significantly different from all drug groups, while there was no significant difference between vincristine and paclitaxel.

In Figure 2, a mild functional deficit was noted after injection of paclitaxel in the absence of a nerve injury, but function quickly returned to baseline after 1–2 weeks. This correlates well with prior results using vincristine. {7} These results demonstrate that injection of paclitaxel in the absence of a neural injury does not affect nerve function.

Rats in the vincristine and paclitaxel groups were walked weekly for 6 weeks and then monthly until 6 months (Table 1). There is a significant main effect of time, meaning that independent from the drug group, there was a significant change in percent functional recovery from pre-op to 6 months ($p < 0.005$). Exploring this change through time with pairwise comparisons shows that pre-operative footprint lengths are significantly different from months 1–4, but not from months 5 and 6. Weeks 1–6 are significantly different from all the other time points. Months 2, 3, and 4 are not significantly different from each other (Figure 3).

DISCUSSION

The goal of this study was to identify an alternative neurotoxic agent that had a higher therapeutic index than vincristine with similar neural inhibitory properties. The data confirmed that vincristine inhibits functional recovery and also indicated that paclitaxel is a viable alternative. Although at 6 months rats treated with either paclitaxel or vincristine recovered some function, neither group fully returned to baseline. Both vincristine and paclitaxel prevent cell division by targeting specific β -tubulin binding sites on microtubules. {14}

Previous results have shown that vincristine injection in the absence of a neural injury does not affect nerve function. Injection of paclitaxel in the absence of a nerve injury also did not result in neural impairment in the present study. This finding indicates that there is a window of opportunity following nerve injury during which neurotoxin injection can prevent unwanted reinnervation; later injections will be ineffective. This was recently confirmed with vincristine in a canine recurrent laryngeal nerve model, {19} which showed that injection 3 months after injury had the same effect as an immediate injection, but by 5 months there was no effect.

It is possible that a higher dose of paclitaxel would give a more complete inhibition of neural recovery, although the 1.2mg group did not have more inhibition than the 0.6mg group. Unfortunately, we were unable to administer a higher dose because concentrations of

paclitaxel CrEL higher than 6mg/mL are not commercially available and we were also limited in the total injection volume by the small size of the rat hindlimb. Additionally, paclitaxel is highly hydrophobic and must be solubilized in cremophore, which may be responsible for nonlinear drug deposition and lower bioavailability.{20,21} There is a form of paclitaxel attached to protein-bound nanoparticles, Abraxane, that comes in a powdered form and is soluble in saline. Due to the increased hydrophilicity, there is higher bioavailability of paclitaxel in this form. We were unable to obtain an unreconstituted vial of Abraxane, but believe that this drug merits further investigation.

Selective prevention of reinnervation of a specific muscle following a nerve injury was originally conceived as a way to reduce laryngeal synkinesis,. {7,12,13,20} Other clinical applications are possible. For example, it might be preferable to favor eye closure over forehead or midface function following facial nerve injury; an intramuscular microtubule inhibitor could be used to achieve this as was shown in a rabbit model. {11} The uses of this new clinical tool are limited only by the imagination of the surgeon.

CONCLUSION

Injection of paclitaxel into the rat gastrocnemius/soleus complex significantly inhibited functional recovery after posterior tibial nerve crush injury. The degree of functional recovery over time was comparable to that seen with vincristine. Further investigations of the clinical application of vincristine and paclitaxel are needed to assess the ability of these neural inhibitors to prevent neural regeneration into a target muscle. Assessment of the ability of paclitaxel to inhibit neural regeneration in the setting of a RLN injury in a canine laryngeal model is currently underway.

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This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the *NIH Guide for the Care and Use of Laboratory Animals*, and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committees (IACUC) of Washington University School of Medicine and the St. Louis VA Medical Center.

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Figure 1. Vincristine and Paclitaxel Inhibit Neural Functional Recovery over 6 weeks

Functional recovery in groups undergoing crush injury to the posterior tibial nerve with immediate post-operative injection of saline or neurotoxin to the gastrocnemius/soleus complex. While the saline rats returned to normal function by 4 weeks, both paclitaxel (n=20) and vincristine (n=30) effectively inhibited recovery.

Figure 2. Paclitaxel Alone Does Not Affect Nerve Function

Vincristine-alone injections have previously been shown not to affect intact nerves. After injection of paclitaxel in the absence of nerve injury (n=9), there was a slight decrease in function, but it quickly returned to baseline after 1–2 weeks. Thus, any reinnervation that has already occurred at the time of paclitaxel or vincristine injection will not be blocked by it.

Figure 3. Vincristine and Paclitaxel Demonstrate Prolonged Inhibition of Functional Recovery over 6 months

Both the paclitaxel (n=20) and vincristine (n=22) groups continued to show only partial function through 6 months of follow-up. There was no difference between the two groups.

Table 1

Results at 6 weeks and 6 months for experimental groups.

Agent	Dose	Nerve Rx	6 week results				6 month results			
			n	FR	±SD	p	n	FR	±SD	p
Paclitaxel	0.6 mg	crush	10	0.73	0.07	<0.001	10	0.73	0.13	<0.001
	1.2 mg	crush	10	0.72	0.06	<0.001	10	0.87	0.18	0.03
	0.6–1.2 mg	crush	20	0.72	0.06	<0.001	20	0.80	0.17	<0.001
	1.2 mg	no crush	9	1.01	0.04	NS				
Vincristine	0.1 mg	crush	30	0.71	0.08	<0.001	22	0.75	0.24	0.001
Saline	0.2 ml	crush	14	1.00	0.09	*				
	0.2 ml	no crush	5	0.99	0.05	NS				

* p-values determined with t-test, comparing with saline-crush 6 week result. Paclitaxel 0.6mg and 1.2mg groups were statistically equivalent; their combined results are shown in italics. Vincristine-no crush data was reported previously (ref. 7), and matched the saline and paclitaxel-no crush groups.

Rx, treatment; FR, functional recovery; SD, standard deviation; NS, not significant.