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The Effects of Potential Neuroprotective Agents on Rat Facial Function Recovery Following Facial Nerve Injury

Kalpesh T. Vakharia, MD1, Robin W. Lindsay, MD2, Christopher Knox1, Colin Edwards1, Doug Henstrom, MD1, Julie Weinberg1, Tessa A. Hadlock, MD1, and James T. Heaton, PhD3

Abstract

Objective. To evaluate whether a series of pharmacologic agents with potential neuroprotective effects accelerate and/or improve facial function recovery after facial nerve crush injury.

Study Design. Randomized animal study.

Setting. Tertiary care facility.

Methods. Eighty female Wistar-Hannover rats underwent head restraint implantation and daily conditioning. Animals then underwent unilateral crush injury to the main trunk of the facial nerve and were randomized to receive treatment with atorvastatin (n = 10), sildenafil (n = 10), darbepoetin (n = 20), or a corresponding control agent (n = 40). The return of whisking function was tracked throughout the recovery period.

Results. All rats initiated the return of whisking function from nerve crush by day 12. Darbepoetin-treated rats (n = 20) showed significantly improved whisking amplitude and velocity across the recovery period, with several days of significant pairwise differences vs comparable control rats (n = 16) across the first 2 weeks of whisking function return. In contrast, rats treated with sildenafil (n = 10) and atorvastatin (n = 10) did not show significant improvement in whisking function recovery after facial nerve crush compared to controls. By week 8, all darbepoetin-treated animals and comparable nerve crush control animals fully recovered whisking function and were statistically indistinguishable.

Conclusion. Among the 3 potentially neuroprotective agents evaluated, only darbepoetin administration resulted in accelerated recovery of whisking parameters after facial nerve crush injury. Further efforts to define the mechanism of action and translate these findings to the use of darbepoetin in the care of patients with traumatic facial paralysis are needed.

Keywords

facial paralysis, atorvastatin, sildenafil, darbepoetin, facial nerve crush injury

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Facial paralysis can be a debilitating condition, with significant functional and aesthetic implications. Management of this condition can be directed toward its underlying cause, toward addressing its functional consequences, or both. In most treatment algorithms, there are few medical options. The discovery of an agent that could facilitate or accelerate facial nerve recovery after crush or transection injury would be invaluable. Our laboratory has developed a rat model of facial nerve injury, whereby facial function is objectively quantified by the tracking of whisker movements during recovery.1 This model lends itself to the evaluation of pharmacologic interventions after facial nerve manipulation. Herein, we investigate the effect of 3 pharmacologic agents on the rate and extent of facial function recovery after facial nerve crush injury.

Many pharmacologic agents have been shown experimentally to have a beneficial effect on nerve regeneration.2-4 Among these agents, several are currently in clinical use for other indications, and establishing their benefit in nerve injury models would pave the way for new indications. Statins, for example, are inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA)
do so through the neurogenic potentiating effects of cGMP. Sildenafil were to promote peripheral nerve regeneration, it may also play a role in promoting neurogenesis. PDE5 is an important enzyme involved in the hydrolysis of cGMP, and PDE5 inhibition increases intracellular cGMP concentration. If sildenafil were to promote peripheral nerve regeneration, it may do so through the neurogenic potentiating effects of cGMP.

Darbepoetin is an analogue of erythropoietin (Epo) and is an erythropoiesis-stimulating agent. These drugs have been shown to promote red blood cell survival, stimulate hematopoiesis and angiogenesis, and promote proliferation of smooth muscle fibers and vessel endothelium. They are approved for the treatment of patients with anemia related to chronic renal insufficiency or the administration of chemotherapy. Numerous studies have reported that Epo administration benefits nerve recovery after central and peripheral nerve injury. Epo has been shown to prevent axonal degeneration after nerve injury and to facilitate the return of erectile function after cavernous nerve transection injury in the rat model.

On the basis of these observations, we hypothesized that atorvastatin, sildenafil, and darbepoetin might accelerate facial function recovery after facial nerve crush injury in rats. If effective, these agents could be safely transitioned to use in human clinical practice for the management of facial nerve injuries.

**Materials and Methods**

**Head Fixation and Behavioral Adaptation**

The research protocols for this study were approved by the Massachusetts Eye and Ear Infirmary animal care/research committee. Female Wistar-Hannover rats (Charles River Laboratories, Wilmington, Massachusetts) weighing 200 to 250 grams were used in accordance with institutional guidelines for animal care and use. After animals acclimated to our facility, they were handled for several days prior to surgical manipulation. Animals were anesthetized with an intramuscular injection of ketamine (50 mg/kg; Fort Dodge Animal Health, Fort Dodge, Iowa) and medetomidine hydrochloride (0.5 mg/kg) or dexmedetomidine hydrochloride (0.5 mg/kg; Orion Corporation, Espoo, Finland). They underwent surgical insertion of a lightweight titanium head implant that provided a set of 4 external attachment points for rigid head fixation. After implantation, animals were conditioned to a body restraint apparatus by brief daily placement into a fitted sack. By the third week, head restraint was added to the daily conditioning regimen. After the third week, the animals were sufficiently conditioned to undergo head/body restraint without struggling or signs of stress. Thereafter, presurgical baseline testing was performed.

**Facial Nerve Crush Surgical Procedure**

Animals were anesthetized, and the left infra-auricular area was shaved and steriley prepared. The left facial nerve was approached through an infra-auricular incision in each animal. Left facial nerve exposure involved incision, removal of the parotid gland, and visual identification of the main trunk of the facial nerve as it emerged anterior to the posterior belly of the digastric muscle. Nerve crush was conducted in a manner that has been previously validated, proximal to its trifurcation, for 30 seconds using a jeweler’s microforceps, and the crush injury was repeated for an additional 30 seconds in the same location. The wound was then irrigated with saline and closed in a single layer with absorbable suture, and the anesthetic was reversed with a subcutaneous injection of 0.05 mg/kg of atipamezole hydrochloride. Animals were allowed to recover on a warming pad and were monitored for food and water intake, postoperative discomfort, and weight gain. Through visual inspection, complete loss of whisking function was verified on postoperative day 1.

**Administration of Compounds**

Facility and personnel constraints limited us to testing a maximum of approximately 20 rats per day, so each drug was initially tested in a pilot group of 10 rats run simultaneously with 10 vehicle-administered rats to control for day-by-day variation in testing environment such as weather-related factors, personnel changes, and so on. Drugs that appeared to affect facial nerve recovery were then tested in a second set of 10 treated and 10 control animals. In this investigation, only 1 drug (darbepoetin) appeared to affect nerve recovery and was therefore tested a second time, which accounts for the discrepancy in group sizes among the 3 drugs under study.

For animals receiving atorvastatin (n = 10), a single tablet of atorvastatin was dissolved in methanol and saline (80 mg atorvastatin dissolved in 46 mL saline with 24 µL methanol). Animals received atorvastatin (10 mg/kg) administered by oral gavage twice daily. The dose of atorvastatin was titrated to 10 mg/kg given twice daily, beginning 4 days prior to facial nerve crush surgery. A corresponding control group (n = 10) was treated with the same mixture of methanol and saline (no drug), administered by oral gavage twice daily at the same time points.

For the sildenafil-treated animals (n = 10), a single tablet of sildenafil (100 mg) was crushed, weighed, and mixed in dough. Animals were given oral doses of sildenafil (4 mg/kg) on the day of facial nerve crush surgery and for the first 6 postoperative days (n = 10). A comparable control group (n = 10) was given 1 gram of drug-free dough at the same time points (n = 10).

For the darbepoetin-treated animals (n = 20), the drug was administered at a 10-mcg/kg dose subcutaneously, once per week, for a total of 4 weeks. The initial dose of darbepoetin was administered approximately 1 hour prior to facial nerve crush surgery. A corresponding control group (n = 20) was treated with an equivalent volume of saline subcutaneously at the same time points.

**Functional Recovery Testing**

Baseline whisking evaluation was performed preoperatively, and initial postsurgical evaluation was performed 6 days after animals recovered from facial nerve manipulation. Our previously validated testing apparatus was employed to monitor...
whisking recovery. Briefly, on the day of testing, animals were placed in the body restraint device, C-1 whiskers were marked bilaterally using polyimide tubes (SWPT-045, SWPT-008; Small Parts, Inc, Logansport, Indiana), and then they were placed into the monitoring apparatus. The horizontal movement of the marked C-1 whiskers was independently tracked using commercial laser micrometers (MetraLight, Santa Mateo, California) and a data acquisition computer. A computer-controlled air valve was used to deliver 10-second sustained flows of scented air toward the snout to elicit whisking behavior at 2 random time points during each 5-minute data recording session per animal. The apparatus recorded whisking kinematic data at 1 kHz sampling rate with 12 bits of resolution.

Data and Statistical Analysis

Automated Whisking software, which was used in previous studies, calculated whisking amplitude and velocity for all whiskers in each testing session. The 3 whiskers of largest amplitude were selected, and the amplitude and velocity of these most vigorous whiskers were averaged for each animal, for each day of testing, on each side of the face. If the animal had fewer than 3 measurable whiskers during a testing period (ie, a whisk <3 degrees), a zero was entered for each missing data point. On any given testing day, an animal may have been excluded from analysis if it exhibited significant distress or discomfort. A group average of each whisking parameter was calculated. Statistical analysis software (Minitab, State College, Pennsylvania) was used to perform omnibus 2-way analyses of variance (ANOVAR) to look for main effects (for each drug vs control and day of recovery) from day 12 of the nerve-crush injury and was excluded. All other animals underwent testing throughout the duration of the study, and the data were used unless an animal was unable to be tested on a particular day because of stress.

Animals demonstrated signs of initial whisking function recovery starting on postoperative day 12. There was a rapid recovery phase from postoperative day 12 to day 18 for all groups. After postoperative day 18, whisking recovery began to plateau. Administration of either atorvastatin (n = 10) or sildenafil (n = 10) failed to significantly influence whisking amplitude (P = .882 and P = .711, respectively) or velocity (P = .761 and P = .952, respectively) compared to their respective control groups (atorvastatin control, n = 9; sildenafil control, n = 10) according to ANOVA tests for days 13 to 31. Figure 1a-d shows whisking recovery for these drugs vs their controls across time, revealing similar patterns of recovery.

Administration of darbepoetin in an initial group of 10 rats caused a statistically significant improvement in whisking amplitude (P < .001) and velocity (P = .005) vs controls based on an ANOVA for days 13 to 36. This led us to test a second group of 10 darbepoetin-treated rats and 10 simultaneous vehicle-treated controls, which we combined with data from the first group and likewise found a statistically significant improvement in whisking amplitude (P < .001) and velocity (P = .001) vs controls based on an ANOVA for days 13 to 36. Post hoc pairwise tests were then performed on the combined data across days 13 to 21 and week 8 of recovery. Patterns of whisking amplitude and velocity recovery for the combined darbepoetin-treated rats (n = 20) and their controls (n = 16) are presented in Figure 2, along with the pairwise statistical test results. The drug-treated and control animals were statistically indistinguishable by the end of the testing period at week 8 (Table 1).

Discussion

A number of reports have identified the beneficial effects of neurotrophic factors such as brain-derived neurotrophic factor and nerve growth factor on the injured nerve. Despite promising findings with various agents, no pharmacologic agent (with the exception of corticosteroids) has been deemed safe or efficacious enough to be used in clinical practice. This study evaluates 3 safe, Food and Drug Administration (FDA)-approved drugs in current clinical use for other conditions: atorvastatin, sildenafil, and darbepoetin. All 3 agents have demonstrated potential for use in the setting of peripheral nerve injury. To our knowledge, this is the first report evaluating these 3 agents in the rat facial nerve without any noted side effects. Animals that had not conditioned appropriately to the testing apparatus were excluded. For the control animals, there were a total of 5 exclusions: 1 dough-receiving animal (from control group of sildenafil), 3 subcutaneous injection animals (from control group of darbepoetin), and 1 gavage animal (from control group of atorvastatin). In the drug-receiving groups, there were 2 exclusions, both in the atorvastatin group. A single subcutaneous injection animal (from control group of darbepoetin) had an ineffective crush injury and was excluded. All other animals underwent testing throughout the duration of the study, and the data were used unless an animal was unable to be tested on a particular day because of stress.

Results

All animals recovered without infection from the head mount implantation and nerve crush surgeries and exhibited normal cage behavior throughout the study, including social interactions, grooming, and maintaining or gaining weight in an appropriate manner. Corneal ulcerations were noted in 4 animals in the control groups and in 1 animal in the atorvastatin group. These were treated with ophthalmic antibiotic drops and healed rapidly with no apparent long-term effect. All animals tolerated administration of drug or control vehicle
model. We found a modest but potentially useful benefit to the administration of darbepoetin following facial nerve crush injury.

Crush injury to a peripheral nerve results in disruption of nerve fibers with subsequent Wallerian degeneration and axonal regeneration. The crush model is a reliable model of axonotmesis. Numerous predictable cellular and molecular changes occur in crush-injured neurons and in their microenvironment, and these events provide targets for potential pharmacologic intervention. Within weeks after injury, most axons have regenerated and have undergone remyelination, eventually attaining complete functional recovery within several months. The predictable regeneration that occurs after crush injury allows evaluation of the effect of different pharmacologic agents on the speed and extent of functional recovery within a limited time window during the relatively rapid recovery process. Overall, the rat facial nerve model serves as an excellent paradigm for the study of peripheral nerve injury, the results of which can have direct clinical implications.

Reports in the literature suggest that statin administration will result in improved facial function after facial nerve injury. Numerous studies have demonstrated the beneficial effects of statin administration on both central and peripheral nerves after injury. Atorvastatin was found to be neuroprotective, promote neuronal migration and plasticity, and improve functional neurologic recovery in mice subjected to stroke.

Simvastatin was shown, in rat central nervous system explants, to counteract myelin-associated neurite outgrowth inhibition and promote neurite outgrowth. Gholami et al in 2007 demonstrated that administration of simvastatin prior to ischemic reperfusion injury of the sciatic nerve resulted in improved observer-rated limb function recovery and went on to identify histological features of neuroprotection after administration of simvastatin, in the context of ischemic reperfusion injury of the sciatic nerve. Our results in the rat facial nerve crush injury model do not corroborate these previously reported benefits.

Sildenafil, an FDA-approved, orally available drug with a low side effect profile, was originally designed to combat erectile dysfunction. Investigators have explored its possible neuroregenerative effects based on the known positive effects of nitric oxide (NO) on neural tissue. Studies have shown that increasing NO levels, or increasing cGMP levels with sildenafil, induces neurogenesis and promotes functional recovery of the central nervous system after stroke. Despite the supporting evidence, the administration of sildenafil, after facial nerve crush injury, showed no significant benefit in facial function recovery.

Although sildenafil and atorvastatin administration showed no benefit, darbepoetin administration showed a significant benefit in whisking function recovery after facial nerve crush injury. Darbepoetin has 3 times the half-life of recombinant

Figure 1. Line graphs showing the effect of treatment with atorvastatin (a, b) and sildenafil (c, d) on whisking amplitude and velocity following facial nerve crush injury. (a, b) There is no difference in whisking amplitude or velocity in the atorvastatin-treated animals (n = 10; plots a-b) compared to matched controls (n = 9) or in the sildenafil-treated animals (n = 10; plots c-d) compared to the matched control animals (n = 10). POD, postoperative day.
human Epo (rHuEpo).\textsuperscript{27} This longer half-life allows for less frequent dosing while achieving an equivalent beneficial effect on anemia related to chemotherapy administration.\textsuperscript{28} Epo and its receptor have been discovered to be expressed in the central and peripheral nervous system. After peripheral nerve injury, Epo production is increased in Schwann cells.\textsuperscript{13} Erythropoietin has been found in the in vitro and in vivo models of central and peripheral nerve injury to have a protective and regenerative effect.\textsuperscript{12} Although the mechanism by which Epo exerts its effect on nerves is under investigation, it is believed that by binding to its receptor, it activates numerous downstream pathways that may be responsible for its neuroregenerative potential.\textsuperscript{12} Epo was found to reduce glutamate toxicity, inhibit neuronal apoptosis, and limit axonal degeneration after nerve injury.\textsuperscript{12,29} In rats after transection of the cavernous nerve, administration of rHuEpo and darbepoetin

Figure 2. Line graphs showing the effect of darbepoetin treatment on whisking amplitude (a) and velocity (b), following facial nerve crush injury, along with a table of associated P values. (a) Whisking amplitude during recovery was statistically higher in animals receiving darbepoetin (n = 20) compared to control animals (n = 16) on postoperative days 15, 18, and 21 when tested across days 13 to 21. (b) Whisking velocity during recovery was statistically higher in animals receiving darbepoetin compared to control animals on postoperative days 15, 18, and 21.

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resulted in improved erectile function. Administration of Epo after crush injury to the sciatic and facial nerves has been reported to improve functional recovery. Our findings in the current study corroborate previous work on Epo, as well as darbepoetin. We found that darbepoetin-treated animals had a modest but statistically significant improvement in whisking kinematics at most postoperative time points during the window of rapid recovery after crush injury, compared to their control counterparts. Testing darbepoetin treatment effects in the context of a nerve cut and repair injury, which is much more chronically debilitating than crush injury, might produce a more robust effect because of the greater opportunity for improvement.

Results from the present study of drug effects on recovery of whisking function should be expanded to include anatomical correlates of these behavioral findings. Darbepoetin may not have hastened the arrival of axons to the whisker pad in this study but may have improved whisking function by affecting the manner or degree to which regenerating axons innervated their muscle targets. Anatomical correlates of these behavioral findings would help elucidate the mechanism by which tested drugs influence nerve regeneration and should be less prone to the day-to-day variation that can occur in behavioral assessments (eg, animal reaction to facility staff variation, slight temperature, humidity or barometric pressure fluctuation, colony temperament).

Conclusion
This study demonstrates that darbepoetin, but not atorvastatin or sildenafil, facilitates facial function recovery after facial nerve crush injury in rats. These findings (1) extend to the previous facial nerve work that has shown Epo and darbepoetin to be beneficial when administered after central and peripheral nerve injury and (2) establish the foundation for future work that may result in the incorporation of darbepoetin into the management of patients presenting with facial paralysis after traumatic facial nerve injury.

Author Contributions
Kalpesh T. Vakharia, design, acquisition of data, manuscript production, and final approval; Robin W. Lindsay, design, acquisition of data, manuscript production, and final approval; Christopher Knox, design, acquisition of data, manuscript production, and final approval; Colin Edwards, design, acquisition of data, manuscript production, and final approval; Doug Henstrom, design, acquisition of data, manuscript production, and final approval; Julie Weinberg, acquisition of data, manuscript production, and final approval; Tessa A. Hadlock, design, acquisition of data, manuscript production, and final approval; James T. Heaton, design, acquisition of data, manuscript production, statistical analysis, and final approval.

Disclosures
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