Effects of Corticosteroids on Functional Recovery and Neuron Survival After Facial Nerve Injury in Mice

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Objectives: To assess the effects of corticosteroid administration on functional recovery and cell survival in the facial motor nucleus (FMN) following crush injury in adult and juvenile mice and to evaluate the relationship between functional recovery and facial motoneuron survival.

Methods: A prospective blinded analysis of functional recovery and cell survival in the FMN after crush injury in juvenile and adult mice was carried out. All mice underwent a unilateral facial nerve crush injury and received 7 doses of daily injections. Adults received normal saline or low-dose or high-dose corticosteroid treatment. Juveniles received either normal saline or low-dose corticosteroid treatment. Whisker function was monitored to assess functional recovery. Stereologic analysis was performed to determine neuron and glial survival in the FMN following recovery.

Results: Following facial nerve injury, all adult mice recovered fully, while juvenile mice recovered slower and incompletely. This corresponded to a significantly greater neuron loss in the FMN of juveniles compared with adults. Corticosteroid treatment slowed functional recovery in adult mice. This corresponded with significantly greater neuron loss in the FMN in corticosteroid-treated mice. In juvenile mice, corticosteroid treatment showed a trend, which was significant at several time points, toward a more robust functional recovery compared with controls.

Conclusions: Corticosteroid treatment slows functional recovery and impairs neuron survival following facial nerve crush injury in adult mice. The degree of motoneuronal survival corresponds with functional status. In juvenile mice, crush injury results in overall poor functional recovery and profound cell loss in the FMN. With low-dose corticosteroid treatment, there is a significantly enhanced functional recovery after injury in these mice (P < .05).


Facial nerve injury is a common clinical presentation secondary to trauma, infection, neoplasia, or ablative surgery. The facial nerve is vulnerable to injury owing to its tortuous course in the temporal bone and its complex course in the soft tissues of the face. Severity of peripheral nerve injury ranges from transection, resulting in complete loss of efferent input and muscle paralysis, to crush injury. During a crush injury, the epineurium remains intact, serving as a medium for nerve regeneration to the target tissue and resulting in greater muscle reinnervation and improved motoneuronal survival. Motoneuronal viability allows for synthesis of critical proteins and factors required for successful cell regeneration. In addition to severity of injury, neuronal regeneration depends on a number of other factors including location of the injury, therapeutic interventions, and age at the time of injury. The facial nerve injury model in rodents is a well-established paradigm for studying peripheral nerves. In this model, the facial nerve is either transected or crushed after exiting the skull base from the stylomastoid foramen. Advantages of this model include ease of accessibility of the facial nerve, lack of vital function loss, and possibility of functional recovery assessment after injury. Whisker movement has been previously used to assess functional recovery after facial nerve crush injury. For functional recovery to proceed, the cell body of the facial motoneuron must remain viable. The cell body is located in the facial motor nucleus (FMN) in the caudal region of the ventrolateral pons within the central nervous system.
METHODS

ANIMALS

Permission for experimentation was granted by the Administrative Panel on Laboratory Animal Care at the Stanford University School of Medicine, Stanford, California. All C37BL/Ka mice were ordered from the Stanford University in-house colony. They were housed in designated holding facilities and maintained on a 12-hour light-dark cycle. Animal husbandry was performed by the Veterinary Service Center. Adult mice were entered into the study at 5 weeks of age (P35) and pups at postnatal day 7 (P7). All adults were male. All pups were weaned at day 21 (P21).

ANIMAL GROUPING AND TREATMENT

Adult mice were divided into 3 groups. Each mouse received daily intraperitoneal injections starting immediately after the facial nerve crush injury for 7 days. Each group was treated with either normal saline (n=6) or 1 of 2 dexamethasone doses diluted in normal saline. The corticosteroid treatment groups received 1-mg/kg body weight/d (n=6) or 10-mg/kg body weight/d (n=6) dexamethasone. Complete blood cell (CBC) count analysis with differential was conducted on 2 normal saline and 2 high-dose corticosteroid-treated (10 mg/kg body weight/d) adults. This assay was performed by the Veterinary Service Center accredited laboratory. These animals underwent surgery and received treatment but were not part of the study groups.

Pups were divided into 2 groups. Each mouse also received daily intraperitoneal injections starting immediately after the facial nerve crush injury for 7 days. Each group was treated with either normal saline (n=7) or dexamethasone (1-mg/kg body weight/d). The 10-mg/kg body weight/dose was tested on the pups but proved to be too toxic for this age group with limited survival.

The experimenter was blinded to the treatment each cage received until all data had been collected and analyzed. Cages were color coded, and each mouse underwent ear tagging for identification at the time of surgery.

SURGICAL PROCEDURES

Mice underwent surgery at age P7 or P35. Adult mice were anesthetized with a mixture of ketamine and xylazine. Pups were anesthetized using inhaled isoflurane. Surgery was not started until the mice were areflexic, and this level of anesthesia was maintained throughout the procedure. A curvilinear infraauricular incision was made and the facial nerve was identified. The nerve trunk was crushed distal to the auricular branch with the tips of jeweler’s forceps (Dumont forceps) for a 30-second interval. This resulted in an approximately 2-mm eneural gap at the crush site. Epineurium was noted to be intact at the completion of each crush injury. The skin incision was closed with cyanoacrylic glue. Crush injury was always on the left. After skin closure, each mouse received the first intraperitoneal injection according to cage. All mice recovered on a temperature-controlled heating pad until deemed ready to return to the litter.

MEASUREMENT OF FACIAL NERVE FUNCTION

Adult mice were observed for 27 days postoperatively. Pups were observed for 37 days postoperatively. Observation intervals ranged from daily to every 3 days. Mice were examined individually for whisker function according to the following scale: a grade of 0 was recorded for no detectable movement, 1 for detectable motion, 2 for significant (but asymmetric) voluntary motion, and 3 for symmetric voluntary motion. Whisker movement was assessed over a 2-minute time frame using exposure to food. Each observation session was videotaped in a standardized fashion. Scoring was done at the initial observation and using the recordings at 2 separate time points following completion of the experiment by a blinded investigator. The mean of the 3 scores was used.
TISSUE PROCESSING

All animals were given a lethal dose of carbon dioxide. The brains were immediately dissected free from the skull and placed in phosphate-buffered 4% paraformaldehyde. Tissue was kept on a mixer in fixative for 48 hours. Brains were then treated with 20% glycerol and 2% dimethyl sulfoxide to prevent freeze artifacts and multiply embedded (19 mice brains per block) in a gelatin matrix using MultiBrain Technology (NeuroScience Associates, Knoxville, Tennessee). After curing, the block was rapidly frozen by immersion in isopentane chilled to −70°C with crushed dry ice and mounted on a freezing stage of an AO 860 sliding microscope. The MultiBrain block was sectioned coronally at 60 µm. All sections cut were collected sequentially into a 4 × 3 array of containers filled with Antigen Preserve solution (50% phosphate-buffered saline, pH 7.0, 50% ethylene glycol, and 1% polyvinyl pyrrolidone) for sections to await thionine (Nissl) staining.

STEREOLOGIC ANALYSIS

Nissl stain–positive nuclei and cell bodies were quantitatively and qualitatively evaluated for neuronal and glial numbers. Neuronal and glial counts were conducted on the FMN bilaterally. Brain areas were defined anatomically by atlas and agreement of 3 neuroscientists. Every second section of the FMN (Bregma −5.68 to −6.48) was selected from a random initial sort to ensure random overall sampling, yielding from 6 to 8 slides per animal. The FMN is composed of the nucleus proper, the dorsomedial subnucleus, dorso intermediate subnucleus, dorso lateral subnucleus, lateral subnucleus, ventral intermediate subnucleus, and the ventromedial subnucleus. The FMN is bordered anteriorly by the dorsal periolivary region; medially, laterally, and superiorly by the perifacial zone; anteriorly and inferiorly by the caudal periolivary nucleus; and rostrally by the Botzinger complex and nucleus ambiguous. The use of MultiBrain Technology for the histological procedures assured that brains embedded in gelatin prior to sectioning possessed enough variance to provide a random and unbiased sampling of each of the 19 brain sections on each slide. The optical dissector method and the Stereologer software package were used to quantify Nissl stain–positive cells and glia. The study used a Nikon Eclipse 80i microscope (Nikon Corporation, Tokyo, Japan), linked to a Sony 3CCD Color Digital Video Camera (SONY Corporation, Tokyo, Japan), which operated an Advanced Scientific Instrumentation MS-2000 motorized Stage input into a Dell Precision 650 Server (Dell Computer Corp, Austin, Texas) and a high-resolution plasma monitor. The area of interest was defined using a 1.3-cm aperture dry lenses (original magnification ×4), and the stereologic analysis was performed at high magnification (original magnification ×100) with a 1.4-cm aperture oil immersion lenses (yielding an original magnification of ×3600), allowing clear visualization of the nucleolus and precise definition of the cell walls. When the areas of interest were identified, they were precisely outlined and checked against an atlas. The inclusion grid was randomly applied by the software, and high-resolution microscopy was used to count neurons and glia.

STATISTICAL ANALYSIS

Analysis of adult functional data was performed using a nonlinear regression least-squares fit model. This allowed computation of the time at which a 50% maximal functional (EC50) score was achieved. The EC50 scores could then be compared using 95% confidence intervals. The raw data for the functional recovery in adults were also analyzed between days 0 and 15 using a 2-tailed t test. Analysis of pup functional data was performed using both a 2-tailed t test and a nonlinear regression semilog plot. The latter analysis allowed computation of the slope of each curve. The slopes could then be compared using 95% confidence intervals. The 2-tailed t test was also used to compare the recovery between the adults and pups treated with normal saline to assess age dependence. Statistical comparisons of the neuron survival data were made by means of paired and unpaired t tests.

RESULTS

EFFECT OF CORTICOSTEROID TREATMENT ON WHITE BLOOD CELL COUNT

To demonstrate that dexamethasone treatment resulted in a degree of immunosuppression, CBC counts with differentials were carried out on 2 normal saline–treated adults and 2 high-dose corticosteroid–treated (10-mg/kg body weight/d) adults. As given in Table 1, corticosteroid treatment resulted in a 28% decrease in total white blood cell count, a 58% decrease in lymphocyte percentage and a 71% decrease in absolute lymphocyte count.

FUNCTIONAL RECOVERY FOLLOWING FACIAL NERVE CRUSH INJURY IN ADULT MICE

Adult mice were examined every 1 to 3 days for 27 days after unilateral crush injury. Observations began the day after surgery. Whisker function was scored according to the system detailed in the “Methods” section. At the beginning of the observation period, there was no detectable whisking noted on the crushed side in all mice. In adult mice, whisker function recovered fully by postoperative day 15. This functional status remained stable until the end of the observation period (Figure 1A). The normal saline–treated mice demonstrated a significantly (P < .05) higher functional raw score at 7, 9, and 11 days. To test the hypothesis that corticosteroids influence speed of functional recovery, the time to achieve an EC50 score was analyzed using the computed curves discussed previously (Figure 1B). The corticosteroid-treated mice recovered significantly slower than the control group.

FUNCTIONAL RECOVERY FOLLOWING FACIAL NERVE CRUSH INJURY IN JUVENILE MICE

Pups were examined every 1 to 3 days for 37 days after unilateral crush injury. Observations began the day af-

<table>
<thead>
<tr>
<th>Table 1. Complete Blood Cell Counts</th>
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<tr>
<td><strong>Cell Counts</strong></td>
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<tr>
<td>White blood cells</td>
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<tr>
<td>% Neutrophils</td>
</tr>
<tr>
<td>% Lymphocytes</td>
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<tr>
<td>Neutrophil, absolute, cells/µL</td>
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<td>Lymphocyte, absolute, cells/µL</td>
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Abbreviation: Dex, dexamethasone.
We observed age-dependent functional recovery whereby whisker functional recovery is seen starting at postinjury day 9 and maintained throughout the observation period (P < .005) (Figure 3).

**FACEAL MOTOR NEURON SURVIVAL AFTER CRUSH INJURY IN ADULT MICE**

The next objective was to evaluate the relationship between functional recovery and neuron survival following crush injury. We were additionally able to test the effect of varying doses of corticosteroids on the FMN and whether this correlated with functional outcome. In normal saline–treated adults, there was no difference between neuron (P = .26) or glia (P = .46) survival on the lesioned vs control (unlesioned) sides (Figure 4A and B).

**Figure 5**A and B shows sample photomicrographs of sectioned adult brainstem tissue. In adults treated with low-dose corticosteroids (1-mg/kg body weight/d), there was significantly less neuron survival on the lesioned side compared with the control side (P = .05). In these mice there was no difference in glia survival (P = .30) (Figure 4B). In adults treated with high-dose corticosteroids (10-mg/kg body weight/d), there was again significantly less neuron survival on the lesioned side compared with the control side (P = .005). In these mice, fewer glia survived on the lesioned side (P = .02) (Figure 4B). The increased level of neuron survival in the normal saline group compared to the 2 corticosteroid groups corresponds with the faster functional recovery noted previously. In the adult groups, corticosteroid treatment after lesioning resulted in both slower recovery and reduced neuronal survival. In the high-dose treatment group, reduced glia survival was also noted.

**FACEAL MOTOR NEURON SURVIVAL AFTER CRUSH INJURY IN JUVENILE MICE**

Analysis of the FMN in the pup groups correlated with the poor overall functional recovery. The pup groups show significantly reduced neuron numbers across all measured parameters (Figure 4C and D). Figure 5C and D shows sample photomicrographs of sectioned pup brainstem tissue. There was no observable difference in percentage of cell survival between pups treated with corticosteroid or normal saline (Table 2). In parallel with the pup functional recovery results, there was a significant decrease in the percentage of neuron and glial survival in the FMN compared with adults (Table 2).

**COMMENT**

The immune system has the potential to play a neuroprotective and/or neurodestructive role following central nervous system or peripheral nervous system injury. Therefore, it is critical that therapeutic immune modulators be applied in a way that maximizes the neuroprotective effects of the immune system. In this study, we examined the effects of corticosteroid therapy following facial nerve crush injury in adult and juvenile mice. We observed age-dependent functional recovery whereby adult but not juvenile mice recover completely and at a
rapid rate. Our findings corroborate with previous studies examining functional recovery in mice. We further report an age-dependent decrease in postinjury neuronal survival in agreement with a study by Kuzis et al. While these 2 age-dependent phenomena of neuronal survival and functional recovery have been reported independently, to our knowledge, this is the first study examining these 2 factors concomitantly. Indeed, we detect a direct correlation between neuronal survival and functional recovery, whereby pups in general have severe neuronal loss in the control groups and consequently do not recover fully. Conversely, adult mice display a much less profound loss in neuronal number after injury and are observed to recover fully and at a much more rapid rate than their juvenile counterparts.

The age-dependent observation of neuronal survival has led to the proposal of a “developmental switch” during early postnatal periods. One hypothesis that has been proposed to elucidate this phenomenon is the notion of immature cellular machinery involved in injury response. For instance, immature motorneurons do not display typical chromatolytic responses.

In the present study, we further report the effects of corticosteroids on cell survival and functional recovery. Doses of corticosteroid were chosen based on prior studies in mice that looked specifically at the role of dexamethasone in the inhibition of interleukin 2 secretion. The length of therapy was chosen to ensure sufficient systemic exposure to corticosteroids to allow for the immunosuppressive effects to act and also to avoid glucocorticoid side effects.

![Figure 2](http://archfaci.jamanetwork.com/pdfaccess.ashx?url=/data/journals/faci/5702/) Figure 2. Pup whisking recovery: A, raw data; B, nonlinear regression semilog plot. Pups were observed until 37 days after surgery with incomplete functional recovery. Panel A demonstrates that functional recovery of the dexamethasone (Dex) group is significantly ($P < .05$) higher at 20, 31, and 37 days after surgery using a 2-tailed $t$ test. Panel B shows a nonlinear regression semilog plot of functional recovery of pup whisking as recorded after surgery. This model demonstrates that low-dose Dex administration resulted in faster recovery compared with normal saline administration. Error bars indicate SEM in panel A and 95% confidence interval variance in panel B. *$P < .05$.

![Figure 3](http://archfaci.jamanetwork.com/pdfaccess.ashx?url=/data/journals/faci/5702/) Figure 3. Adult vs pup whisking. This plot compares whisking values of the normal saline–treated pup and adult groups, highlighting the difference in functional recovery. It is evident from this plot that functional recovery is age dependent. Adults recover much faster and to maximum functionality compared with pups. Pups do not recover fully. Error bars indicate 95% confidence interval variance.

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ticoid withdrawal if stopped abruptly. We hypothesize that immune suppressors, such as dexamethasone, will lead to reduced functional recovery and low cell survival. This is based on prior research that has documented that functional recovery is at least partially dependent on a functional immune response. While we observe a trend in agreement with our hypothesis, we also detect that response to dexamethasone is age-dependent. In juvenile mice, low-dose corticosteroid administration results in a trend, which was significant at several time points, toward more robust recovery. Interestingly, however, corticosteroid therapy does not rescue the number of neurons or glia lost after injury in juvenile mice. In contrast, in adult mice, both functional recovery and neuronal survival decreased with corticosteroid administration following injury. In addition, decreased functional recovery and cell survival in adult mice after injury was noted to be dependent on the corticosteroid dose administered. These data support the hypothesis that certain doses of corticosteroids cause progressive immunosuppression and interfere with functional recovery and cell survival.

It is important to highlight the differences between the potential immunoprotective and inhibitory roles of corticosteroids. Previously, other investigators have demonstrated a protective role for corticosteroids after peripheral nerve damage. Al-Bishri et al demonstrated that 24-hour corticosteroid courses augment functional recovery following sciatic nerve crush injury in the rat, the mechanism of which was thought to involve reduction of edema immediately after injury. Our data, however, highlight the immunosuppressive effects of prolonged high-dose therapy that hinder functional recovery and neuronal survival. This is further supported by studies demonstrating the transient ability of lymphocytes to rescue neurons during the first few weeks after injury.

Corticosteroids are potent immunosuppressant agents, acting through both genomic and nongenomic means to suppress the immunologic response. Corticosteroids also demonstrate immunomodulatory effects, including the decrease in number of circulating CD4+ T cells and re-
duced transcription of interleukin 1, resulting in reduced lymphocyte activation. Current evidence suggests that corticosteroids reduce T-cell cytokine production, proliferation, and differentiation through nongenomic mechanisms by reducing T-cell receptor signaling.

The question then arises of the mechanism of cell survival after peripheral nerve injury. One of the proposed models of immune response to peripheral nerve injury postulates that CD4+ T cells are activated in an antigen-specific manner in the periphery. These cells are then hypothesized to cross the blood-brain barrier and become reactivated through microglia and thus promote FMN survival. This so-called dual compartment concept of antigen presentation fits within our proposed hypothesis of corticosteroids leading to reduced cell survival and therefore reduced functional recovery. Corticosteroids reduce T-cell proliferation and T-cell receptor signaling, thereby potentially lowering the neuroprotective roles of CD4+ T cells. Our data demonstrate a reduction in the number of neurons and glia. In the adults, glial survival is reduced by corticosteroid treatment and varies with corticosteroid dose. Reduced glial survival correlates with

Table 2. Average Percentage of Cell Survival

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<tr>
<th>Treatment Group</th>
<th>Adults</th>
<th>Pups</th>
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<tbody>
<tr>
<td></td>
<td>Neurons</td>
<td>Glia</td>
</tr>
<tr>
<td>Normal saline, % (SEM)</td>
<td>94.2 (0.15)</td>
<td>96.0 (0.07)</td>
</tr>
<tr>
<td>1-mg/kg Dex, % (SEM)</td>
<td>88.90 (0.15)</td>
<td>107 (0.16)</td>
</tr>
<tr>
<td>10-mg/kg Dex, % (SEM)</td>
<td>80.7 (0.11)*</td>
<td>86.7 (0.16)</td>
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Abbreviation: Dex, dexamethasone; NA, not applicable.

* P < .005 when compared with adult values.

* P < .05 when compared with normal saline.

Figure 5. Sample photomicrographs of adult and pup thionine (Nissl)-stained brainstem sections. Nissl-stained sections from treated adults, both contralateral (A) and ipsilateral (B) sections, demonstrating nuclear staining. Sample brainstem sections from contralateral (C) and ipsilateral (D) sides of pups. Scale bar=200 µm for all panels.
The reduction in functional recovery, as one would expect in this model. In addition to reactivating CD4+ T cells, glia are central nervous system macrophage precursors that phagocytose debris from dying neurons.6

Taken together, our study demonstrates for the first time to our knowledge, a direct relationship between neuronal survival and functional recovery after facial nerve injury. As hypothesized, corticosteroid treatment reduces functional recovery and cell survival in adult mice. In contrast, functional recovery in pups showed a significant improvement (P < .05) using low-dose corticosteroid treatment. Crush injury resulted in massive neuronal and glial loss in both corticosteroid-treated and control pup groups. Corticosteroid administration did not have an effect on cell survival in these groups. These results suggest that the age-dependent phenomenon of cell survival after crush injury is due to a fundamental difference in either cellular sensitivity to injury-response mechanisms or a difference in the injury-responsive mechanism itself (or both). This is further supported by the age-dependent response to corticosteroid administration.

This study raises the question that it may indeed be possible to overtreat with corticosteroids following crush injury, given the known dependence of recovery on a functioning immune response. To further elucidate this relationship, careful analyses of different doses of corticosteroids administered at different time points after injury must be evaluated. In addition, selective immunomodulators could be used to delineate further the role of the immune response in recovery.

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REFERENCES