

# Nerve growth factor reduces apoptotic cell death in rat facial motor neurons after facial nerve injury

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### ABSTRACT

**Objectives:** To assess the effects of nerve growth factor (NGF) on motor neurons after induction of a facial nerve lesion, and to compare the effects of different routes of NGF injection on motor neuron survival.

**Methods:** This study was carried out in the Department of Otolaryngology Head & Neck Surgery, China Medical University, Liaoning, China from October 2012 to March 2013. Male Wistar rats (n = 65) were randomly assigned into 4 groups: A) healthy controls; B) facial nerve lesion model + normal saline injection; C) facial nerve lesion model + NGF injection through the stylomastoid foramen; D) facial nerve lesion model + intraperitoneal injection of NGF. Apoptotic cell death was detected using the terminal deoxynucleotidyl transferase dUTP nick end-labeling assay. Expression of caspase-3 and p53 up-regulated modulator of apoptosis (PUMA) was determined by immunohistochemistry.

**Results:** Injection of NGF significantly reduced cell apoptosis, and also greatly decreased caspase-3 and PUMA expression in injured motor neurons. Group C exhibited better efficacy for preventing cellular apoptosis and decreasing caspase-3 and PUMA expression compared with group D ( $p < 0.05$ ).

**Conclusion:** Our findings suggest that injections of NGF may prevent apoptosis of motor neurons by decreasing caspase-3 and PUMA expression after facial nerve injury in rats. The NGF injected through the stylomastoid foramen demonstrated better protective efficacy than when injected intraperitoneally.

Multiple etiologies can lead to injury of facial peripheral nerves and result in facial paralysis. Although surgical management of a paralyzed face often produces a favorable outcome, the slow rate of facial nerve regeneration following certain injuries may lead to degeneration of motor neurons and permanent loss of function, thus hindering facial recovery. Nerve growth factor (NGF) is recognized as a trophic molecule that is critical for survival of sympathetic and sensory neurons. Nevertheless, the effects of NGF on motor neurons following facial nerve injury remain unclear, and the most efficacious route for NGF administration remains unknown. It has been

reported that axotomy induces apoptotic cell death of motor neurons.<sup>1</sup> In addition, activation of apoptosis is associated with increased caspase-3 activity as well as enhanced activity of the pro-apoptotic gene, PUMA. The PUMA, a BH3-only protein, binds to Bcl-xL and disrupts interaction between cytosolic p53 and Bcl-xL. Dissociation of p53 from Bcl-xL leads to transcriptional activation of p53, and allows induction of apoptosis.<sup>2,3</sup> Additionally, PUMA may also induce apoptosis through a p53 independent pathway.<sup>4</sup> Based on such evidence, we investigated the effects of NGF injections on motor neurons following induction of a facial nerve lesion in rodents, and compared the neuro-protective efficacies achieved when administering NGF using different routes of injection. Additionally, we explored the involvement of caspase-3 and PUMA in the process of neuro-protection.

**Methods. Reagents.** Mouse NGF for injection was provided by Wuhan Hiteck Biological Pharma Co., Ltd. (Wuhan, China). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) kits were purchased from NanJing KeyGen Biotech Co., Ltd., Nanjing, China. Anti-PUMA, anti-caspase-3 primary antibodies and streptavidin-biotin-peroxidase complex (SABC) immunohistological staining kits (SA1022) were purchased from Wuhan Boster Biological Technology Co., Ltd., Wuhan, China.

**Animals and experimental design.** The experiment was carried out in the Department of Otolaryngology Head & Neck Surgery, China Medical University, Liaoning, China from October 2012 to March 2013. A total of 65 male Wistar rats (200-250 g each) were provided by China Medical University (Shenyang, China). The animals were randomly assigned to one of 4 experimental groups using a random number table: group A, healthy control group (n = 5); group B, facial nerve lesion model + normal saline (n = 20); group C, facial nerve lesion model + NGF injection through the stylomastoid foramen (n = 20); group D, facial nerve lesion model + intraperitoneal injection of NGF (n = 20). All protocols used for animal studies were approved by the Institutional Animal Use Committee of China Medical University. Briefly, animals were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 mL/kg body weight). Then, one side of the

**Disclosure.** This work was supported by grants from Liaoning Province Natural Science Foundation (Grant No. 20072097).

facial nerve was cut at the stylomastoid foramen, and a one cm segment distal to the transaction was excised to impede regeneration of the nerve. Successful model establishment was confirmed by disappearance of the blink reflex. For NGF treatment, animals in groups C and D were injected once daily with one  $\mu\text{g}$  NGF (in 0.1 mL normal saline) either intraperitoneally or through the stylomastoid foramen until the time of tissue sample collection. In group B, animals were injected with normal saline solution rather than NGF. Samples of brain tissue were collected at 7, 14, 28, or 42 days following establishment of the facial nerve lesion model.

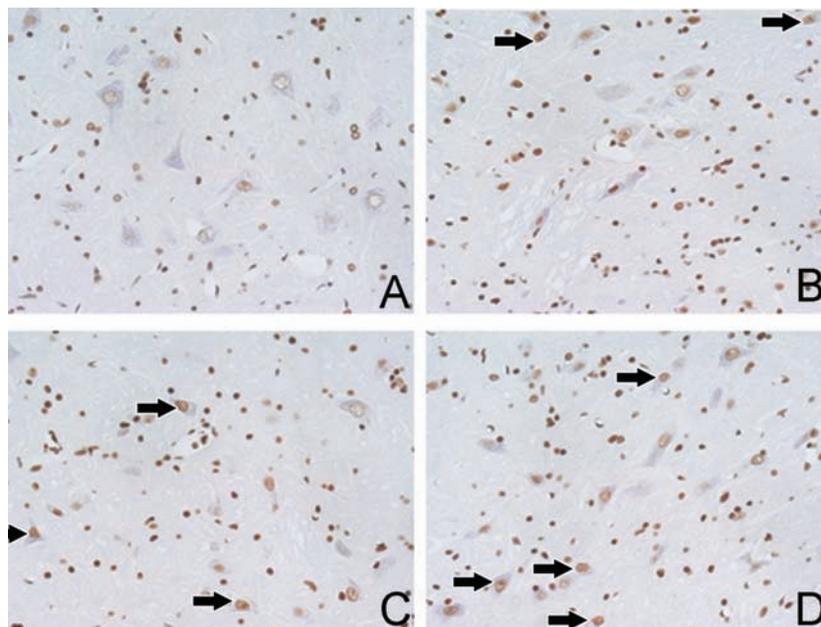
**Evaluation of cell apoptosis.** Apoptotic cell death was detected using the TUNEL assay according to the manufacturer's instructions. Five stained sections of nerve tissue were randomly selected from each rat. A total of 10 randomly selected fields ( $\times 400$  magnification) were analyzed and the number of showing brown-yellow staining (indicative of apoptosis) was counted. Data reflect results obtained from the tissues of 5 animals from each experimental group.

**Immunohistochemistry.** The SABC immunohistochemistry to detect protein expression of PUMA and caspase-3 was performed according to the manufacturer's instructions. Tissue sections were stained with anti-caspase-3 or anti-PUMA primary

antibody (1:500 dilution), followed by staining with 3,3'-diaminobenzidine. The PUMA-positive staining was identified when cells showed a brown-yellow stain in their cytoplasm. Caspase-3-positive staining was identified when cells showed a brown-yellow stain in their cytoplasm or nucleus. Images were examined using an Olympus microscope (BX45-92P05, Olympus, Tokyo, Japan), and a total of 10 randomly selected fields ( $\times 400$  magnification) were analyzed. Protein expression of PUMA was further quantitated using Image-Pro Plus 6.0 software, and results are reported as optical density values. The number of caspase-3-positive cells was determined, and data were calculated using results obtained from 5 animals from each experimental group.

**Statistical analysis.** Data were analyzed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) 17.0 software and plotted as the mean  $\pm$  SD. Comparisons between 2 groups of animals were made using the Tukey test or Tamhane's T2 test.  $P < 0.05$  was considered statistically significant.

**Results.** The NGF administration decreased apoptotic cell death following induction of a facial nerve lesion. Figure 1 and Table 1 shows that barely any apoptotic cells could be observed in motor neurons from healthy control rats. However, following lesion induction in the facial nerve, the number of apoptotic



**Figure 1** - Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining of apoptotic cells. Two weeks following induction of a facial nerve lesion, brain sections of the pontomedullary sulcus region obtained from different groups of animals were stained with TUNEL. Healthy brain tissue was used as a control. Representative figures of group A (A), group B (B), group C (C), and group D (D) are shown. TUNEL staining  $\times 400$ . Apoptotic cells are indicated by arrows.

**Table 1** - Number of apoptotic cells at indicated time following induction of a facial nerve lesion.

Groups	Day 0	Day 7	Day 14	Day 28	Day 42
A	2.16 ± 1.32	—	—	—	—
B	—	18.40 ± 1.43	26.20 ± 1.75	13.10 ± 1.20	9.30 ± 0.67
C	—	11.00 ± 1.25*	15.60 ± 0.97**	7.20 ± 1.03**	4.60 ± 0.84**
D	—	13.70 ± 1.06*	20.30 ± 0.82*	8.50 ± 1.27*	5.70 ± 1.16*

Samples were analyzed using the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay. The average number of apoptotic cells was calculated. \* $p < 0.01$  compared with group B (day 7,  $p = 0.000$ ; day 14,  $p = 0.000$ ; day 28,  $p = 0.000$ ; day 42,  $p = 0.000$ ); † $p < 0.05$  compared with group D (day 7,  $p = 0.000$ ; day 14,  $p = 0.000$ ; day 28,  $p = 0.020$ ; day 42,  $p = 0.012$ ) A - healthy controls, B - facial nerve lesion model + normal saline injection, C facial nerve lesion model + NGF injection through the stylomastoid foramen, D facial nerve lesion model + intraperitoneal injection of NGF

**Table 2** - Effects of nerve growth factor on caspase-3 expression in injured facial nerve at indicated times following induction of facial nerve lesion.

Groups	Day 0	Day 7	Day 14	Day 28	Day 42
A	15.90 ± 2.33	—	—	—	—
B	—	84.70 ± 3.47	118.20 ± 3.65	60.40 ± 5.23	40.30 ± 5.74
C	—	53.8 ± 5.07**	78.30 ± 4.22**	26.90 ± 4.18**	20.0 ± 3.83**
D	—	64.10 ± 3.38*	96.40 ± 6.59*	38.50 ± 3.92*	27.20 ± 4.44*

Samples were immunostained with anti-caspase-3 antibody, and the average number of caspase-3-positive cells was calculated. \* $p < 0.01$  compared with group B (day 7,  $p = 0.000$ , day 14,  $p = 0.000$ , day 28,  $p = 0.000$ , day 42,  $p = 0.000$ ), † $p < 0.01$  compared with group D (day 7,  $p = 0.000$ , day 14,  $p = 0.000$ , day 28,  $p = 0.000$ , day 42,  $p = 0.002$ ). A - healthy controls, B - facial nerve lesion model + normal saline injection, C facial nerve lesion model + NGF injection through the stylomastoid foramen, D facial nerve lesion model + intraperitoneal injection of NGF

cells was greatly increased at day 7, peaked at day 14, and gradually declined after day 28 following injury ( $p < 0.01$ ). Daily injections of NGF significantly reduced cell apoptosis in the model group as compared with rats in the model group receiving saline injections ( $p < 0.01$ ). Moreover, group C exhibited better efficacy for preventing cell apoptosis than group D ( $p < 0.05$ ).

The NGF administration decreased caspase-3 and PUMA expression in injured motor neurons. Following injury, the number of caspase-3-positive cells was greatly increased at day 7, peaked at day 14, and gradually decreased after day 28 ( $p < 0.01$ ) (Table 2). Similar results were obtained from analysis of PUMA expression. In addition, NGF injection remarkably reduced both caspase-3 and PUMA levels in injured motor neurons; however, group C showed better efficacy for decreasing caspase-3 and PUMA expression ( $p < 0.01$ ) when compared with group D. This data suggest that stylomastoid foramen injection of NGF may reduce cell apoptosis by decreasing expression of caspase-3 and PUMA.

**Discussion.** Facial nerve axotomy induces slow and long-lasting neurodegeneration, accompanied by apoptosis of motor neurons,<sup>1</sup> and the potential efficacy of NGF for preventing facial nerve lesion-

induced apoptosis in motor neurons is still unclear. Being a trophic molecule, NGF provides a regulatory link between targeted organs and the nerve cells that innervate them.<sup>5</sup> The NGF promotes projection of neuronal fibers in both the peripheral and central nervous systems, and contributes to neuronal regeneration.<sup>6</sup> We demonstrated that NGF prevented the apoptosis of motor neurons after induction of a facial nerve lesion.

Caspase-3 belongs to the cysteine protease family and serves well-established functions in the execution of apoptosis.<sup>7</sup> We observed enhanced caspase-3 expression in motor neurons following induction of a facial nerve lesion. In addition, injections of NGF greatly decreased the caspase-3 activity induced by facial nerve injury. The change in caspase-3 expression was in accordance with the level of PUMA, implying a potential association between caspase-3 and PUMA in execution of motor neuron apoptosis. The PUMA binds to the Bcl-2 family of pro-survival proteins through its BH3 domain, and triggers cell apoptosis via diverse mechanisms.<sup>3,4,8</sup> All these pathways may function to increase mitochondrial permeability, release cytochrome C, initiate the caspase cascade, and activate caspase-3 and cell apoptosis. Therefore, caspase-3 might be linked to PUMA.

Here, NGF injections through the stylomastoid foramen demonstrated better protective efficacy than

intraperitoneal injections, suggesting that local NGF administration might be more effective than systemic application. However, NGF's effects when used to treat an old injury occurring several months prior to its injection are unknown.

In summary, our study demonstrated that NGF prevented motor neuron apoptosis induced by facial nerve injury in rodents, and that such protection was possibly mediated through down-regulation of PUMA and caspase-3. The limitation of the study is that it was an animal experiment. Further study is required to study how NGF will affect human facial motor neurons.

Received 9th April 2014. Accepted 23rd November 2014.

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