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# Enhanced Reinnervation of the Paralyzed Orbicularis Oculi Muscle After Insulin-like Growth Factor-I (IGF-I) Delivery to a Nerve Graft

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**ABSTRACT**

Facial Paralysis (FP) remains today one of the most disturbing peripheral nerve disorders. The present study utilized the rat model of FP and examined a dual approach of combining the current microsurgical treatment of the cross-facial nerve graft (CFNG) with local administration of insulin-like growth factor-I (IGF-I). The efficacy of this combined treatment approach was assessed by motor endplate analysis of the reinnervated orbicularis oculi muscle (OOM). Local administration of IGF-I (50  $\mu\text{g/ml}$ ) to the CFNG demonstrated a 61% increase in the number of endplates in the reinnervated OOMs compared to the OOMs reinnervated with CFNG + vehicle. These results indicate that the local therapeutic augmentation of IGF-I levels at the coaptation site(s) of the CFNG may in fact enhance reinnervation of muscle and recovery of function in general.

## INTRODUCTION

Aggressive microsurgical treatment is currently the treatment of choice for facial paralysis (FP).<sup>1-6</sup> Unfortunately; microsurgical approaches alone do not completely restore function to denervated facial muscles. Since the introduction of the rat model of FP in 1994<sup>7</sup> we have systematically examined the behavioral and histomorphometric sequella of the cross-facial nerve graft (CFNG) treatment of FP.<sup>8-10</sup> However, treatment of FP with a CFNG alone does not restore full muscle function due to a significant decline in axonal carry-through across the two coaptation sites.

It has been over a century<sup>11</sup> since the first proposal of the role of trophic factors in nerve regeneration, and this has led to the identification of a plethora of potential growth factors that could be considered for this rat model of FP.<sup>12</sup> Among these growth factors, insulin-like growth factor-I's (IGF-I) role in enhancing peripheral nerve regeneration and muscle reinnervation has been well documented.<sup>13-21</sup> These data led us to examine the role of IGF-I in the rat model of FP.<sup>22</sup> This study demonstrated that local administration of IGF-I (50 µg/ml) to the CFNG produced a faster blink response and a stronger degree of eye closure. In addition, it was demonstrated that the IGF-I treated CFNG showed a 22% increase in the mean number of axons crossing the distal coaptation along with an increased mean nerve fiber diameter and myelin thickness.

The present study utilized the same design as previously described<sup>22</sup> and examined the target; more specifically the endplate profile of the reinnervated OOM following treatment with a) vehicle treated CFNG or b) IGF-I treated CFNG. It was hypothesized that IGF-I treatment of a CFNG would enhance the number of motor endplates of the reinnervated OOM and play an important role in restoring eye blink function

IGF-I efficacy of OOM reinnervation was determined by motor endplate analysis. Endplate analysis is an excellent indicator of muscle reinnervation as well as a widely used technique in evaluating the efficacy of different methods of reinnervating muscle<sup>23-27</sup>. OOM evaluation and motor endplate analysis was performed between the 1) Normal rat OOM 2) OOM reinnervated with a vehicle treated CFNG and 3) OOM reinnervated with an IGF-I treated CFNG.

## **MATERIALS AND METHODS**

### **A. ANIMALS**

Twenty adult male Sprague Dawley rats (350 - 450 g) were divided into three groups.

A) Normal OOM group (N=5). These animals received no surgical manipulation.

B) The vehicle treated group consisted of normal rats that received a unilateral facial nerve axotomy just distal to the stylomastoid foramen. A CFNG was performed and an osmotic pump containing a vehicle (1 mM acetic acid) solution (N = 5) was implanted.

C) The IGF-I treated group consisted of animals that similarly received unilateral facial nerve axotomy, and subsequently were treated with a CFNG plus IGF-I (50 µg/ml) infused locally by a similar pump (N = 10). Animals in groups B and C received the following surgical treatments.

### **B. SURGICAL PROCEDURES**

Rats were anesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine (intraperitoneal injection) and operated on with the aid of a surgical operating microscope

(Zeiss). The right facial nerve trunk and its branches were explored and identified through a preauricular incision. The temporal branch of the facial nerve (VII-T) to the orbicularis oculi muscle (OOM) was identified using intraoperative nerve stimulation (Fig. 1).

The right facial nerve trunk was transected just distal to the stylomastoid foramen producing a complete right facial paralysis (Fig. 2). Two hemoclips were then placed around the proximal nerve stump to prevent reinnervation. The left saphenous nerve (3 cm in length) was then harvested as a graft and tunneled subcutaneously to the contralateral side. The saphenous nerve graft was then coapted to both the left and right VII-T using 11-0 (Ethicon) sutures with a 30-micron needle (Fig. 2). An ALZET (# 2004) osmotic pump (200  $\mu$ l reservoir volume, releasing 0.25  $\mu$ l/h over four weeks) fitted with silastic tubing (PE-60) catheter was positioned adjacent to the proximal coaptation of the CFNG and anchored in place with three 11-0 epineurial sutures (Figs. 2, 3). Group B rats had their pump filled with a vehicle solution (1 mM acetic acid), while Group C animals had their pumps filled with IGF-I (50  $\mu$ g/ml). Skin closure was accomplished with 6-0 polypropylene (Prolene) sutures.

Following nine weeks, the entire CFNG was explored. The osmotic pump was removed; the entire graft (CFNG) was harvested including the proximal and distal coaptations, processed for both light and electron microscopy. These histomorphometric analyses were previously described<sup>22</sup>. In addition, behavioral assessment of the reinnervated eye sphincter using the blink test has been previously reported.<sup>22</sup>

### C. HISTOCHEMISTRY

The OOMs were harvested under the operating microscope and mounted on corkboard so that the muscle was flat for longitudinal sectioning. Specimens were then embedded in a tissue-freezing media (OCT compound), frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$ . The frozen OOMs were then sectioned in a serial manner at  $30\ \mu\text{m}$  on a cryostat and stained with a modified acetylcholinesterase (AChE) stain. Briefly, the specimens were: a) incubated at  $37^{\circ}\text{C}$  (pH 7.2) in an AChE solution (3% 5-bromoindoxyl acetate, 0.2% 200 proof ethanol, 16% potassium ferrocyanide, 35% potassium ferricyanide, 23% calcium chloride, and 21% distilled water) for 24 hours b) rinsed with deionized water c) 0.5% potassium ferricyanide for 8 min. d) rinsed with deionized water e) fixed in formal saline solution for 30 min f) washed in deionized water for 20 min. g) dehydrated and coverslipped.

### D. ANALYSIS

Quantitative assessment of the OOM motor endplate profile was performed using the Zeiss Universal light microscope. No sampling was done in the analysis but rather a complete and total count of all the MEPs. MEP analysis was performed blindly by a research assistant who was unfamiliar with the treatment groups of each sample.

Statistical evaluation of the mean number of MEPs in each group was examined using paired t-test comparisons and compared with the endplate profile of the normal OOM

## E. ETHICAL TREATMENT OF ANIMALS

All animals were housed in facilities approved by the American Association for the Accreditation of Laboratory Animal Care. This study was approved by the Animal Care and Use Committee of Eastern Virginia Medical School and carried out according to NIH guidelines. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques.

## RESULTS

### A. QUALITATIVE RESULTS

OOM specimens were stained with AChE to reveal their motor endplate profile. Longitudinal 30  $\mu\text{m}$  thick sections of the OOMs are illustrated across treatment groups in figure 4. Specifically, morphological assessment of the vehicle treated OOMs (Fig. 4c, 4d) in contrast to the normal OOMs (Fig. 4a, 4b) displayed only subtle differences. IGF-I treated OOMs showed slightly more diffuse endplate staining, and many of these endplates did not display the same dark staining as normal control specimens.

### B. QUANTITATIVE RESULTS

Histomorphometric analysis was performed on all OOM specimens. Assessment of the mean total number of endplates between groups is illustrated in Fig. 5. Paired t-test comparisons of the mean total number of endplates between the normal OOM and the vehicle treated CFNG (Fig.5) revealed a significant decrease ( $p < 0.012$ ); whereas no significant difference was observed between the normal OOM and the IGF-I treated

CFNG ( $p > 0.05$ ). In addition, t-test comparison between the vehicle treated CFNG and the IGF-I treated CFNG revealed a significant difference ( $p < 0.028$ ) in the mean total number of endplates.

## DISCUSSION

Presently, the best treatment strategy for FP involves the use of microsurgical techniques and a series of CFNGs. In an attempt to further enhance the degree and strength of recovery, many research approaches are today being investigated. One of these approaches focuses on the role of the neurotrophic environment of the peripheral nervous system. Specifically, previous studies have highlighted IGF's critical role in this milieu of compounds.

The present study examined the effect of IGF-I treatment of the graft (CFNG) on the endplate profile of the reinnervated OOM. Outcome was assessed by motor endplate analysis. Histomorphometric analysis of the number of motor endplates in the OOM revealed an overall greater number of endplates present in the IGF-I (CFNG + IGF-I) treated animals. IGF-I treated animals showed a 61% increase in the number of endplates compared to the vehicle treated animals. Interestingly, the IGF-I treated reinnervated OOMs did not differ with the normal OOMs in their number of endplates.

These data provide further support that IGF-I plays an important role in the regeneration and treatment of FP and support our previous findings<sup>22</sup>. This previous study<sup>22</sup> demonstrated that IGF-I enhanced axonal regeneration within a nerve graft, producing a 22% increase in the mean number of axons. Comparison and correlation between the present OOM motor endplate data and the number of axons present in the

distal graft of the CFNG<sup>22</sup> reveals a positive correlation and approximately a 3:1 ratio respectively. Thus, for every axon reaching the distal end of the graft there were three motor endplates in the OOM. Although it is possible of course that some of these axons are proprioceptive (sensory), it may be hypothesized that a significant number of these endplates are not functional. This hypothesis may be supported by a) the behavioral data<sup>22</sup> where eye closure was improved with IGF-I treatment of the CFNG but at nine weeks was still only 80% complete; and b) the qualitative assessment of the endplates in the IGF-I treated animals revealed that some endplates were diffuse in their staining and of less intensity. This diffuse endplate staining is a characteristic of immature and reinnervated MEPS<sup>28</sup> and muscle fibers strongly influence endplate size and terminal arborization<sup>29</sup>.

The present findings are in agreement with previous studies in the literature. Specifically, the greater number of MEPS in the reinnervated muscle compared to the number of axons reaching the muscle. Tomas et al.<sup>30</sup> recently reported that paralyzed muscle fibers might, somehow spontaneously induce an increase in motor nerve terminal sprouting and therefore a larger motor unit than normally expected. In addition, Park et al.<sup>31</sup> demonstrated that reinnervation of the denervated soleus muscle gave rise to axons reinnervating several different muscle fibers and thus a greater number of endplates than axons and thus supported our findings. Furthermore, when axons reach denervated muscle fibers specialized molecules (neural agrins) induce acetylcholine receptors to cluster under the axon endings and thus may help create new motor endplates<sup>32</sup>

In conclusion, the present study demonstrated that IGF-I is involved in facilitating muscle reinnervation and in enhancing the motor endplate profile of the reinnervated OOM. The present data on the role of IGF-I in treating FP is encouraging. Clearly, further studies are required to define the precise efficacy of IGF-I in combination with CFNG procedures as treatment strategies for FP. Aside from the concentration of IGF-I administered, future studies will examine the potential efficacy of multiple sites of administration, including both proximal and distal coaptations as well as the target muscle.

Other substances besides IGF-I have also been examined for their potential therapeutic use in muscle reinnervation and recovery of function from paralysis. These substances include FK506<sup>33</sup>, Leukemia inhibitory factor<sup>34</sup>, and NT-3<sup>35</sup>. In the future, gene therapy techniques may provide new opportunities in treatment. More concisely, forthcoming studies will examine and compare the viral vector approach of delivering IGF-I alone or in combination with other growth factors (GDNF<sup>36</sup>, NT-3<sup>37</sup>, BDNF<sup>38</sup>, CNTF<sup>39</sup>), in the treatment of FP. Agents that facilitate nerve regeneration such as these mentioned have the potential to limit the extend of motor endplate loss and muscle atrophy seen with prolonged denervation.

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## FIGURE LEGENDS

Figure 1. Facial nerve microdissection in a rat. (T = temporal; Z = zygomatic; B = buccal; M = madibular; C = cervical; PA = posteroauricular; VII = facial nerve trunk.)

Figure 2. Diagram of the facial nerve anatomy in the rat illustrating the site of the axotomy, CFNG, and placement of the IGF-I pump catheter. (Reproduced with permission from Thanos PK, et al.: Insulin-like growth factor-I promotes nerve regeneration through a nerve graft in an experimental model of facial paralysis. *Restor Neurol Neurosci* 15:57, 1999.)

Figure 3. An Alzet (# 2004) osmotic pump attached to a silastic catheter. (Reproduced with permission from Thanos PK, et al. *Restor Neurol Neurosci* 15:57, 1999.)

Figure 4. MEP staining in a longitudinal section of OOM. A) Normal OOM (200X). B) Normal OOM (400X). C) CFNG + vehicle (200X). D) CFNG + vehicle (400X). E) CFNG + IGF-I (200X). F) CFNG + IGF-I (400X).

Figure 5. Mean total number of MEP +/- SEM. Group 1 = normal. Group 2 = CFNG + vehicle. Group 3 = CFNG + IGF-I