Anatomical study of the masseteric and obturator nerves: Application to face transplant and reanimation procedures

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ABSTRACT

Introduction. The masseteric nerve (MN) and the anterior branch of the obturator nerve (ON) that innervate the transferred gracilis muscle have proved highly efficient for reanimating paralyzed facial muscles when muscle-transfer is required. Previous researchers have published the total axonal load for myelinated fibers in both nerves. However, the real motor axonal load has not been established.

Materials and Methods. We performed the study on 20 MN and 13 ON. The segments of the MN and the ON were embedded in paraffin, sectioned at 10μm and stained following a standard immunohistochemical procedure using anti-choline acetyltransferase (ChAT) to visualize the motor fibers.

Results. The MN has a higher axonal load than the ON. There were statistically significant differences between the axonal load of the proximal segment of the MN and the ON. These findings confirm that end-to-end anastomoses between the MN and the ON should preferably use the proximal segment. However, masseteric nerve neurotomy should ideally be performed between the proximal and distal segments, preserving innervation to the deep fascicles.

Conclusions. Our results show that the MN is ideal as a donor motor nerve for

reinnervating transplanted muscle for dynamic reanimation of the paralyzed face. The neurotomy should ideally be performed between the first and second collateral branches of the MN.

Introduction

Facial paralysis (FP) is considered a devastating condition. It seriously affects a key point of human identity and the ability to interact socially, leading to serious consequences for patients (Van Swearingen et al., 1999; Bradbury et al., 2006). Therefore, integral treatment of FP has always been a challenge for reconstructive surgeons since the natural smile, spontaneous expressions or eye closure must be restored *ad integrum.*

The reanimation of FP includes a variety of surgical procedures aimed at restoring the functionality, symmetry and aesthetics of the face. The masseteric nerve has recently been selected as a motor source because of its lower associated morbidity rate and greater ease of activation for the patient (Klebuc, 2015; Biglioli et al., 2017). Its has proved highly efficient for reanimating paralyzed facial muscles, transferring directly to the facial nerve (Wang et al., 2012; Angspatt and Pannanusorn, 2017), but other reconstructive surgical strategies are required depending on the timing of the facial paralysis. In cases with intrinsic facial muscle atrophy presenting after more than 24 months of evolution, muscle-transfer is required, and the choice of recipient nerve is of the utmost importance (Lee et al., 2008).

Among candidates, the masseteric nerve (MN) is important, and so is the anterior branch of the obturator nerve (ON) that innervates the transferred gracilis muscle. Moreover, for many surgeons, this is the ideal muscle to use for dynamic restoration of FP (Zucker et al., 2000; Woollard et al., 2010; Fattah et al., 2011; Bianchi et al., 2012; Fattah et al., 2012).

Histomorphometric studies have been conducted to establish the total numbers of myelinated fibers in the masseteric and the obturator nerves. However, the reported results are variable: 1543 fibers/mm^2 (Coombs et al., 2009); 2775 fibers/mm^2 (Borschel et al., 2012) and 5289 fibers/mm^2 (Snyder-Warwick et al., 2015). These differences can be explained because the different authors quantified all the myelinated fibers, both motor and sensory. Furthermore, the motor axonal load could change along the nerve trajectory. To visualize motor fibers specifically, it is necessary to use anti-choline acetyltransferase (ChAT) antibody. To the best of our knowledge, this technique has not been used to study the MN and ON motor fibers. Therefore, we have undertaken this study on a reliable sample of human cadavers.

Materials and methods

We performed the study on 20 masseteric nerves (eleven right and nine left) and 13 obturator nerves (seven right and six left) from 11 formalin-fixed cadavers from the Centre for Body Donations at the Complutense University of Madrid (six males and five females, with a mean age of 73 years at death).

To visualize the masseter muscle and the MN, the skin and facial superficial fascia were removed. First, the parotid gland and the facial nerve were removed in order to identify different fascicles of the masseter muscle. The superficial fascicle of the muscle was detached from the zygomatic arch to observe the entry zone of the MN through the mandibular notch. The course of MN inside the muscle was carefully dissected to determine the total number of collateral branches.

To obtain the anterior branch of the ON in the gracilis muscle, the anteromedial thigh region was dissected. All the skin and the superficial fascia were removed over the region to expose the investment fascia. The sartorius muscle was detached from its proximal attachment, and the femoral triangle was dissected free to reveal the femoral vessels and their branches, the pectineus and the adductor longus muscles. The anterior division of the ON to the gracilis muscle was located, and a segment of the nerve as it entered the muscle was harvested.

We divided the MN into four segments and examined them immunohistochemically. The first segment included the main trunk up to the first branch to the superficial belly; the second segment ran between the origins of the first and second branches; the third

was the first branch itself; and the fourth segment was the rest of the MN distal to its second branch (Figure 1).

These different segments and the anterior branch of the ON were embedded in paraffin, sectioned at 10μm and stained following a standard immunohistochemical procedure using anti-choline acetyltransferase antibody to visualize the motor fibers (Pascual-Font et al., 2013). Briefly, after treatment with sodium citrate buffer (10.2 mM, pH 6.1) for 20 min at 95ºC for antigen retrieval, the nerve sections were blocked with horse serum (10%) and BSA (3%) in Tris-phosphate buffer containing 0.3% Triton X-100. They were then incubated for three days with goat anti-ChAT primary antibody (1/75; AB144P; Millipore, Eugene, Oregon, USA), which characterizes motor fibers. After several washings, the sections were incubated for one day with biotin-conjugated horse anti-goat antibody (1/200; Vector Laboratories, Peterborough, UK). To visualize the ChAT, an ABC-peroxidase kit (Vector) using DAB-nickel staining (Vector) was used. The slides were dehydrated and mounted with DPX. The immunohistochemical procedure was performed in duplicate on all samples; as a negative control, one of the slides was not incubated with the primary antibody. The sections were viewed and photographed using a Nikon Eclipse 800M microscope equipped with a Nikon DMX1200 (12 megapixel) digital camera. ChAT-positive axons were counted using ImageJ software (NIH, Bethesda, Maryland). Individual myelinated ChAT fibers were identified by black staining.

IBM SPSS (International Business Machines Corp. SPSS Statistics Premium Faculty Pack 21.0 ®) was used for statistical analyses. For descriptive statistics we calculated

the mean, median, range and standard deviation. The non-parametric Mann-Whitney U test was used to compare medians in relation to the size and variance of the samples. Statistical significance was accepted at $p<0.05$.

Results

The number of ChAT-positive axons presumptively identified as motor axons in each of the different segments of the MN is summarized in Table 1. The first segment of the MN contained a mean of 640 ± 283 ChAT-positive and 749 ± 235 ChAT-negative axons. The second segment contained a mean of 527 ± 221 ChAT-positive and 548 ± 134 ChAT-negative axons. The first branch showed 423 ± 298 ChAT-positive and 444 ± 177 ChAT-negative axons. Finally, in the distal segment just after giving off the second branch to the superficial belly, there were 216 ± 301 ChAT-positive motor axons and 233 ± 187 for ChAT-negative fibers (Table 1, Figure 1).

The means and standard deviations of the axonal count of the anterior branch of the ON supplying the gracilis muscle were 352 ± 193 ChAT-positive and 392 ± 188 ChAT-negative axons (Table 1, Figure 2). All these numbers and the percentages of ChAT-positive axons in both the MN and ON are summarized in Table 1.

Owing to the dispersion of the chosen variable (ChAT-positive and -negative axons), we used the median as a frequency measure in our statistical analysis. Using the nonparametric Mann-Whitney test, the median of the ChAT-positive axons in each segment of the MN (proximal, middle, distal and first branch) was compared to the median of the ChAT-positive axons in the ON. There were statistically significant differences ($p<0.05$) between segment I of the MN and ON and between segment IV of the MN and the ON (Figure 3).

Discussion

Methods used to classify the sequelae of FP are based on the evolution time of the paralysis and on the degree of functionality of the remaining muscles of the face (May, 1979; Gousheh and Arasteh, 2011).

The period between the injury to the nerve and the possibility of restoring the innervation to the muscles is critical and should be given special consideration by the reconstructive surgeon. After two years of definitive FP the facial muscles are atrophied, so the best solution is to transfer new functional muscle groups (Lee et al., 2008). Currently, the dynamic techniques of microsurgical transfer of neurotized muscles are the gold standard after long-lasting FP (Conley, 1979).

Nevertheless, it is very important to choose a suitable recipient nerve for connecting axons from the motor nerve to the transferred muscle. Using the contralateral facial nerve (cross face) we can achieve a spontaneous nerve impulse (Anderl, 1979; Baker and Conley, 1979; Gary-Bobo et al., 1980), but the functional results could be far from acceptable to the patients (Bae et al., 2006). When the contralateral facial nerve is used, nerve grafts are required to join it to the paralyzed side of the face, reaching at least 50% of the axonal load. The number of axons able to enter the transferred muscle is directly related with the functional outcomes (Harrison, 1985; Rayment et al., 1987; Frey et al., 1991).

Since the beginning of the 20th century, the MN has been used for dynamic reconstruction of the paralyzed face, producing spontaneous movements that indicate an

important degree of neural plasticity (Lifchez et al., 2005; Manktelow et al., 2006).

While the MN has been mainly indicated for dynamic restoration in treating Möbius syndrome cases, it is currently used for reinnervation after free muscle transfer (Manktelow et al., 2006). These authors concluded that the innervation was remarkable, with a range in direction of motion of the upper lip and oral commissure similar to that in the healthy part of the face. Their results were subsequently corroborated (Bianchi et al., 2009), and similar findings have been published describing direct anastomosis of the MN to the distal branches of the facial nerve (Coombs et al., 2009; Wang et al., 2014). The use of this nerve in cases of incomplete facial palsy has been also reported (Klebuc, 2011).

On the other hand, the gracilis muscle is constant, simple to dissect, with a medium volume and minimal morbidity of the donor zone. Moreover, surgeries can be performed by two surgical teams (Harii et al., 1976; Manktelow and Zuker, 1984). The nerve is identified easily and its length and diameter make it suitable for microsurgical procedures (Lasso et al., 2004; Frey et al., 2008).

To our knowledge, only three studies have analyzed the number of myelinated fibers in the MN and compared it to the ON (Coombs et al., 2009; Borschel et al., 2012; Snyder-Warwick et al., 2015). These studies used the detection of myelinated nerve fibers as a surrogate for axonal count. The samples used in two of these studies (Coombs et al., 2009; Borschel et al., 2012) correspond to those in the proximal segment of our study; that is, to the entry of the nerve into the masseter muscle before it gives off the first intramuscular branch. The myelinated fibers results reported by the abovementioned

authors are quite different, as mentioned earlier. These differences can be explained because the authors quantified all the myelinated fibers, both motor and sensory.

A nerve can include several different types of cholinergic axons, that is, those containing acetylcholinesterase. Motor axons from the nerve fibers innervating skeletal muscles are one type and are the object of study in this research. However, acetylcholinesterase can also be present in other types of nerve fiber, such as preganglionic sympathetic and parasympathetic fibers in the autonomic nervous system, or postganglionic fibers in the sympathetic system innervating sweat glands and blood vessels.

On the basis of the locations of the samples we have studied, near the muscle, we ruled out the possibility that these fibers were preganglionic neurons (sympathetic or parasympathetic) or postganglionic sympathetic fibers innervating sweat glands. However, other types of cholinergic axons could have been present in the nerves that we studied, such as sympathetic postganglionic neurons ending in the blood vessels of some of the skeletal muscles (type C unmyelinated fibers). To distinguish these type C cholinergic fibers from our AChE-positive motor axons, we relied on the presence of the myelin sheath in the motor axons of the MN and on the size of the nerve fibers. We concluded that all the AChE-positive fibers we quantified were motor axons.

In order to guarantee adequate reinnervation of the transplanted muscles, fast axonal growth from the donor motor nerve to the reinnervated neuromuscular plate is essential. Recent research on nerve regeneration and muscle transplantation proves that increasing muscle movement is directly linked to more extensive regeneration of motor axons,

which translates into a higher motor axonal count. Identifying AChE by immunohistochemical techniques guarantees accurate determination of the percentage of motor fibers in nerve fascicles, which allows us to establish the effective capacity and compatibility of any nerve as a motor nerve. We found significant statistical differences between AChE-positive axons in the 1st and 4th segments of the MN and the AChE-positive axons in the ON. The first segment had practically twice as many AChE-positive motor axons as the ON. This leads us to consider the MN in this segment as an ideal motor nerve for reinnervating transplanted muscle because it guarantees a significant motor axonal count. The clinical explanation for the significant statistical difference in the fourth segment is the significant reduction of AChE-positive axons in this part of the MN. In the 2nd and 3rd segments of the MN there are no significant statistical differences. However, the number of AChE-positive motor axons in these segments is still greater than in the ON. In other words, the motor axonal count is still greater than in the ON. This is why we consider the 2nd and 3rd segments of the MN equally ideal for transplanted muscle reinnervation.

Conclusions

These findings confirm that end-to-end anastomoses between the masseteric and the obturator nerves should preferably use the proximal and middle segments and the first branch itself, and reveal that the MN is ideal as a donor motor nerve for reinnervating transplanted muscle for dynamic reanimation of the paralyzed face. However, the masseteric neurotomy should ideally be performed on the middle segment, preserving

the innervation to the deep fascicles.

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Ethical statement:

All procedures used in this study were in accordance with the ethical standards of the 1964 Helsinki Declaration and its later amendments. The authors have no conflict of interest to declare.

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Figure legends

Figure 1. Right (A) and left (B) isolated masseter muscles with superficial fascicles (s) sectioned and pulled back to visualize the course and relationships of the MN. The first and second collateral branches are labeled 1b and 2b respectively. Proximal, middle and distal segments are located, respectively, before the first branch, between the first and second, and distal to the second. d: deep fascicle of the masseter muscle, hb: horizontal branch of the mandible, m: masseter muscle, t: temporal muscle, vb: vertical branch of the mandible, za: zygomatic arch, (*): masseteric artery.

Figure 2. Immunohistochemical labeling for choline-acetyltransferase (ChAT) in axial sections of four different segments of the MN. The black stain revealed the motor axons. A: Segment I; main trunk. B: Segment II; the MN between the first and second branches. C: Segment III; the first branch to the superficial belly. D: Segment IV, just after the origin of the second branch to the superficial belly. The motor axons (ChAT positive) are stained black.

Figure 3. Immunohistochemical labeling of choline acetyltransferase (ChAT) in axial sections of the ON anterior branch. The black stain revealed the motor axons. A: Section of the nerve just before entering the gracilis muscle. B: Detail of one of the nerve fascicles to the gracilis muscle.

Figure 4. Graph boxes showing and comparing the median (A) and the mean (B) of ChAT-positive (ChAT+) axons in the different segments of the MN (I-IV) and in the

branch of ON to the gracilis muscle. * and **, Statistically significant differences ($p<0.05$ and $p<0.005$ respectively).

Nerve	Segment	ChAT+	ChAT-	Total	% of ChAT+
Masseter	proximal	640±289	749±235	1390±290	45±16
	middle	527±221	548±134	1074±240	48±12
	distal	216±301	233±187	450±472	38±15
	first branch	423±298	444±177	868±355	47±17
Obturator nerve		352±193	392±188	744±290	46.7±15.3

Table 1. Mean numbers of motor axons in the masseteric (MN) and the obturator (ON) nerves. ChAT+: number of axons with choline acetyltransferase (ChAT-positive axons); ChAT-: number of axons without choline acetyltransferase (ChAT-negative axons); % of ChAT+: percentage of axons in the nerve or segment that were ChAT-positive.