Maturation of the neuromuscular junction in masseters of human fetus

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Abstract

Objectives: The aim of the present investigation is to examine if the histological maturation of the neuromuscular junction in the masseters of human fetuses has already begun by the 12-th week of gestation or not. Material and Methods: Twenty-four masseter muscles from 14 human fetuses at gestational age 12 weeks were divided into two groups. In the first group, muscle sections were stained with Bielschowsky and Holzer stains for examination of neurofibrils and glial cells respectively. In the second group, rhodamine and fluorescein conjugated alpha-bungarotoxin were used to detect nicotinic receptors and anti-GAD for neuronal terminals. Results: It was observed the presence of one axon for each end-plate and glial cells spread over a branched axon. The nicotinic receptors clustered in the neuromuscular junction, neuronal terminals and large oval nucleus were detected. Conclusions: These observations suggest that the maturation of the neuromuscular junctions of the masseter muscles in the human fetuses has already begun at the 12-th week of gestation.

Keywords: neuromuscular junction, masseter muscle, facial development, fetal growth.

Introduction

In the human fetus, maturation of the muscle structures begins with the fusion of myoblasts to form muscles fibers. At the end of the third month of gestation the typical grooves of the skeletal muscle appear. The patterns of muscle formation are controlled by connective tissues, the original mesoderm of the arches forms the muscles of the face and the muscle cells migrate together with nervous and the arterial components [1].

It is known that the myogenesis in vertebrates is controlled by a variety of signals and regulatory events at the gene level including somite formation, cell determination, early cell migratory events, and myogenic differentiation [2]. The nervous and muscle cells can form synaptic components by themselves. These cells organize their own differentiation, but the synaptic differentiation needs intercellular specialization [3].

It has been established [4] that there are three characteristics of muscle development which allow us to know the mechanism of synaptic formation. First, the nerve and muscle organize their own differentiation. The initial contact of the myotube and motor neuron is random. The synaptic specialization site is not predetermined. Second, the neuromuscular junction is not merely an immature stage of the complete motor plate, but it has all the elements. Only the synaptic cleft in posterior stages suffers expansion, the nerve sends a signal to the muscle, and begins the first step of postsynaptic differentiation. Third, the neuromuscular development consists in that the major of the synaptic components of the motor neuron and the myotube are developing on independent form.

The maturation of the neuromuscular junction includes the following features: large oval nuclei can be observed on the myofibrils [5], nervous terminal is covered by the extension of glial cells, the receptors for the transmission are clustered in the synaptic membrane and all the axons remain eliminated except the one that matures [6]. When the axonal ending suffers alterations, the synapse maturation is damaged [7]. It has been shown that the glial cells are a major contributor to maturity of the neuromuscular junction [8].

Studies on the development and formation of the end-plate in the muscle have been conducted in some muscles like quadriceps femoris of 9 to 20 weeks in human fetuses [9]. It is also reported that the neuromuscular junction (NMJ) were observed in the ninth week of gestation. This possibly suggests the relationship between the spontaneous movements of human fetuses and maturation of the neuromuscular junction. The movements of human fetuses were studied longitudinally between 21 and 41 weeks of gestation [10]. At 12 weeks of gestation, it was possible to observe the tongue and lips movement [11] and the maturation of the articular disc of the temporomandibular joint in the human fetus [12]. At this stage of gestation suggests masseter muscle maturation and activity.
The purpose of the present study, therefore, is to evaluate the maturation of the neuromuscular junction in the masseter muscle of human fetuses at 12 weeks of gestation.

To detect nicotinic receptors in the nervous system α-bungarotoxin dye was used [13]. In the same way, a mouse anti-GAD has been used to detect nerve terminals [14], in the present investigation we have employed the method described by Holzer [15], together with Bielschowsky’s modified method [16] for the examination of glial cells and neurofibrils respectively.

Material and Methods

Twenty-four masseter muscles of fourteen human fetuses, aborted at 12 weeks of gestation were divided into two groups. The first consisted of seven fetuses that were used for histochemical studies of the masseter muscles by means of the Bielschowsky and Holzer methods. The second group was used for fluorescence and immunofluorescence.

Muscle preparation for histochemical sections

Samples of aborted fetuses were used in accordance with the procedures approved by the Ethical Committee of the Pathological Anatomy Unit at Valencia Central Hospital taking into account the Declaration of Helsinki for Human Research. In no case, a mother requested her fetus. Thus, the Pathological Anatomy Unit gave written consent for this study protocol. No fetus used in this study had any visible evidence of developmental abnormalities or genetic disorders.

Bielschowsky and Holzer methods

The masseter muscles of seven human fetuses were completely removed. These muscles were fixed by immersion in 10% stabilized neutral formalin (40% formaldehyde, NaH2PO4 4.0 g, Na2HPO4 6.5 g, at pH 7.0) and stored at 4°C. All the muscles were embedded in paraffin and serially sectioned in sagittal planes at 8 µm with a rotatory microtome. Eight sections were obtained for each muscle. The sections were deparaffinized and hydrated in distilled water. The Bielschowsky stain method was used for neurofibrils, and histological sections in sagittal planes at 6 µm with Holzer stain method [17] were used for glial cells.

The histological sections were independently assessed by three members of the Pathological Anatomy Unit at Valencia Central Hospital. Each section was cut and mounted on a previously numbered glass slide. Their report was performed according to the following criteria: presence or absence of axon ending in the neuromuscular junction, number of the axon ending in the neuromuscular junctions, presence or absence of the zones stained with Holzer methods for glial cells in the myofibrils under study.

Fluorescence

To detect nicotinic receptors with the α-bungarotoxin stain, the masseter muscle fiber groups of seven human fetuses were fixed with 4% formaldehyde in 0.1 M sodium phosphate buffer (PBS). All the muscles fibers were separated in each muscle, numbered and put in Eppendorf tubes with PBS 0.2 M (pH 7.2) for 45 minutes. They were washed three times at 15 minutes intervals with PBS. The fibers of each muscle (previously mounted on glass slide) were divided into two groups. The first group was incubated in the dark with rhodamine-conjugated α-bungarotoxin for six hours at room temperature. The second group of muscle fibers was incubated with fluorescein conjugated α-bungarotoxin for six hours in the darkness too. Both groups were analyzed using confocal laser scanning microscope to detect the presence of nicotinic receptors in the neuromuscular junction.

Immunocytochemical labeling

Double labeling was performed for immunostaining. The myofibrils of the masseter muscles of the 12-week fetuses were fixed with 4% paraformaldehyde for 40 minutes in PBS. The myofibrils were incubated with rabbit anti-Gad 1:1000 with fluorescein for immunolabeling for six hours to detect neuronal terminals, and in the same group of muscle fibers rhodamine-conjugated α-bungarotoxin (1 mg/mL, Sigma Aldrich) 1:1000 was used for labeling postsynaptic acetylcholine receptors.

Confocal laser scanning microscopy

A Fluoview 200 laser confocal system (ver. 1.3) was used to obtain high-spatial resolution images of fluorescently-labeled materials. The confocal system was controlled through manufacturer-supplied software (Fluoview 2.1 program). The 633 nm line of an argon ion laser was used for rhodamine excitation and the 490 nm line for fluorescein excitation. Images were obtained using an oil-immersion lens (60×10 numerical aperture), and were digitized at 8-bit resolution into 512×512 pixel arrays. Series of optical sections were taken at 4.0 to 4.4 µm steps. The aperture setting of the confocal pinhole was 100 µm. The typical images are shown in Figures 1–6.

Results

Neurofibrils and glial cells

Black stained neurofibrils (Figure 1) were observed by means of the Bielschowsky method.

Figure 1 – Only one axon reaches the end-plate.

Only one neurofibril contact with end-plate was observed in each myofibril of the studied fetuses. In
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In many cases the axonal terminal presented branching, but in no case the branching had contact with others end-plates. The neuromuscular junction was not observed in the same position in the myofibrils.

In all the sections, an intensely stained violet axonal terminal was observed. This intensely stained area occupied the entire neuromuscular junction (Figure 2).

**Nicotinic receptors**

As mentioned earlier, in order to localize neuromuscular junctions, postsynaptic acetylcholine receptors in two independent groups were stained with rhodamine-conjugated α-bungarotoxin (Figure 3) and fluorescein-conjugated α-bungarotoxin (Figure 4) respectively.

In both cases, the nicotinic receptors showed a homogeneous intensity. Moreover, the samples stained with rhodamine-conjugated α-bungarotoxin reveal the presence of large oval nucleus (Figure 5).

**Nicotinic receptors and neuronal terminals**

A double fluorescence labeling was used: (a) with fluorescein-conjugated anti-GAD to locate the nerve terminals, and (b) with rhodamine-conjugated α-bungarotoxin that binds to post-synaptic acetylcholine receptors (Figure 6).

These receptors were observed on the periphery zone of the muscle fibers as red stained structures. In the same way, the zone over the acetylcholine receptors was stained. Intense green color suggests the presence of neuronal terminals.

**Discussion**

The present study provides histological evidence that the maturation of the neuromuscular junction in the masseter muscles in human fetuses has already begun at 12 weeks of gestation. The NMJ is the only example of a synapse between a nerve and a muscle. The develop-
ment of the NMJ begins in the uterus and the muscle fibers are characterized by large nucleus [5]. In this study, we observed the presence of large oval nuclei on different areas of the muscle fibers.

Many studies on small mammals have shown that some changes occur in the NMJ between the fetus and the adult during development [18]. The major histogenetic events include: the neurulation, proliferation, migration and differentiation process [19].

In normal innervated muscle, the nicotinic receptors are a homogeneous population, which is concentrated in the synaptic folds under the presynaptic terminal [20], the quantities of nicotinic acetylcholine receptors measured by α-bungarotoxin binding on the surface of the mouse skeletal muscle increase during their terminal differentiation in culture [21]. Other stain like Holzer method has been used to detect glial cells about the synaptic folds under the presynaptic terminal [20], similar studies have been carrying out in central nervous system for morphological analysis of glial cells [23].

The NMJ in a series of steps that involve the exchange of signals among its three cellular components: nerve terminal, muscle fiber, and Schwann cell, any motor axon can form NMJ with any muscle fiber [24]. Using restrain mouth opening, by suture, revealed that restricted fetal temporomandibular joint movement influences the process of endochondral bone formation of condylar cartilage [25]. The information on the development of the neuromuscular junction and the activity of masticatory muscles could be used to evaluate normal growth of human fetuses. In the fetuses studied here, the development of structures such as oval nuclei, nicotinic receptors, nerve terminal, glial cells, one axon for end-plate and muscle fibers was clearly visible. The presence of these structures is a sign of histological maturation of the neuromuscular junction in the masseter muscles.

Conclusions

The results of this study showed the presence of acetylcholine postsynaptic receptors, axon, glial cells, axonal terminals, and large oval nucleus in the peripheral zone of muscle fiber in the masseter muscles of human fetus aborted at 12 weeks old. These findings substantiate the conclusion, namely the maturation of NMJ in masseter muscle begun at the 12-th week of gestation.

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References

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