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Morphological and histochemical study of the masseter muscle after occlusal alteration

JOÃO PAULO MARDEGAN ISSA*, RODRIGO TIOSSI*, AND MAMIE MIZUSAKI IYOMASA**

* Graduate student, Faculty of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil.
** Professor, Faculty of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil.

Key words: masseter muscle, morphology, histology, histochemistry

ABSTRACT: The aim of this study was to evaluate alterations in the masseter muscle of 30 male guinea-pigs submitted to occlusal alteration. The animals were divided into 2 equal groups, the control group (C) only submitted to surgical stress, and the occlusal altered group (T) submitted to teeth extraction. Each group was subdivided into 3 groups, with 5 animals, for the following studies: macroscopy and vessels distribution, light microscopy and histochemical analysis, with animals' perfusion 2 months after surgery. Data were submitted to statistical analysis. Macroscopically, an anteroposterior orientation of the muscular fibers was found and statistical difference between C and T groups in width ($p<0.05$). Microscopically, C and T groups showed polygonal muscular fibers with variable diameters, and on the left side of the T group these differences were more pronounced. Histochemically, in both groups, the prevalence of intermediate reactivity fibers and several high reactivity fibers spread out in the deeper area was observed, with no significant differences between superficial fibers on both sides of C and T groups. It was possible to conclude that the masseter muscle in guinea-pigs was sensible to functional chewing alteration.

Introduction

Functional disorders that affect structures of the stomatognathic system are among the major concerns in dentistry nowadays, where injuries to the masticatory muscles caused by various disorders are steadily increasing (Clark *et al.,* 1981; Close *et al.,* 1995; Harriman, 1996; Mason, 2005; Turker, 2006). Electromyographic studies have revealed that fatigue, discomfort and pain in the masticatory muscles are frequently associated with occlusal alterations and loss of the posterior teeth (Möller *et al.,* 1984; Michelotti *et al.,* 2006). Muscular pain has also been related to ischemia resulting from impaired blood flow due to prolonged muscle contraction (Bakke, 1993; Clark *et al.,* 1981; Möller *et al.,* 1984; Fukura *et al.,* 2004); however, studies regarding the specific morphological muscular alterations resulting from malocclusion are scarce. Although reports have revealed the effects of some types of occlusal alteration on masticatory muscles in experimental animals (Bani *et al.,* 1999; Maeda *et al.,* 1990; Miehe *et al.,* 1999), only few investigations regarding the morphological alterations that occur in the masseter muscle are available.

In the masticatory strength adjustment, not only the direction of the fibers and the diameter of the masticatory muscles section are determinant factors, but also fibers composition is important (He *et al.,* 2004). Through histochemical methods, different kinds of muscular fibers can be distinguished according to its

Address correspondence to: Dr. João Paulo Mardegan Issa.
Faculdade de Odontologia de Ribeirão Preto-USP
Departamento de Morfologia, Estomatologia e Fisiologia
Av. Café S/N, Ribeirão Preto, CEP: 14040-904, SP, BRASIL
Fax: (+55-16) 36330999. E-mail: *jpmissa@yahoo.com.br*
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enzymatic content. In reactions for myofibril ATPase, at pH 9.4, type I fibers present clear coloration which indicates low ATPase activity and slow contraction time, while type IIA fibers, of dark coloration, indicate high ATPase activity correlating to fast contraction time (Eriksson and Thornell, 1983; Bakke, 1993). Type II fibers are subdivided into type IIA, mitochondria rich, and type IIB, mitochondria poor. Type I fibers are responsible for functional maintenance, not quickly fatiguing, not sensitive to lack of oxygen, showing an important amount of myoglobin and surrounded by thin capillary; this fiber type requires an abundant ATP supply and have an intense aerobic metabolism. Type II fibers have fewer myoglobin, high ATPase activity and obtains its energy through glycolytic way (Miehe *et al.,* 1999).

Thus, the aim of this study was to analyze macroscopical, biometrical, microscopical and histochemical modifications in the masseter muscle of guinea pigs, induced by occlusal alteration in order to provide a better understanding of the effects of functional disorders on the structures of the stomatognathic system.

Material and methods

This study used 30 adult male guinea pigs *(Cavia porcellus)*, with an average weight of 450g, maintained at the University animals house, under controlled temperature, with commercial chow and *ad libitum* water availability. Half of the animals received occlusal alteration induction and the other half was considered as control. Both groups were divided into three subgroups with five animals, for the following studies: morphologic and vessels distribution, light microscopy and histochemical analysis. This work was approved by the Ethics Committee on the Use of Animals in Experimentation of the University (CEUA).

1. Induction of occlusal alteration

The animals were anaesthetized with tribromoethanol (0.25g/kg body weight) and submitted to extraction of the upper molars from the left hemiarch, with animals receiving the Pentabiotic (24000 IU) antibiotic as preoperative prophylaxis. After asepsis and disinfection of the surgical site with PVPI, teeth extraction was performed with anatomical tweezers and 3S hollenback. The extraction area was compressed to stop bleeding (Chompret maneuver). After surgery, the animals received anti-inflammatory and analgesic drugs (sodium diclofenac- 0.04mL/100g) and were maintained in appropriate cages for two months.

2. Specimen preparation for macroscopical and biometrical analysis

After two months, animals from both groups were anaesthetized with urethane (1.5g/kg body weight) and intracardially injected with 0.9% physiological saline to wash vessels, followed by perfusion with the same amount of 10% formalin in sodium phosphate buffer at pH 7.4. Arterial repletion was performed by injection of diluted latex in 50% distilled water. Animals were kept immersed in fixative for later dissection and macroscopical and biometrical analysis.

3. Specimen preparation for light microscopy analysis

The animals were treated as described above until the perfusion step with 10% formalin in sodium phosphate buffer at pH 7.4. The masseter muscle was then removed and immersed in the same fixative solution. After fixation, specimens were submitted to routine processing, embedded in paraffin and cut into 6μm thick sections at frontal direction. The sections were stained with hematoxylin-eosin, analyzed under a light microscope and photographed by a photomicroscope.

4. Specimen preparation for histochemical analysis

The masseter muscle was carefully removed, immediately weighed, according to Scherle (1970) method, and then orthogonal cleavages were made, which were destined for the histochemical study. Masseter muscle samples were washed in physiological saline and immersed in N-Hexane at -70ºC for 1 minute. After this period of time, the material was stored in a freezer at -20ºC until transferred to a cryostat microtome at -20ºC, where the 10μm thick cuts were made. Some cuts were dyed in hematoxylin-eosin and others submitted to reactions for the following enzymes: Succinate Dehydrogenase (Nachlas *et al*., 1957), Nicotinamide Adenine Dinucleotide Diaphorase (Novikoff *et al.,* 1961), and Myofibril ATPase at pH 9.4 after pre-incubation in an alkaline medium (pH 10.4) and in an acid medium (pH 4.6) (Guth and Samaha, 1969). Based on enzyme reactivity, fibers were catalogued according to Peter *et al.* (1972) nomenclature in: Slow Oxydative (SO), Fast Glycolitic (FG) and Fast Oxydative Glycolitic (FOG).

5. Statistical analysis

For each condition, analysis of variance (ANOVA) was applied for 2 criteria (width and length) to verify group homogeneity. When the ANOVA indicated statistically significant differences, Student's T test was used for individual comparisons (α=0.05). Data were processed using statistical software JMP 6.0 (SAS Institute Inc., USA).

Results

Macroscopy and Biometry

The major axis of the guinea pigs masseter muscle showed an anteroposterior orientation and the fibers of the superficial bundle were arranged anteroposteriorly (Fig. 1). The superficial bundle originated anteriorly at the root of the zygomatic process of the maxilla through a strong cylindroid tendon and was extendedly inserted on the lateral side in the posterior half of the deep bundle, above an aponeurosis, and in the postero-inferior region of the mandible ramus at medial side. The inferior region was inserted above the crest of the posterior process of the mandible ramus, where their fibers intercrossed with the medial pterygoid muscle. The results of the biometric analysis are shown in the Table 1.

The width of the occlusal altered animal muscles, on the left and right sides, showed statistical difference when compared to each other ($p<0.05$).

Microscopy

The superficial bundle of the guinea pigs masseter muscle, control group, showed a strong tendon anteriorly inserted into the lower part of the root in the maxillary zygomatic process. This tendon was thicker in the inferomedial portion and had the shape of a superficial aponeurosis in the anterior portion of the muscle belly. The posterior half of the muscle presented thick septae of dense connective tissue, which did not completely separate the muscle into real bundles, thus characterizing a multipennate muscle. Few muscle spindles were identified in the muscle belly of the superficial bundle, one of them was fused. In the upper deep portion a fasciculus clearly separated by loose connective tissue revealed various spindles, some fused and others not.

The masseter muscle of the control animals showed polygonal muscle fibers comprising fasciculi in the superficial region of the muscle (Fig. 2A) and an endomysium rich in blood vessels. No difference was observed between the right (Fig. 2A) and the left side (Fig. 2B).

It was observed on the right masseter muscle, from the occlusal altered group, a polygonal and round muscle fibers in the fascicule, superficial region. Despite of this difference in shape, the fibers diameter was similar in

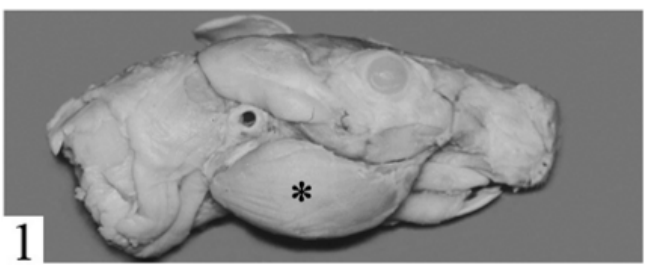

FIGURE 1. A general view of the right masseter muscle, superficial area (*), of the occlusal altered group, showing some altered fibers.

TABLE 1.

Means (mm) and standard deviations (SD) of the masseter muscle dimensions using t-test.

Parameter	Group	Left masseter	Right masseter
Length	Control	42.26±0.72	42.28±1.12
	Treated	42.98±0.96	42.64±0.70
Width	Control	24.62±1.35	24.22±1.89
	Treated	22.72±1.13*	26.14±1.21*

*- $p<0.05$

TABLE 2.

Muscular volume (cm^3) according to Scherle method

Animals	Groups			
	Treated		Control	
	Left Masseter	Right Masseter	Left Masseter	Right Masseter
1	1.26	1.63	2.27	2.19
2	2.64	3.39	3.28	3.28
3	1.02	1.36	1.48	1.48
4	1.06	1.48	1.78	1.77
5	0.98	1.72	1.23	1.15

the control and in the occlusal altered groups (Figs. 2C and 2D). In contrast, on the left side, although the fascicule in the superficial region was similar to those of the control group, altered fibers containing a central nucleus, degenerating fibers (Figs. 2E and 2F) were observed deep in the masseter muscle, from this same group, and round fibers of variable diameters were clearly visible.

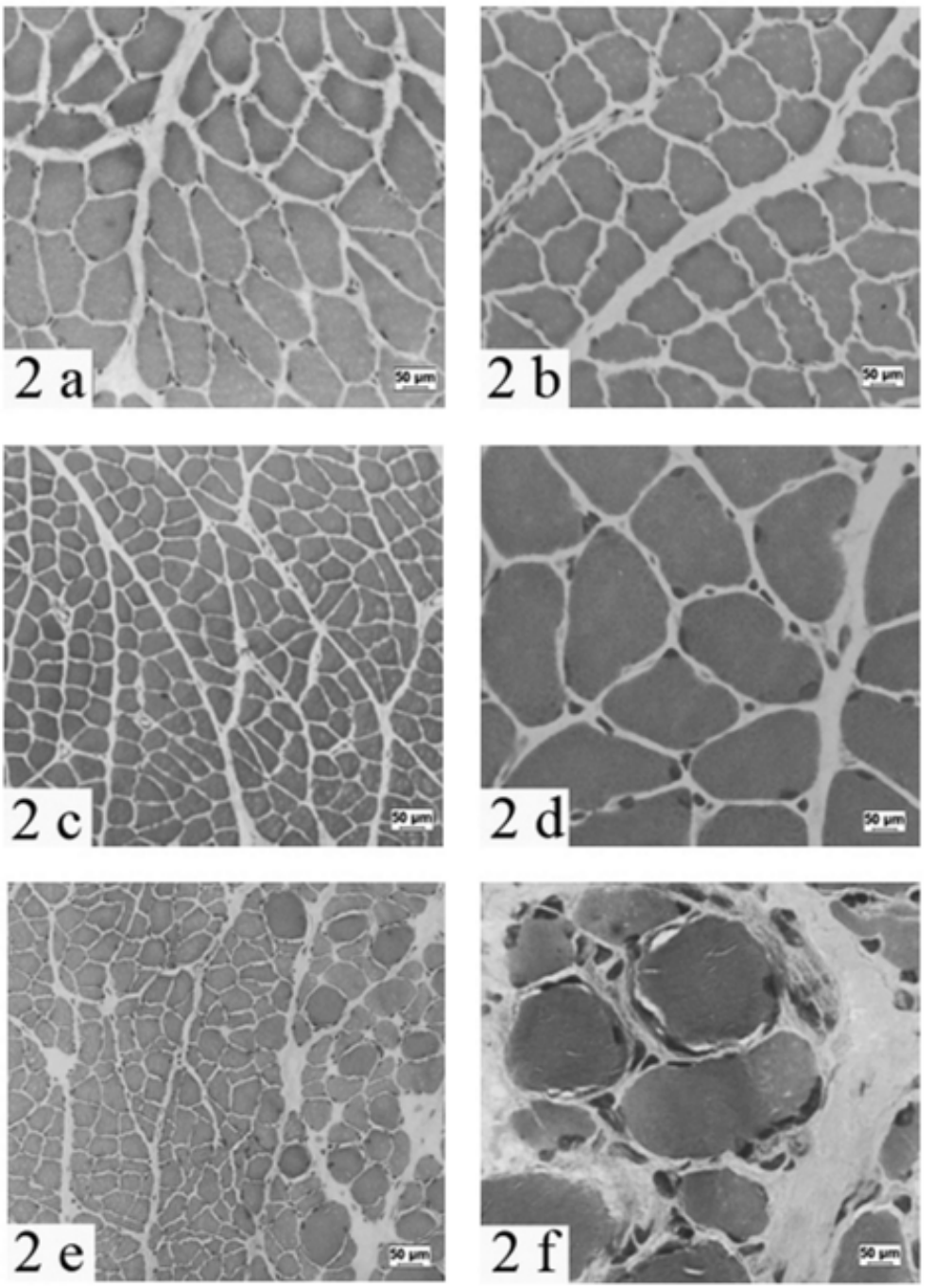

FIGURE 2. A. Right masseter muscle, control group, showing the polygonal aspect of the fibers (X 250 of original magnification). **B.** Left masseter muscle, control group, showing the polygonal aspect of the fibers (X 250 of original magnification). **C.** Polygonal muscle fibers in the fascicule of the right masseter muscle, control group (X 125 of original magnification). **D.** Polygonal muscle fibers in the fascicule of the right masseter muscle, occlusal altered group (X 500 of original magnification). **E.** Altered fibers with central nucleus on the left side of the occlusal altered group (X 125 of original magnification). **F.** High magnification of anterior view showing these characteristics (X 500 of original magnification).

Histochemistry

Regarding Scherle method analysis, no significant differences were found between groups or sides. It is important to mention that, considering the occlusal altered group, all values found for the right side (hyperfunction) were higher than the values found on the left side (Table 2).

Reactions with acid ATPase in the masseter muscle of the control group showed fibers with intermediate reactivity and with low reactivity, although with a different intensity of reaction between them (Fig. 3A). On both sides, the fibers showed low or intermediate reactivity on the superficial region from the middle portion of the muscle. Deep in the muscle, some high reactivity small fibers were observed (Fig. 3B).

The reaction with NADH and SDH evidenced in the control group, fibers with intermediate reactivity varying in small quantity to the reactivity degree (Figs. 3C and 3D); however, it was observed, deep in the muscle, scarce fibers with intense reactivity (Fig. 3D). No significant difference was observed between left and right sides.

In guinea pigs submitted to surgery, the masseter muscle on the right side, after incubation with acid ATPase, revealed intermediate reactivity fibers on the superficial region from the middle portion of the muscle and some with high reactivity mosaic-shaped spread in a highly restricted area, deeper in the muscle (Figs. 3E and 3F).

The reaction with NADH and SDH evidenced the fibers with intermediate reactivity, varying in small amount the reactivity degree in the superficial region from middle portion of the right masseter muscle (Figs. 3G and 3H), in occlusal altered group.

In the middle portion of the left masseter muscle, on animals submitted to surgery, it was found on the superficial region, fibers with intermediate and high reactivity (Figs. 3I and 3J) and in depth, fibers with intermediate reactivity and scarce fibers with high reactivity (Figs. 3K and 3L).

Discussion

The present macroscopical analysis revealed an irregular lozenge-like morphology of guinea pigs masseter muscle, with the superficial fibers being arranged in an anteroposterior direction. The superficial bundle resembled that of rats (Greene, 1959), and was partially separated from the deeper bundles by thick septae of connective tissue, as also observed by another study (Rowlerson *et al.,* 1988).

Despite of the morphological differences between human and guinea pig masseter muscles, especially in terms of fiber arrangement in the superficial bundle, some common characteristics have been described in classic literature, such as tendinous aponeurosis lining up to one third of the masseter muscle as well as tendinous plates alternating with muscle bundles which create a highly complex intimate structure. These characteristics promote a reduction on the length of the contractile elements at the same time as the number of muscle fibers increase (Kubo *et al.,* 2006). Fiber length, multipennate arrangement and the large amount of tendinous tissue characterize guinea pigs masseter muscle as a potent muscle, confirming human studies (Sato *et al.,* 1992; Van Eijden *et al.,* 1997; Ohnuki *et al.,* 1999), which showed that the architectural characteristics of the masseter are adequate for force production (Tortopidis *et al.,* 1998).

The biometrical data obtained after occlusal alteration revealed a significant decrease in width of the masseter muscle on left side compared to the control group and width increase of the occlusal altered group when was compared to the control group; these values suggest functional alterations induced by the occlusal alteration. Diet consistency influences the development of the masticatory system, and a solid diet leads to weight increase to the mandibular bone and to the masseter muscle (Kuboyama and Moriya, 1995; Langenbach *et al.,* 2003), while animals receiving a liquid or soft diet show a decrease in the masseter muscle weight; data demonstrate changes in the muscle response to stimuli, i.e., hypertrophy and atrophy due to disuse (Ciochon *et al.,* 1997). In the present study, the reduced thickness of the masseter muscle observed in the group that was performed the occlusal alteration indicates atrophy of some bundles due to disuse and induced by teeth extraction. The absence of change in muscle length between the altered and control groups suggests that the function of the superficial bundle was not modified by this type of induced occlusal alteration due to the anteroposterior orientation of its fibers.

Microscopical analysis of the masseter muscle on the side that suffered teeth extraction showed round fibers of variable diameters, large quantities of altered fibers and the presence of macrophages, especially in deeper bundles (Rowlerson *et al.,* 2005). The superficial bundle of both, ipsilateral and contralateral muscles of the occlusal altered animals, did not show significant alterations compared to the control group. Maeda *et al.* (1990), reported that a liquid diet and unilateral

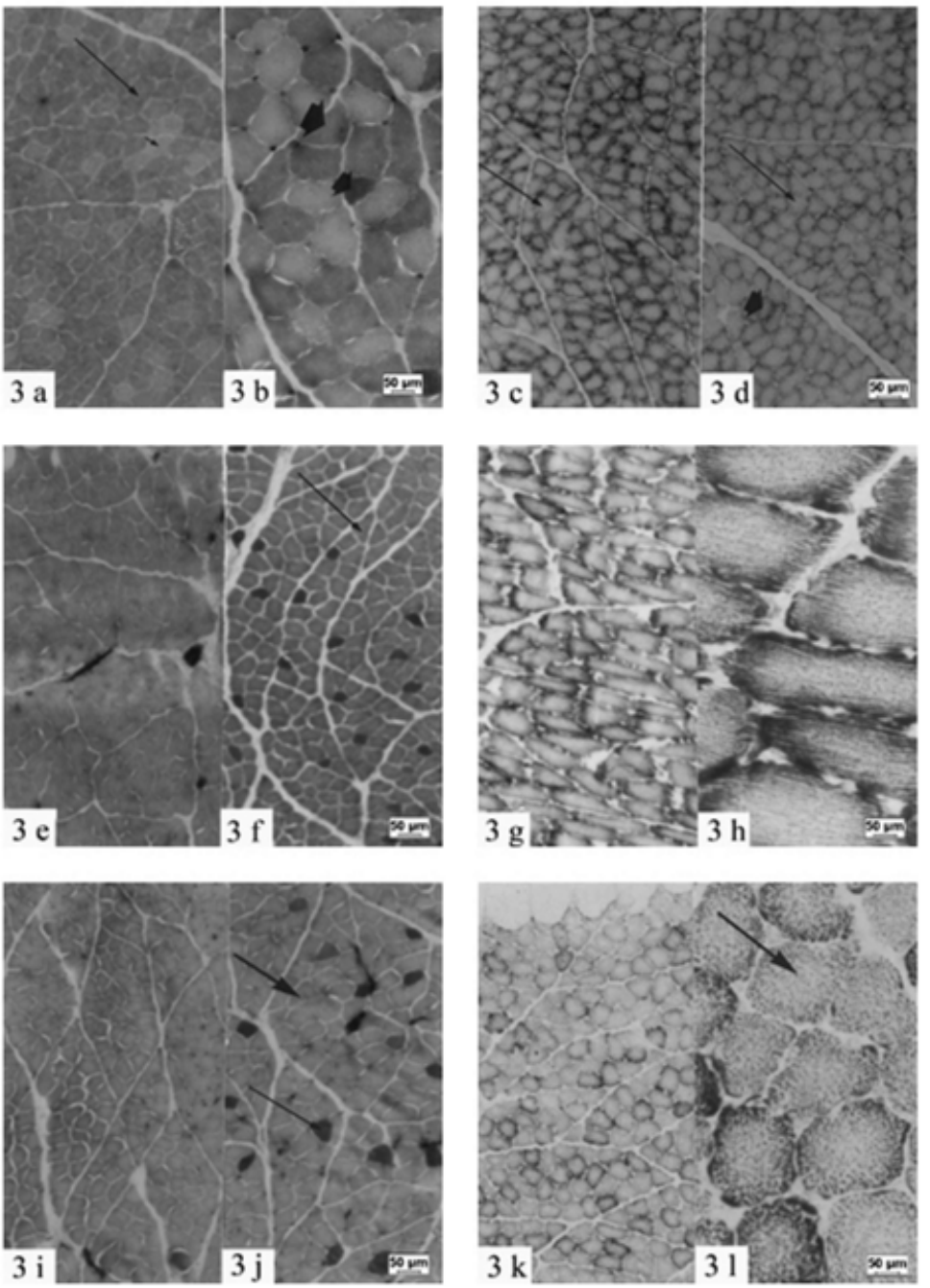

FIGURE 3. A. Fibers with intermediate reactivity (arrow) and with low reactivity (short arrow) in the superficial region, middle portion, of the masseter muscle, in both sides of the control group (X 62.5 of original magnification). ATPase reaction. **B.** Small fibers with intermediate reactivity (long arrow) and scarce fibers with high reactivity (short arrow) in the depth region of the right masseter muscle (125 of original magnification). ATPase. **C.** Fibers with intermediate reactivity (arrow) in the superficial portion of the right masseter muscle, control group (X 62.5 of original magnification). SDH reaction. **D.** Depth portion of the right masseter muscle, control group, with intermediate reactivity of the fibers (long arrow) and scarce fibers with intense reactivity (short arrow) (X 62.5 of original magnification). SDH reaction. **E.** Fibers with intermediate reactivity (arrow) in the middle portion, superficial region, of the right masseter muscle, occlusal altered group (X 500 of original magnification). ATPase reaction. **F.** Fibers with intermediate reactivity (arrow) and high reactivity, in the depth region, middle portion, of the right masseter muscle of the occlusal altered group (X 62.5 of original magnification). ATPase reaction. **G.** Fibers with intermediate reactivity, in the middle portion, superficial region of the right masseter muscle, occlusal altered group (X 125 of original magnification). SDH reaction. **H.** High magnification of anterior view showing the different levels of the fibers reactivity (X 500 of original magnification). SDH reaction. **I.** Fibers with intermediate reactivity on the superficial region, middle portion of the left masseter muscle, occlusal altered group (X 125 of original magnification). ATPase reaction. **J.** Fibers with intermediate reactivity (thick arrow) and high reactivity (thin arrow), in the depth region, middle portion of the left masseter muscle, occlusal altered group (X 250 original magnification). ATPase reaction. **K.** Fibers with intermediate reactivity on the superficial region, middle portion, of the left masseter muscle, occlusal altered group (X 125 of original magnification). SDH reaction. **L.** Fibers with intermediate reactivity (arrow) and scarce fibers with high reactivity, in the depth region, middle portion, of the left masseter muscle, occlusal altered group (X 500 of original magnification). SDH reaction.

extraction cause a reduction on the extrafusal diameter, and later, intrafusal fibers. The similar microscopic characteristics observed on the right side of the occlusal altered animals, the side not submitted to teeth extraction, and the muscles of control animals, in both sides, showing that the sensory stimuli of the periodontal ligament play an important role in the maintenance of the masseter muscle homeostasis (Bani *et al.,* 1999; Brinkworth and Turker, 2005).

The types of fibers that characterize the masseter muscle of a guinea pig described in the literature are type IIA (Rowlerson *et al.,* 1988; Ohnuki *et al.,* 2000), type IIB (Ohnuki *et al.,* 2000) and type I (Rowlerson *et al.,* 1988). The histochemical methods used in various studies show a remarkable difference in the composition of masseter fiber types between humans and animals. The most common aspect of the human masseter muscle is the presence of type I fibers, and on animals is the presence of type IIA fibers, despite the presence of other types of fibers in different proportions in both species (Tuxen and Kirkeby, 1990; Tuxen and Rostrup, 1993).

Regarding the operated guinea pigs, reactions with acid ATPase, NADH and SDH did not present significant differences on the superficial region of the right and left masseter muscle when compared to the control group. However, the high reactivity fibers located in the depth of the left masseter muscle showed between themselves a great number of various diameters and different intensity of reaction, when compared to the right muscle. Alteration in the composition of the muscular fibers from one kind to another suggest the ability to adapt themselves to muscle hyperactivity, fatigue resistance and to develop signs and symptoms of mandibular dysfunction (Eriksson and Thornell, 1983), which is an adaptive response to the new functional demand (Ohnuki *et al.,* 2000).

The present study permitted to conclude that the reflexes of occlusal alteration in the masseter muscle are installed 60 days after teeth extraction in this experimental animal model.

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