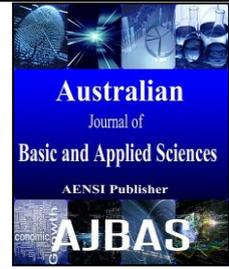




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**Effect of Retrusive Displacement of the Mandible and Increase of the Occlusal Vertical Dimension on Mandibular Bone Density and the Masticatory Muscles of Wistar Rats**

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**ABSTRACT**

The aim of this study was to investigate the biological changes of the mandible bone tissue and the morphology of the masticatory muscles fibers of Wistar rats subjected to malocclusion. Forty-eight male rats (Wistar) were divided in the VD group subjected to bilateral increase of occlusal vertical dimension, MR group subjected to retrusive displacement of the mandible and control group. Bone density assessment in the hemimandible was carried out through standardized digital radiographies and biopsies were done in the superficial portion of the masseter muscle and in the posterior region of the temporalis muscle for histochemistry analysis. For bone density, the base of the mandible decreased 1% in VD group and increased 5,2% in MR group. For histochemistry evaluation there were predominance of FG fibers (44,3%) in all muscles and groups studied, followed by SO fibers (29,2%) and FOG fibers (26,5%). The experimental induction of malocclusion proved to be able to cause significant modifications in the mandible bone density and in the morphology of the temporalis and masseter muscles.

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**INTRODUCTION**

Considered a public health issue that affect people’s quality of life (Azuma *et al.*, 2008; Liu *et al.*, 2009; Shivakumar *et al.*, 2010), malocclusion causes overloads on the teeth and periodontium leading alterations on the structure, microarchitecture and density of the orofacial musculoskeletal tissue (Harrel and Nunn, 2001; Bani and Bergamini, 2002; Konstantynowicz *et al.*, 2007; Mavropoulos *et al.*, 2007; Magalhães *et al.*, 2010; Lepley *et al.*, 2011).

Masticatory muscles and teeth are able to transfer physical stimulus to the mandible and maxilla, modulating the bone strain during the masticatory process (Mavropoulos *et al.*, 2004b; Mavropoulos *et al.*, 2007; Magalhães *et al.*, 2010; Lepley *et al.*, 2011). Thus, mechanical stress is an important regulating factor to establish bone morphology. Either the orofacial development or

bone loss may be influenced by the decreasing of the mechanical stimulus induced by functional disorders or even by the aging process (Bresin and Kiliaridis, 2002; Kunii *et al.*, 2008; Ormianer and Palty, 2009). Bone alterations like low levels of mineral density on the maxillary bones are considered effective markers for diagnosing the development of diseases, such as osteoporosis, periodontitis, teeth loss, temporomandibular disorders and malocclusion (Harrel and Nunn, 2001; Tözüm and Taguchi, 2004; Konstantynowics *et al.*, 2007; Mohlin *et al.*, 2007; Vlasiadis *et al.*, 2008).

Despite of the importance of some types of malocclusion like modifications in occlusal vertical dimension and mandibular retrusion associated with morphofunctional alterations, sometimes having considerable impact on the stomatognathic system, there are scarce literature about them. So, they have become clinically important for diagnosis and

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treatment in dentistry (Cholasueksa *et al.*, 2004; Ormianer and Palty, 2009)

Based on the relation between malocclusion and anatomofunctional modifications in the masticatory structures, the present study analyzed the biological changes of the mandible bone tissue and the morphology of the masticatory muscles fibers of Wistar rats subjected to malocclusion.

## MATERIAL AND METHODS

### **Animals and Procedures:**

This study was accepted by the Institutional Animal Care and Experimental Use Committee (1216-1/2009). For the experimentation, 48 male Wistar rats, 8-14 weeks old, with average weight of 413g were employed. Water and food were available *ad libitum* for environmental adaptation.

The animals were divided in 3 groups (n=16 in each group), in the VD group, the animals were subjected to bilateral increase of the occlusal vertical dimension by attaching a 0.9 mm thick orthodontic wire to the occlusal surface of the upper molars with resin (Figure 1) (Bani and Bergamini, 2002). In the MR group, the animals were subjected to retrusive displacement of the mandible by placing an appliance (2 mm thick x 4 mm diameter x 4 mm height) in the upper incisor teeth, made of orthodontic bands cemented with resin (Figure 1) (Cholasueksa *et al.*, 2004). And in the control group (CO), the animals were not subjected to any occlusal alteration.

Before placing the occlusal appliances, the animals were weighed and anesthetized intramuscularly with Ketamine-Xylazine (100 mg/kg each). After 14 days of the occlusal alteration, the animals were weighed and killed by inhalation of carbon dioxide. Hemimandible and segments of the left masseter and temporalis muscle were resected for bone density and histochemical analysis (Bani and Bergamini, 2002).

### **Bone Density Analysis:**

Bone density assessment in the hemimandible was carried out through standardized radiographies obtained by the paralleling technique using the Gendex DC-X-ray unit generator (Gendex Dental Systems<sup>®</sup>, Des Plaines, IL, USA) operated at 65 Kvp, 10 mA, 0.13 exposure impulses per second, 20 cm focus-film distance with cilindric collimation in phosphor-coated plates Digora OpTime (Soredex Orion Corporation<sup>®</sup>, Helsinki, Finland). An aluminum scale was added to all X-ray expositions and the films were processed using the same automatic processor Gendex DC-X-ray unit generator (Gendex Dental Systems<sup>®</sup>, Des Plaines, IL, USA).

Following this procedure, radiographies were digitized by intraoral PSP System Digora OpTime digital phosphor plate system (Soredex Orion

Corporation<sup>®</sup>, Helsinki, Finland). Densitometric values were obtained using mean grey values in pixels and converted to millimeters of aluminum (mmAl). The analyses were performed in a squared area (0.7 x 0.7 mm), standard for the three mandibular regions: alveolar process (close to lower incisor teeth) (AP), ramus (R) and base (B) of the mandible (Figure 2) (Mavropoulos *et al.*, 2004a).

### **Histochemistry Analysis:**

Biopsies were done in the superficial portion of the masseter muscle and in the posterior region of the temporalis muscle, identically in all animals. Serial cryosections (10  $\mu$ m) were performed and stained using nicotinamide adenine dinucleotide - tetrazolium reductase (NADH-TR) (Dubowitz *et al.*, 1985; Andreo *et al.*, 2005)

The Cryosections were observed (x20 magnification) by light microscope (DMLP Leica Microsystems<sup>®</sup>, GmbHm Germany) equipped with a digital camera DFC 280 (Leica Microsystems<sup>®</sup>, GmbH, Germany) and were digitalized using Image Manager - IM50 software (Leica Microsystems<sup>®</sup>, GmbH, Germany).

Three serial sections from each muscle were selected for analysis. One-hundred myofibers from each muscle were identified as being either dark blue for slow-oxidative (SO), or intermediate blue for fast-oxidative glycolytic (FOG), or light blue fast-glycolytic type (FG). Calibrated ImageJ software (Version v1.40g National Institutes of Health – NIH, USA), was employed to measure myofiber cross-sectional area, and randomly selected (Peter *et al.*, 1972; Papadopoulos *et al.*, 2007; Bani and Bergamini, 2002).

For data reproducibility assessment, all analyses were repeated three times by the same examiner following a seven-days interval with the same technical conditions. The intra-examiner reliabilities evaluated by intraclass correlation coefficient (ICC) were 97%, 98%, 97% and 96% for groups, analyzed bone regions, total fibers and area measurements, respectively (Hewitt and Stringer, 2008).

### **Statistical Analysis:**

Densitometric and morphometric mean values were converted into aluminum millimeters (mmAl) and square micrometers ( $\mu$ m<sup>2</sup>), respectively. Data were submitted to Shapiro-Wilks test for normality and two-way analysis of variance (ANOVA) followed by Tukey post hoc multiple comparison test, presented as means  $\pm$  SEM with significance level at 5%.

### **Results:**

A significant body weight (g) decrease was observed in VD group (initial weight of  $427.8 \pm 48.1$  g; final weight of  $372.6 \pm 52$  g) and MR group (initial weight of  $422.9 \pm 38.5$  g; final weight of  $418.4 \pm 47.2$  g) when compared with CO group

(initial weight of  $417 \pm 38.3$  g; final weight of  $418.3 \pm 51.5$  g) (Table 1).

**Table I:** Values in gram for body weight (initial and final) in the groups.

Groups	Initial	Final	Mean Values (%)
VD	$427,8 \pm 48,1$	$372,6 \pm 52$	13%
MR	$422,9 \pm 38,5$	$418,4 \pm 47,2$	1%
CO	$417 \pm 38,3$	$418,3 \pm 51,5$	0,4%

The data were expressed as in Mean  $\pm$  Standard Deviation. VD- occlusal vertical dimension group; MR- retrusive displacement of the mandible group; CO- control group.

#### Bone density:

The results for bone density were significant ( $p \leq 0,05$ ). The base of the mandible decreased 1% in VD group ( $0,454 \pm 0,020$  mmAl) and increased 5,2% in MR group ( $0,482 \pm 0,025$  mmAl) compared to CO group ( $0,458 \pm 0,020$  mmAl). The density increased

in the ramus of the mandible and in the alveolar process in both VD and MR groups (Table 2).

The ramus and the base of the mandible showed the lowest and highest bone density in almost all groups ( $p \leq 0,05$ ). However, the alveolar process exhibited higher densitometric values than the mandibular basal bone only in VD group (Table 2).

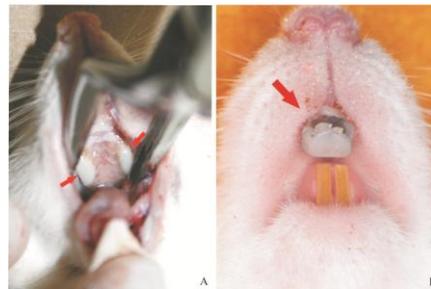
**Table II:** Values in mmAl for radiographic bone density in the groups and mandibular areas analyzed.

Area (mmAl)	Groups			Mean Values
	VD	MR	CO	
Base	$0,454 \pm 0,020$	$0,482 \pm 0,025$	$0,458 \pm 0,020$	$0,465 \pm 0,022$
Ramus	$0,426 \pm 0,023$	$0,441 \pm 0,030$	$0,425 \pm 0,021$	$0,431 \pm 0,025$
Alveolar Process	$0,457 \pm 0,020$	$0,478 \pm 0,020$	$0,456 \pm 0,020$	$0,464 \pm 0,020$
Mean Values	* $0,446 \pm 0,020$	$0,467 \pm 0,026$	* $0,446 \pm 0,021$	

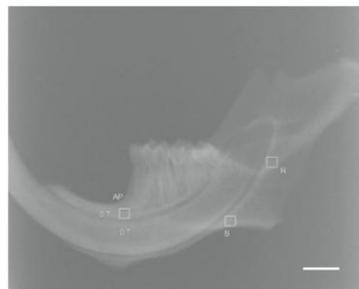
The data were expressed as in Mean  $\pm$  Standard Deviation, (ICC = 98%).

\* Not differ by ANOVA two way and Tukey post hoc,  $p > 0,05$ .

VD- occlusal vertical dimension group; MR- retrusion of the mandible group; CO- control group.



**Fig. 1:** Methods for occlusal alteration, (A) orthodontic wire fixed bilaterally on the occlusal surface of the upper molars with resin in VD group (red arrows). (B) Orthodontic band placed on the upper incisor teeth of MR group (red arrow).



**Fig. 2:** Left view of rat mandible with its respective squared areas (0.7 x 0.7 mm) selected and analyzed by ImageJ software (scale bar = 90  $\mu$ m). AP- alveolar process; B- mandibular base; R- mandibular ramus.

#### NADH-TR Histochemistry:

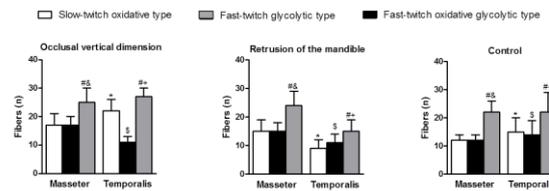
The results for histochemistry evaluation were significant ( $p \leq 0,05$ ) with the predominance of FG fibers (44,3%) in all muscles and groups studied, followed by SO fibers (29,2%) and FOG fibers (26,5%) (Figure 3). The largest number of FG fibers

was found in masseter and temporalis muscles of the VD group (42% - 46%) followed by MR (42,7% - 43%) and CO (42,69% - 43%) groups (Figure 3).

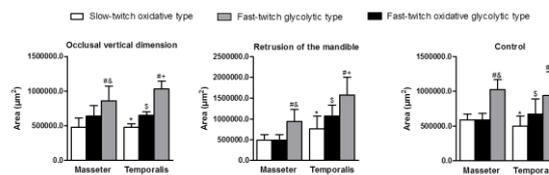
The SO fibers content for the masseter and temporalis muscles were, respectively, 37% and 29% in VD group; 28.83% and 26% in MR group and

28,73% and 29% in CO group. Although FOG fibers were, generally, observed in smaller numbers in all muscles and groups, in the masseter muscle they had comparable quantities to SO fibers (Figure 3). The

values for FOG fiber in the masseter and temporal muscles were, respectively, 29% and 17% in VD group; 29,47% and 31% in MR group and 29,58% and 28% in CO group (Figure 3).



**Fig. 3:** Percentage of muscle fibers (ICC = 97%) in the experimental groups.



**Fig. 4:** Mean values (ICC = 96%) of fiber cross-sectional areas in  $\mu\text{m}^2$  in the experimental groups. \* Not differ by ANOVA two way and Tukey post hoc,  $p > 0,05$ .

FG fiber areas ( $1.090.195 \pm 278.659 \mu\text{m}^2$ ) were significantly higher ( $p < 0,05$ ) in all groups and muscles studied than FOG fiber areas ( $685.686 \pm 150.262 \mu\text{m}^2$ ) and SO fibers areas ( $549.886 \pm 140.164 \mu\text{m}^2$ ) (Figure 4). FG fibers areas values in the temporalis muscle were  $1.576.603 \pm 424.636 \mu\text{m}^2$  in MR group,  $1.031.097 \pm 110.476 \mu\text{m}^2$  in VD group and  $939.171 \pm 340.281 \mu\text{m}^2$  in CO group. The values in the masseter muscle were  $1.027.277 \pm 139.624 \mu\text{m}^2$  in CO group,  $939.744 \pm 287.503 \mu\text{m}^2$  in MR group and  $859.743 \pm 214.965 \mu\text{m}^2$  in VD group (Figure 4).

SO fiber areas were larger in the masseter muscle ( $481.231 \pm 129.356 \mu\text{m}^2$ ) than in the temporalis muscle ( $476.389 \pm 53.596 \mu\text{m}^2$ ) in VD group. However, in MR group, the result was the opposite ( $490.546 \pm 132.620 \mu\text{m}^2$  in the masseter muscle and  $765.922 \pm 298.665 \mu\text{m}^2$  in the temporalis muscle). There was no significant difference of SO fiber areas in these both muscles of CO group ( $p > 0,05$ , Figure 4).

FOG fiber areas were larger in the temporalis muscle of MR group ( $1.070.003 \pm 262.033 \mu\text{m}^2$ ) than CO group ( $674.985 \pm 215.672 \mu\text{m}^2$ ) and VD group ( $651.023 \pm 47.690 \mu\text{m}^2$ ). In the masseter muscle, they were larger in VD group ( $637.320 \pm 151.982 \mu\text{m}^2$ ) than CO group ( $590.514 \pm 91.487$ ) and MR group ( $490.271 \pm 132.708 \mu\text{m}^2$ ) (Figure 4).

The temporalis muscle showed significantly ( $p \leq 0,05$ ) larger area values ( $853.647 \pm 236.310 \mu\text{m}^2$ ) of all fiber types compared with the masseter muscle ( $696.865 \pm 143.080 \mu\text{m}^2$ ). Intergroup comparisons of the temporalis muscle fiber areas demonstrated that they were larger in MR group ( $1.137.509 \pm 328.445 \mu\text{m}^2$ ), followed by VD group ( $719.503 \pm 147.189 \mu\text{m}^2$ ) and CO group ( $703.928 \pm 233.296 \mu\text{m}^2$ ) ( $p \leq 0,05$ ). Among the masseter muscles, the largest area

values were found in the CO group ( $735.131 \pm 104.642 \mu\text{m}^2$ ), followed by VD group ( $715.276 \pm 140.321 \mu\text{m}^2$ ) and finally by MR group ( $640.187 \pm 184.277 \mu\text{m}^2$ ) (Figure 4).

MR group showed the largest area of all identified muscle fibers ( $888.848 \pm 256.361 \mu\text{m}^2$ ), followed by CO group ( $719.529 \pm 168.969 \mu\text{m}^2$ ) and VD group ( $717.389 \pm 143.755 \mu\text{m}^2$ ), except for FOG fibers which had smaller areas in CO group ( $632.750 \pm 153.580 \mu\text{m}^2$ ) comparing with VD group ( $644.172 \pm 99.836 \mu\text{m}^2$ ) (Figure 4).

### Discussion:

The occlusal alterations induced led the animals to develop parafunctions of the masticatory system, limiting the masticatory capacity, causing pain and inflammation in the affected regions (Cao *et al.*, 2009; Ormianer and Palty, 2009). Such aspects justify the body weight loss in the experimental groups, being the increase of the occlusal vertical dimension the most aggressive method, since the animals from VD group lost more weight than MR group.

Physical limitation caused by the interposition of the orthodontic wire in the occlusal surface prevented the animals from touching all teeth when closing their mouths. This disorder, induced by muscular hypofunction, decreased the transfer of masticatory forces to the bone tissue, attenuating bone apposition and increasing the absorption sites (Cao *et al.*, 2009). This explained the bone density decrease in the base region of the mandible of VD group compared with CO group.

The ramus of the mandible was the least affected by mechanical stress during the mastication, demonstrating the smallest index of bone density. The ramus region is the insertion area of the deep

masseter muscle and did not suffer enough functional alterations to disturb the anabolic and catabolic bone events (Kuroda *et al.*, 2003).

Animals subjected to the increasing of the occlusal vertical dimension showed higher bone density in the alveolar process region than in the base region, because those animals developed an inefficient masticatory process, overloading the incisor teeth, transferring higher tension and causing higher bone apposition in the alveolar process region (Kunii *et al.*, 2008).

Still, MR and CO groups showed higher bone mass in the base of the mandible than in the alveolar process. The highest bone density values were found in the MR group, indicating that the retracted mandible is likely to cause difficulties to the mastication, preventing the incisor teeth from performing their regular functions. Such aspect brought a bigger effort to the masseter muscle, creating more intense stimulus on its bone insertion and, consequently, accumulating more bone mass in the base region than in the alveolar process region. It is known that the superficial masseter muscle insertion is the mandibular region with highest tension level, as it is situated far from the movement axis of the temporomandibular articulation (Bresin and Kiliaridis, 2002).

Occlusal disorders led the animals to develop parafunctions of the masticatory system permitting glycolytic adaptations, transforming intermediary twitch fibers to fast twitch ones, verified by the increase in the distribution of FG fibers (Pette and Staron, 2001). This aspect elucidates the largest number of FG fibers in the VD group, in which animals suffered from inefficient mastication and muscular hypofunction induced by modification in the occlusal vertical dimension, proved to be more invasive than the retrusion of the mandible (Cao *et al.*, 2009).

SO fibers were predominant in the VD group. FOG fibers were also majority in the VD group, with similar numbers in the MR and CO. Such differences explained the muscular stress caused by increase in the occlusal vertical dimension in an attempt to restore the anatomical balance of the mandible (Pette and Staron, 2001; Flück and Hoppeler, 2003). This clarifies the larger concentration of SO and FOG fibers in the VD group, while in the MR group, there was also effort increase, but no limitation of work force, as the mouth-closing movement occurred normally with no fiber switch.

FOG fibers were found in lower number in the temporalis muscle, while the masseter muscle had similar amounts of FOG and SO fibers. This fiber characteristic and the presence of larger cross sectional areas reveals or shows that the temporalis muscle tolerates higher overload of constant physical work than masseter muscle fibers. A constant increase in the work load induces oxidative adaptations in FOG fibers, consequently increasing the number of SO oxidative fibers (Uribe *et al.*, 1992; Korfage *et al.*, 2005).

Although the masseter muscle from the control group showed no significant values of SO fibers, they exhibited larger cross-sectional areas than other groups. This can be attributed to aerobic, fatigue-resistant characteristics of the masseter muscle, which enable it to perform low-intensity, dynamic and static twitches for long periods of time. However, the masseter muscles from VD and MR groups showed lower values probably induced by bite-capacity restriction and occlusal disharmony in the animals, which led to functional and metabolic unbalance of the masticatory system (Bani and Bergamini, 2002).

Masseter muscle FOG fibers showed larger ( $637.320 \pm 151.982 \mu\text{m}^2$ ) and smaller ( $490.271 \pm 132.708 \mu\text{m}^2$ ) cross-sectional areas in VD and MR groups, respectively. This happened because of the fact that the masseter muscle (superficial) is related to the protrusion of the mandible movement, which is opposite to the one induced in the occlusal alteration in MR group, decreasing the physical performance of this muscle, consequently reducing the cross-sectional areas of FOG fibers (Hiemäe, 1971).

The temporalis muscles presented fibers with larger cross-sectional areas in animals from the MR group, followed by VD and CO groups. This because the animals could perform the mouth-closing movement with no alteration in the vertical mechanics, but with alteration in the horizontal mechanics during retrusion of the mandible, which is directly related to the posterior portion of the temporalis muscle (Hiemäe, 1971; Cholasueksa *et al.*, 2004; Ohmure *et al.*, 2008). This event did not occur with the VD group, where the occlusal contact was totally eliminated, preventing horizontal movements and keeping only the necessary effort for mandibular stabilization (Bresin and Kiliaridis, 2002).

### Conclusions:

The experimental induction of increased occlusal vertical dimension and the mandible retrusion in rats proved to be invasive method, to be able to cause significant modifications in the quality of bone structure of the mandible and in the anatomical and functional adaptations in the temporalis and masseter muscles.

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