FMUC **FACULTY OF MEDICINE**

UNIVERSITY OF COIMBRA, PORTUGAL

Morphometric Evaluation of Masticatory Muscles on a Experimental Model of Non-Functional Masticatory Movement's

Integrated Master in Dentistry

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1. Abstract

Introduction: The number of studies regarding bruxism and its etiology has dramatically increased in the recent years. However, besides some references to muscle hypertrophy, there are no studies regarding its morphological effects on the muscle tissue.

Objectives: Morphometric Evaluation of Masticatory Muscles of Wistar rats, from an experimental study "in vivo" of Non-Functional Masticatory Movements produced by an induction protocol of stress and administration of amphetamine.

Materials and Methods: We used 40 Wistar male rats at 9 weeks of age, divided in 4 groups: The group I is the negative control group without any experimental manipulation. Group II is the control group of experimental manipulation: intraperitoneal injection of saline and induction of stress. Group III is the group with induction protocol: intraperitoneal injection of amphetamine and induction of stress. Group IV is the group induction and treatment: intraperitoneal injection of amphetamine; induction protocol stress and treatment with acupuncture twice a week in the masseter muscle.

Results: d-Amphetamine potentiated stress-induced increase in Non- Functional Masticatory Movement´s, stress-related behavior and consequently in dental attrition(Group I: 6,4±0,2mm, Group II: 6,8±0,3mm and Group III: 7,2±0,4mm). The acupuncture protocol led to a significant gain in Non- Functional Masticatory Movement´s, in an initial phase of the study and consequently in attrition in Group IV (7,84±0,62mm). While the Group II animals didn't gain weight, amphetamine produced a decrease in body weight between day 0 and 14 in Group III (-21,3±16,4) and acupuncture potentiated this loss in Group IV (-49,37g±29,83g). The Creatine-Kinase serum levels increased time-dependently in both Group II and GIII. Day 14-Group I: 1752,4 U/L; Group II: 2573,2 U/L, Group III: 3416,9 U/L and Group IV : 3291.17 U/L). The present results demonstrate that amphetamine enhances the action of stress as suggested by higher Creatine-Kinase serum on 7 and 14 days when compared to Group II and acupuncture seems not to affect these values.

After morphometric analysis, obtained the following results for the masseter muscle: Group I has 0% of very small fibers, 17.29% of small fibers, 63.66% of medium fibers, 15.29% of large fibers and 3,76% of very large fibers. Group II has 0% of very small fibers, 1,46% of small fibers, 33,82% of medium fibers, 26,76% of large fibers and 37,96 of very large fibers. Group III has 0% of very small fibers, 13,39% of small fibers, 68,20% of medium fibers, 9,62% of large fibers and 8,79% of very large fibers. Group IV has 0% of very small fibers, 4,7% of small fibers, 41,45% of medium fibers, 24,36% of large fibers and 29,49% of very large fibers.

For the temporal muscle, group I has 0% of very small fibers, 18,5% of small fibers,

65,95% of medium fibers, 11,53 of large fibers and 4,02% of very large fibers. Group II has 0% of very small fibers, 17,55% of small fibers, 57,35% of medium fibers, 13,27% of large fibers and 11,48% of very large fibers. Group III has 0% of very small fibers, 52,02% of small fibers, 46,24 % of medium fibers,1,45% of large fibers and 0,29% of very large fibers. Group IV has 0% of very small fibers, 28,24% of small fibers, 68,88% of medium fibers, 2,59% of large fibers and 0,29% of very large fibers.

Discussion/Conclusions: In rats, stress increases the attrition and the Creatine-Kinase values and limits the weight gain. Amphetamine potentiates the effects of stress in attrition and Creatine-Kinase, and generates an increased weight loss. Contrary to what was expected, the acupuncture protocol used did not reduce the incisal attrition, and led to a significant loss of total weight, causing no significant changes in Creatine-Kinase. After morphometric analysis of the masseter and temporal muscles, it was possible to verify that the percentage of muscle fibers varies among different experimental groups and muscles. Large and Very Large fibers increased with stress. Acupuncture seems to attenuate the effect of amphetamine to reduce the Large and Very Large fibers. There are no studies in the literature to compare with this work. For that reason we cannot conclude and did not found an obvious relation between muscle fibers diameter and Creatine-Kinase, attrition and weight loss. Furthers studies should be conducted in this area in order to better understand the mechanisms of masseter hypertrophy, and masticatory muscle fibers diameter changes and stress or dental attrition.

Keywords: Muscle Fibers, Bruxism, Creatine-Kinase, Amphetamine, Stress, Acupuncture.

2. Introduction

The definition of bruxism has evolved over time. At present, is generally considered at the medical literature as a voluntary or involuntary oral habit, conscious or unconscious, which can occur during wakefulness or during sleep, with a rhythmic character or spasmodic[1, 2] of clenching (inter-pressing arcades), gnashing and tooth grinding.[1-12] Although the pathophysiology of bruxism is a complex and controversial issue[4, 13], is now consensual among the authors that its etiology is multifactorial and its regulation is mediated centrally.[1, 3, 4, 13-23] The mechanisms responsible for the evolution of bruxism from a normal condition to a pathological one remain unknown, however it is believed that certain factors such as diseases, intake of alcohol and drugs, conditions relating to personality and stressful events may be involved.[5]

Bruxism is usually defined as a unitary disease entity however, is divided into awake bruxism and sleep bruxism.[2-4] Awake bruxism can be associated with "tics" (medical definition), or with a 'parafunction' that is believed to be associated with life stress caused by familial responsibilities or work pressure.[20] Such suggestions are not strongly evidence based; they are mainly derived from the existing knowledge and the practical experience of clinicians or academics.[20] Usual orofacial activities include functional chewing, swallowing and speaking. Unusual activities (or parafunctions) are non-functional oromandibular or lingual activities that may include, alone or in combination: jaw clenching, bruxism, teeth Grinding (rarely observed during daytime in the absence of medication or a neurological disorder such as tardive dyskinesia), tooth tapping, cheek, lip or tongue biting, nail biting, tongue pushing against teeth, licking lips, tongue protrusion, gum chewing, object biting (e.g. cigarette, pipe, pencil, candy and instrument), hypersalivation ⁄ swallowing, backward or forward or lateral head or jaw posture (e.g. telephone resting on shoulder and computer work).[20] Sleep bruxism is an oromandibular behavior that is defined as a stereotyped movement disorder occurring during sleep and characterized by TG and ⁄ or clenching.[20] Sleep bruxism was recently classified as a "sleep related movement disorder" according to the recent International Classification of Sleep Disorders. It remains to be assessed when sleep bruxism, as a behavior found in the sleep of otherwise healthy subjects, becomes a disorder.[20]

Bruxism is a very common condition in the general population, although there are many conflicting data in the literature regarding its prevalence.[24] This difference may be related to the fact that the inclusion criteria for the studies are very different, particularly in relation to the population studied and the diagnostic criteria used.[2] Regarding age, children have a high prevalence rate however, Lavigne, in 1994, reported a decrease in episodes of

bruxism with age.[1, 18] This condition arises in adolescence, with a prevalence of 14% to 20%, which diminishes over the years.[25] Between 18 and 29 years of age, the prevalence decreases to 13%.[26] In adults, ranging from 5% to 8%[27-29] (with 20% of this percentage corresponds to the type centric and 6% to the eccentric type[21]) and above 60 years of age is 3%. [26, 30]However, the prevalence in the elderly population may be greater than estimated because the acrylic prosthesis may decrease the sounds of gnashing teeth. [26, 30]Many authors do not report any gender differences in the occurrence of bruxism. [19, 26, 31-34]Other authors have reported a higher prevalence of this disease in females.[19, 31, 32] Regarding the different types of bruxism, has a nocturnal bruxism prevalence of around 8% while the daytime is approximately described a prevalence of 20%.[18] In relation to bruxism daytime, some authors claim that this occurs predominantly in females, while the night bruxism has no gender differences. [1]

The etiological factors that have been suggested are divided in local origin, systemic, psychological, occupational, hereditary, idiopathic or factors related to sleep disorders and parasomnias.[4, 13, 19, 20, 31, 35-40 41-44]However, occlusal interferences (in the past) and emotional stress (in the recent decades) have classically been implicated as a major etiological factors. [11, 31, 45-47]Several studies support the involvement of the central dopaminergic system (CDS) in the etiology of bruxism.[48, 49] It has been shown that the administration of apomorphine in rats a dopamine receptor agonist, causes a repeated stimulation of the dopaminergic system which in turn induces the increase of non-functional masticatory activity, predominantly in the transition between sleep and wakefulness. It is believed that the central nervous system is thus associated with the start and acceleration of bruxism, but its influence is not yet completely understood. **[2, 19]**

The number of studies regarding bruxism and its etiology has dramatically increased in the recent years. However, besides some references of muscle hypertrophy, there are no studies regarding its effects on the muscle tissue.

The masticatory muscles are constituted by skeletal muscle. This consists of bundles of very long cylindrical cells and multinucleated, which have transverse striations, and its fibers have rapid and forceful contraction subject to voluntary control.[50]

The muscle fibers also have a complex structure, because these when observed under optical microscope showed transverse striations, by alternating dark and light beams. [50]These can also be of two types: slow contraction fibers or fast twitch fibers. Both types of fibers are present in the masticatory muscles, and the contraction of slow fibers represents a small part of the jaw muscle fibers. [50, 51]

In the literature there are some reference to cases of muscle hypertrophy, however

isn´t something very common. Sannomiya *et al*, in 2006 reported a case of bilateral hypertrophy of the masseter and temporal muscles in a 10 year-old-girl.[52] Although few cases have been documented since then, it has been stated that this disorder is more common than generally recognized, and according to the authors there are several theoretical considerations about the etiology of masseter muscle hypertrophy, but it still remains unclear. [52]Several authors claim that emotional stress results in chronic forceful clenching of the jaws and bruxism, which cause a work hypertrophy of the muscle.[52, 53] Kebede, B *et al* concluded that benign masseteric Hypertrophy is a relatively uncommon condition that can occur unilaterally or bilaterally, and although it is tempting to point to malocclusion, bruxism, clenching, or temporomandibular joint disorders, the etiology, in the majority of cases is unclear. [54]

In fact, not only the percentage of reported cases of masseteric hypertrophy is very low, as also there aren´t many studies on its prevalence. However, it is a very common clinical finding (and also a statistical finding) that the vast majority of bruxers don't develop a marked of masseter hypertrophy, reinforcing the questions about its etiology.

Stress is defined as a real or interpreted threat to the physiological or psychological integrity of an individual, resulting in physiological and / or behavioral changes on its part, is often suggested as an etiological factor in many physical conditions that are poorly understood.[55] The body reacts to stress by increasing its metabolic activity so that it can adapt to the new requirements, which reduces its general resistance and can affect the immune system.[55] The continuous stress can cause tension aches skeletal muscle tension and spasticity, CNS excitation, insomnia and other problems. In the literature a significant number of articles suggest the existence of a relationship between stress and bruxism, but this relationship does not generate consensus.[55] It seems to be a consensual topic that individuals with bruxism have excessive levels of stress or have inadequate mechanisms to deal with stressful situations, presenting symptoms such as headache, neck back and shoulders pain. To Bani D *et al* occlusal alterations may result in changes in the functional performance of masticatory muscles [56]

The main objectives of this study are to perform a morphometric evaluation the Masticatory Muscles of Wistar rats, from an experimental study "in vivo" of Non-Functional Masticatory Movements (NFMM) produced by an induction protocol of stress and administration of amphetamine.

3. Materials and Methods

For the development of this experimental work 40 adult Wistar male rats were used. Animals were within 9 weeks of age at baseline and weighed between 255 and 333g. All animals were previously submitted to a quarantine period of 8 days in the Central Bioterium of the Faculty of Medicine of the University of Coimbra (FMUC), with reviews of health conditions, selection, maintenance and animal welfare ensured by qualified technicians. All protocols were approved by the institution and in accordance with the legislation in force, including the authorization of the Consultative Committee of Animal Welfare and Protection of Animals used for Experimental and/or Scientific purpose of the General Directorate of Veterinary. The protocol was subjected to the technical standards for the protection of the rat as animal trial contained in the Portuguese legislation currently in force (Decree No. 1005/92 of 23 October; Ordinance No. 466/95 of 17 May; Ordinance No. 1131/97 of 7 November) and the legislation of the European Community for Animal Welfare (Directive No. 86/609/EEC of 24 November, 1986). The animals were kept under standard animal care conditions (22ºC temperature, 60-65% humidity and air exchange) and light (12 hours of light and 12 hours of dark). Animals were caged in pairs suitable for its kind, with enough space to move, dry food and water *ad libitum*.

The 40 Wistar rats were randomly assigned to four experimental groups (n=10):

Group I (GI) – negative control group – control group maintained without any experimental manipulation;

Group II (GII) – experimental manipulation group induced with a stress protocol and daily injection of saline (0.9% NaCl) for 14 days. This group served as a control in order to check the pattern of changes produced by handling animals, including the production of Non-Functional Masticatory Movement´s;

Group III (GIII) – group induced with stress protocol and daily injection of amphetamine. The animals were subjected to a daily intraperitoneally injection of d-amphetamine for 14 days, according to a habituation protocol with increasing doses;

Group IV (GIV –) group of induction and treatment - amphetamine was administered by intraperitoneal injection, performed following the same protocol as in Group III and twice weekly acupuncture was performed.

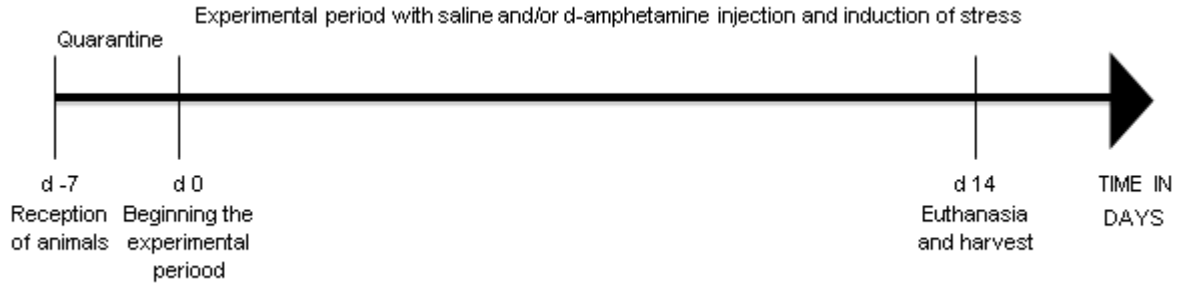

Figure 1- Experimental Design Scheme.

3.1. Weighing the animals

In all experimental groups daily weight was obtained with the aid of a digital scale, being the last weight obtained at the time of sacrifice.

3.2. Anesthesia

The animals were anesthetized before completion of the dental marks, with an anesthetic combination of ketamine hydrochloride 50mg/ml (ketalar ®, Pfizer) and chlorpromazine 25mg/ml (Largatil ®IV, Victory Laboratories) intraperitoneally.

3.3. Dental marks / Incisal attrition

The marks were made on the labial surface of the incisors, in the cervical area of the tooth, with a disc mounted on the handpiece at low speed and with proper irrigation. [11]Consecutively, the distances between the marks and the incisal edge of the central incisors (X1) were measured with the aid of a digital caliber. On the seventh experimental day, as the marks were very close to the incisal edge, new marks were made on the cervical area and the distance between the 1st and 2nd marks (X2) were remeasured. On the last day the distance was measured between the 2nd marks to the incisal edge (X3). The degree of incisal attrition was obtained by summing between the initial measurement mark (X1) and the subtraction between the second mark to the incisal edge (X3) by the distance between the 1st and the 2nd marks (X2).

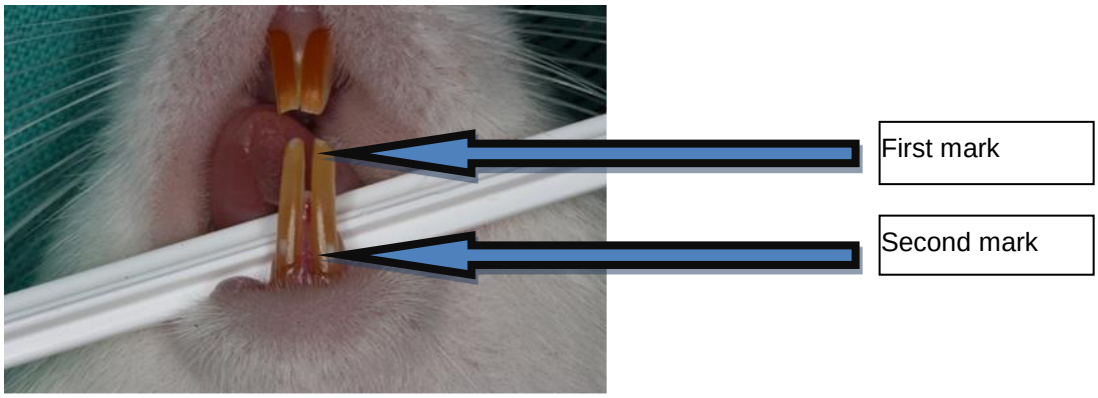

Figure 2- Observation of dental marks.

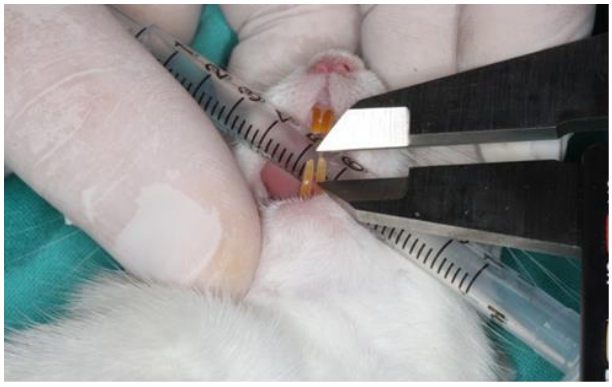

Figure 3- Measuring the distance from the incisal marks with digital caliber.

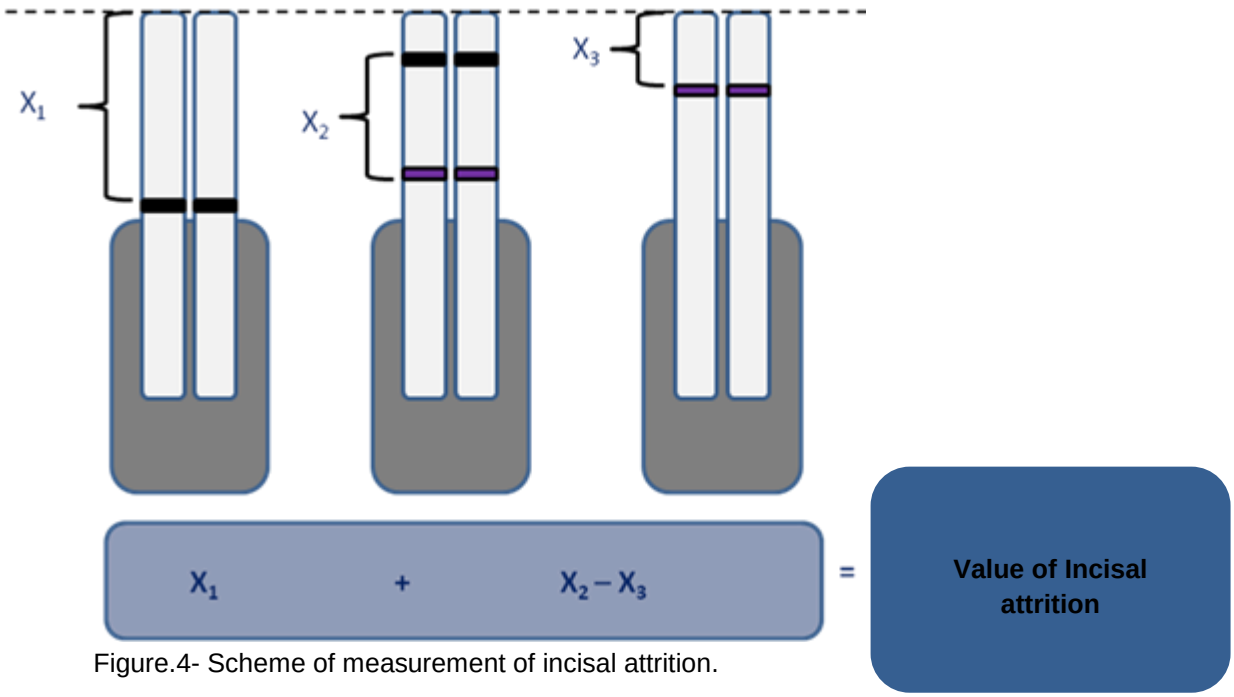

Figure.4- Scheme of measurement of incisal attrition.

3.4. Recovery from anesthesia

After the preparation of the tooth marks made in all experimental groups, the animals recovered in a recovery room, dark, quiet and comfortable during 2h. This is a key period to allow for the comfort and well-being of animals, thereby reducing the magnitude of the metabolic responses to experimental manipulation. Animals were continuously monitored with respect to hypothermia and aspiration of food or water and maintained two animals per cage, although monitored to avoid attacks. The cages were cleaned of any material that could interfere with the airway and free of any material that could be responsible for any injuries early in the recovery of locomotion. Control was performed postoperatively in the following days, through a daily monitoring of any changes to normal habits of food or water intake, body weight, patterns of behavior considered belonging to species and even the presence of clinical signs or abnormal reactions to the species.

3.5. Injection of saline solution and amphetamines

The d-amphetamine was administered intraperitoneally daily, between 9:00 and 11:30 hours. [11]The animals were handled for 15 minutes before each injection (period adaptation). The injections followed a habituation protocol with successively increasing doses (1.6mg/kg to 12.0mg/kg) for 14 days. The d-amphetamine was injected according to the individual weight of the animals daily reassessed. The GII animals were daily administered with serum during the same period. After the injections, the rats were immediately subjected to the stress protocol. The GI animals were not injected or subjected to induction of stress.

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Single daily dose of amphetamine (mg/kg)	0,8	1,6	2,4	3,2	4,0	4,8	5,6	6,4	7,2	8,0	88	9,6	10,4	11,2

Figure.5- Protocol of increasing doses of amphetamine used

3.6. Blood samples

On days 1, 7 and 14 of the experimental procedure, three blood samples were taken between 9:00 and 11:30 a.m. in order to evaluate changes in serum concentration of Creatine-kinase (CK). Blood samples were taken through a puncture of the rats tail vein to a “Eppenddorf” tube (non-heparinized). Approximately 0.4ml of blood were collected and kepted at room temperature for approximately 20 minutes, allowing its coagulation.

Centrifugation of blood was made to obtain serum. This procedure was carried out by qualified technicians of an external laboratory.

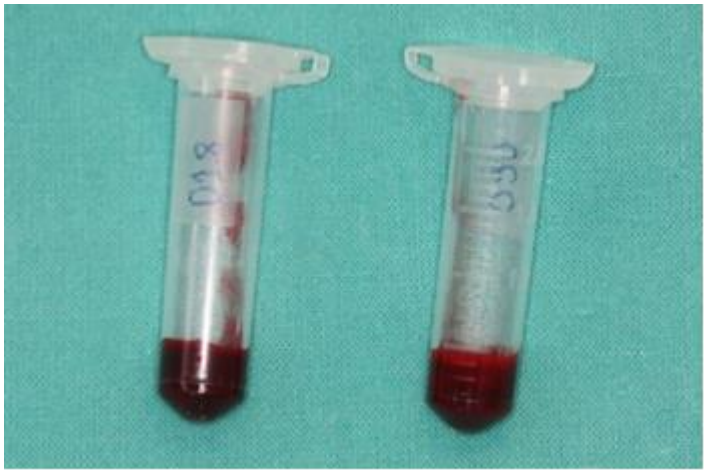

Figure.6- Blood samples in Eppendorf tubes.

3.7. Stressful agents

According to some authors, repeated exposure to the same type of stressor agents leads to a decreased of the adrenal response, from session to session. [57-62]Among the various agents available, protocols of stress were selected: noise (urban noises, predator species and a mixture of both), crowding and body vibration. These were used interchangeably throughout the 14 days of the experiment, to prevent physiological adaptation to repeated stress induced by the same agent.[59] After intraperitoneal injections, stress protocols were performed daily between 9:00 and 11:30 a.m. with duration of 10 minutes except for crowding stressors that lasted for 12 hours.

Day 1 of experimental manipulation - noise protocol 1 (through the use of urban sounds: cars, crowds, etc.);

Day 2 of experimental manipulation – protocol of crowding (by placing 5 mice per cage in competition for food and water for 12 hours);

Day 3 of experimental manipulation - noise protocol 2 (through the use of sounds predatory species: birds, dogs, wolves, etc.);

Day 4 of experimental manipulation - body vibration protocol (using a plaster vibrator);

Day 5 of experimental manipulation- noise protocol 3 (using a mixture of sounds and noises predatory species urban);

Day 6 of experimental manipulation – protocol of crowding;

Day 7 of experimental manipulation – noise protocol 1;

Day 8 of experimental manipulation - body vibration protocol;

Day 9 of experimental manipulation – noise protocol 2;

Day 10 of experimental manipulation - protocol of crowding;

Day 11 of experimental manipulation – noise protocol 3;

Day 12 of experimental manipulation - body vibration protocol;

Day 13 of experimental manipulation - noise protocol 1;

Day 14 of experimental manipulation - protocol of crowding.

3.8. Treatment with acupuncture

Acupuncture was made in GIV three times per week with 10 minutes duration for each animal after the injection of amphetamine. The points used were seven heart (07 Co) and the pericardium six (06 Fr).

3.9. Euthanasia and Animal Necropsy

At the end of the experiment, all surviving animals were euthanized and necropsied according to the established animal care guidelines. Euthanasia was performed by overdose with an association of ketamine 50mg/ml (Ketalar®, Pfizer) and chlorpromazine 25mg/ml (Largatil IV, Victoria Laboratories) followed by cervical dislocation and exsanguination by intracardiac puncture. There was a detailed registration of the information of the macroscopic observations. Major organs were collected, weighed and measured (heart, liver, lungs, kidneys, spleen, thymus, adrenal glands and stomach) and fragments of the masseter, temporalis, diaphragm and muscles of gait (gastrocnemius muscles). While some fragments of harvested organs and muscles were processed for histological studies or, according to the protocol in use at the Institute of Experimental Pathology; other fragments were selected for rapid freezing in liquid nitrogen and subsequent storage at -70ºC.

4. Histopathological and Morphometric Analysis

For each of the masseter and temporal muscles, were taken three photographs of histological sections stained with hematoxylin-eosin using standard sterologic image acquisition with randomization standardized fields photographed. The morphometric analysis was performed with the aid of software developed by the National Institutes of Health (U.S.), Image J bottle (edited by rsbweb version 1.44o) which allowed the measurement of the

cross-sectional area of muscle fibers. Thus, the variation of this parameter can be described using mean, standard deviation and coefficient of variation. Based on these data are defined for the control group with no pathology, and for each of the muscles, five types of muscle fibers according sections observed: Very small fibers, small fibers, medium fibers, large fibers and very large fibers.

Were designated medium fibers, when the fibers area was included in the interval [mean - SD, mean + SD], large fibers when the area was included in the interval [mean + SD, mean +2SD] and small fibers when the area was included in the interval [mean-SD, mean-2SD]. We consider that the muscle fibers are very small, when the value of its area is below the considered small fibers, and the fibers are considered to be very large when its area exceeds the area of the large fibers.

5. RESULTS

5.1. Statistical Analysis

Data were analyzed in Windows ® 7, using the SPSS Statistics program version 19.0 (SPSS Co., Chicago, Illinois). Shapiro-Wilk test was used to check the variables normality (is an alternative to the Kolmogorov-Smirnov test, where sample size is less than or equal to 50). To test the homogeneity of variances the Levene test was applied. In both tests, a p value less than 0.05 was considered statistically significant. For the variables that meet the assumptions of normality and homogeneity of variances, we applied the hypothesis test of mean differences (ANOVA), which is a parametric test. In the case of variables that did not meeting the integrity assumptions of normality and homogeneity of variances, we applied the Kruskal-Wallis, nonparametric ANOVA alternative, for a significance level of 0.05. The Kruskal-Wallis test was used to calculate any statistically significant differences between initial and final total body weights. The Friedman ANOVA test was used, to compare the mean values of Creatine-kinase during the three time periods within groups. The Fisher's post-hoc LSD test was used for comparisons between groups. To evaluate differences of dental attrition between groups the LSD test was used. Subsequently, the resulting data were imported into EXCEL environment (Microsoft Office 2000 ®) and charts representing the parameters were made. The results are represented as mean ± SEM (standard error of the mean).

5.2. Body Weight

The weight variations of the animals during the study period are shown in figure 7. In all experimental groups, the weighing of the animals was performed daily, the last weighing coinciding with the time of sacrifice. Experimental data were analyzed by ANOVA analysis of variance and Kruskal-Wallis test was subsequently used to calculate any statistically significant differences between the initial and final weight of the groups. Subsequently, we used the Fisher's LSD test to calculate statistically significant differences between the weights of the different groups for the same study day. In all groups there was a decrease in weight after experimental manipulation, particularly after injection of anesthetic and performing dental marks. The inflection of this trend started on day 3. The weight on day 2 was lower in average than the initial weight for almost all mice in the study. The weight drop in the control group (26.3 ± 5.2 g corresponding to 8.4% of total mass) was contrary to expectations, higher than in Group II subjected to stress (5.3 ± 3.4 g corresponding to 1.7% of total mass). Group III lost an average of 4.8 g ± 9.16 g (corresponding to 3.3% of total

mass), while Group IV presented the biggest lost weight until day 2 (28.87 g ± 7.73 g , 8.7% of the total mass).We observed a general decrease in the weight of these animals (GIV) until the last day of trial. After 2 days all animals of Groups I and II gradually regained weight. There was an increase in individual animal weight in all these groups, (difference between day 0 and 14). Although no statistically significant differences between the weights of the animals in Group I and Group II in the several days of experience, we can say that there was a more marked weight regain in Group I, not subject to stress (+15.84 ± 14, 66g) than in Group II, subject to stress. (+7.65 ± 15.06 g). In fact the difference between the value of Weight_GI_0 (M = 314.98 ± 14:42, SEM = 4.8) compared to Weight_GI_14 (M = 330.82 ± 25.72, SEM = 8.57) corresponds to a statistically significant difference t (8) = -3241, p = 0.012 (Kruskal Wallis). For the Group II, the weight change is not significant, t (9) = -1606, p = 0.143 (Kruskal Wallis) considering the Weight_GII_0 (M = 304.89 ± 10.86, SEM = 3.4) for the Weight_GII_14 (M = 312.54 ± 08.20, SEM = 6.35).In respect of Groups III and IV there was no recovery of weight as in Groups I and II, and we observed a general decrease in the weight of these animals until the last day of trial. The weight variation, now in the negative direction (weight loss) is significant for the Group III (-21.28 ± 16.43 g) where t (9) = 4.094, p = 0.03 (Kruskal Wallis) but even more significant (-49.37 ± 29.83 g) for Group IV (t (8) = 4.96, p = 0.001 (Kruskal Wallis).

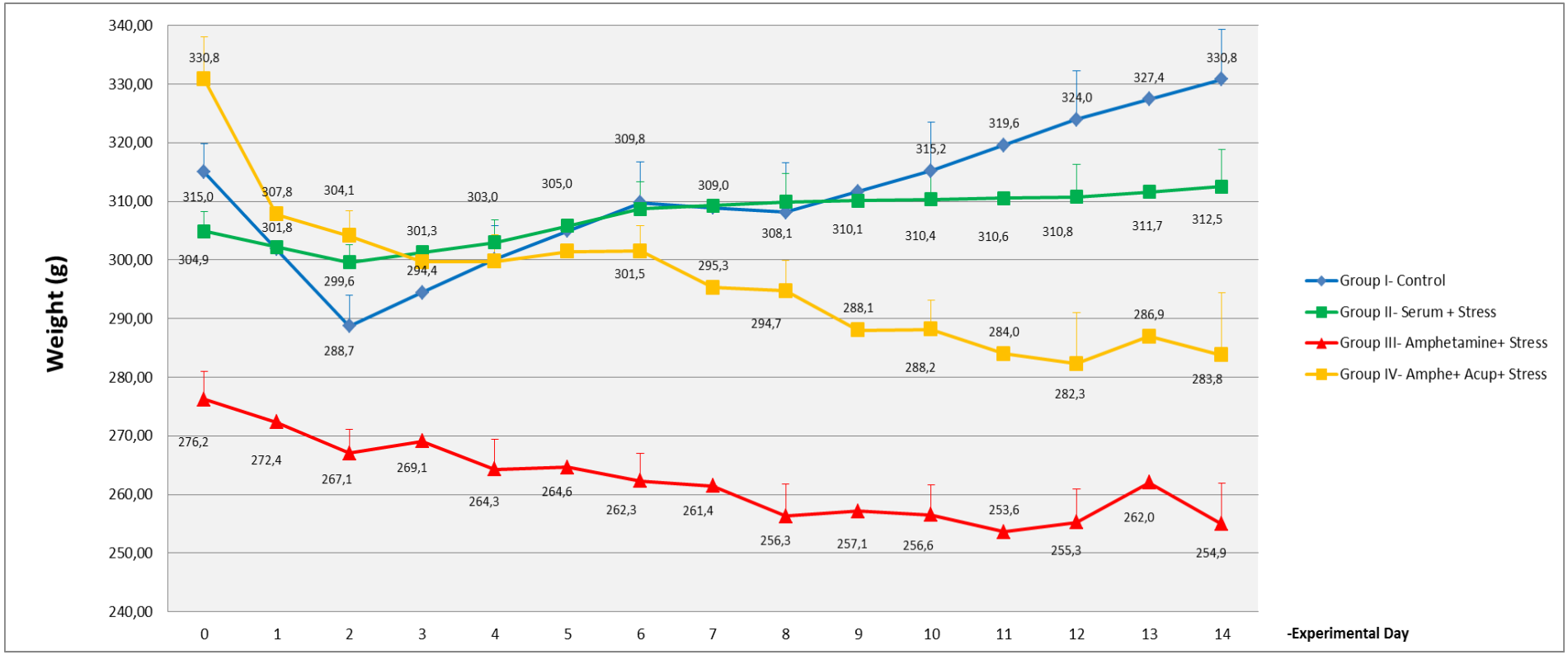

Figure 7- Graph representing the weight variation during the experimental protocol.

5.3. Creatine-kinase (CK)

The results of the serum Creatine- kinase enzyme are shown in figure 8. The results are expressed as enzyme units per liter of serum (U / L). In the first analysis there are no statistically significant differences between the experimental groups (p> 0.05 Fisher's LSD test). After analysis 1 there was a clear increase in CK values in Group II (2297.9 U / L) compared to Group I (1435.35 U / L), and these values were markedly higher in Group III (2705, 3 U / L) and group IV (2811.9 U / L).

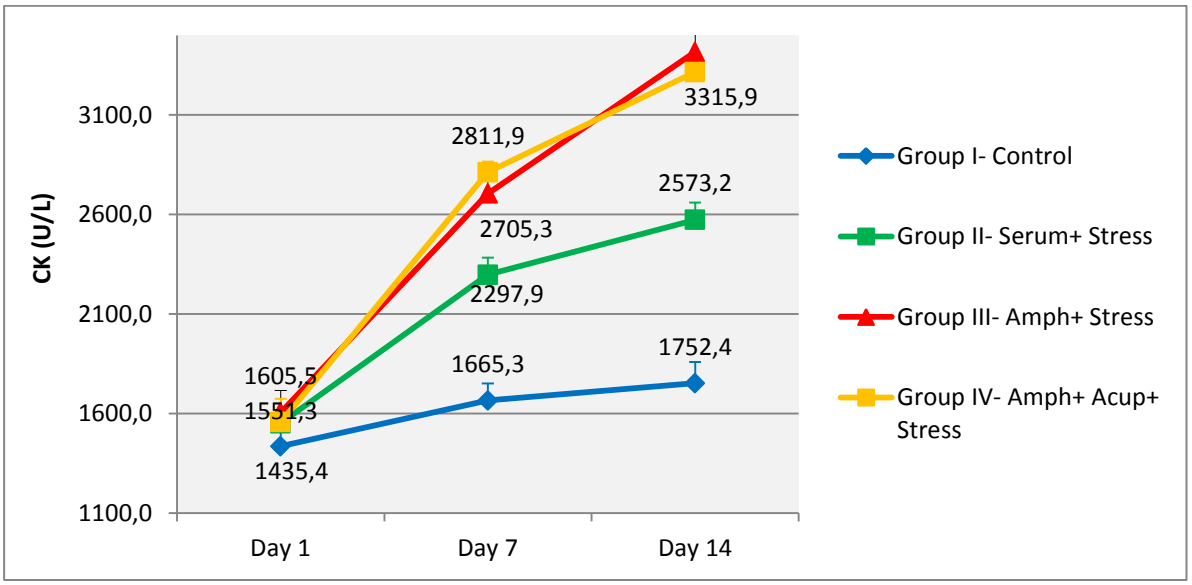

Figure.8- Graph representing the variation of serum CK values, for Analysis 1 (day 1), Analysis 2 (day 7) and Analysis 3 (day 14).

In Analysis 2 there was a small peak value of CK in Group IV (2837.28 U / L), with a value slightly higher than Group III (2705.29 U / L) that diminished in Analysis 3 where group IV (3291.17 U / L) is slightly lower than that of Group III (3416.90 U / L).

CK values in Group II and Group III are significantly higher than those of Group I in the subsequent analyzes, increasing over time, always being statistically different (p <0.001, LSD test). Despite the considerable increase in enzyme expression also in Group IV, there were no statistically significant differences between Group III and IV in all the three periods (p> 0.05, LSD test).

It is observed through the experimental period, a great similarity in the evolution between Group III and Group IV values. Amphetamine potentiates the action of stress at day 7 and 14, although the acupuncture protocol used does not appear to bring any difference.

Analyzing the differences of the CK values to day 14 to day 1, we conclude that these differences are statistically significant (p = 0.000, Friedman ANOVA) for all groups except for

Group I, where the change that occurred over the three measurements is not significant (p = 0.057, Friedman ANOVA).

5.4. Dental attrition

The measurement results of incisal attrition are shown in figure 9. The results are expressed in millimeters (mm). The measurement of the incisal attrition was carried out on the two incisors of the rats. With an average of the values obtained we calculated the attrition value of the group.

The differences of the values of attrition between the four experimental groups were analyzed by LSD test. Group III (7.24 ± 0.41 mm) shows values higher than in Group II (6.82 ± 0.34 mm) and these exhibit a considerably higher attrition than Group I (6.37 ± 0.23 mm).

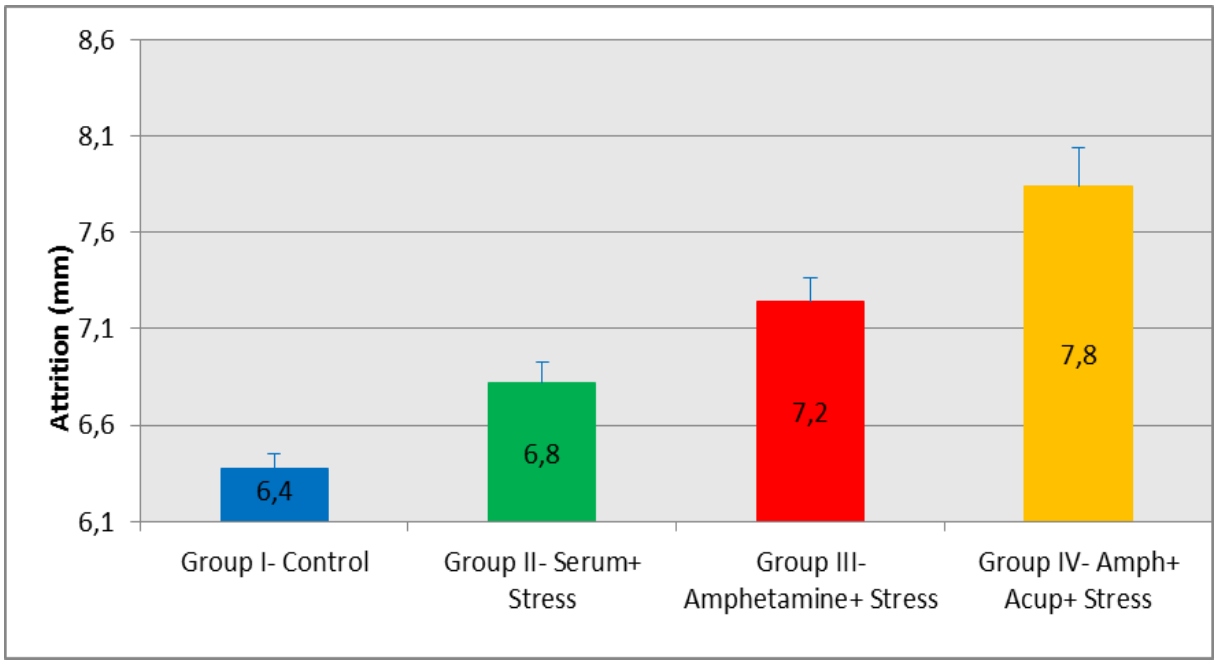

Figure.9- Graph representing the variation of attrition values, according to groups.

Stress produced a statistically significant increase in dental attrition in Group II compared to Group I (p <0.009; LSD test). Additionally, the attrition produced by amphetamine and stress in Group III (7.24 ± 0.41) is significantly higher than in animals subjected to stress only in Group II (6.82 ± 0.34 mm) Group II vs. Group III is equal to p <0.009; LSD test; suggesting that the psychostimulant potentiated the stress effect on this parameter.

However it was not expected to find a higher attrition value in Group IV (7.84 ± 0.62

mm) subjected to amphetamines and acupuncture. These differences are statistically significant (p <0.009, LSD test) among all groups.

5.5. Morphometric Analysis

The analysis of morphometric data was conducted on Excel (Microsoft Office 2010) ®) and graphs were made representing the evaluated parameters. The fibers were classified as: Very small (VS), Small (S), Medium (M) Large (L) and Very Large (VL) and the values were translated into percentages.

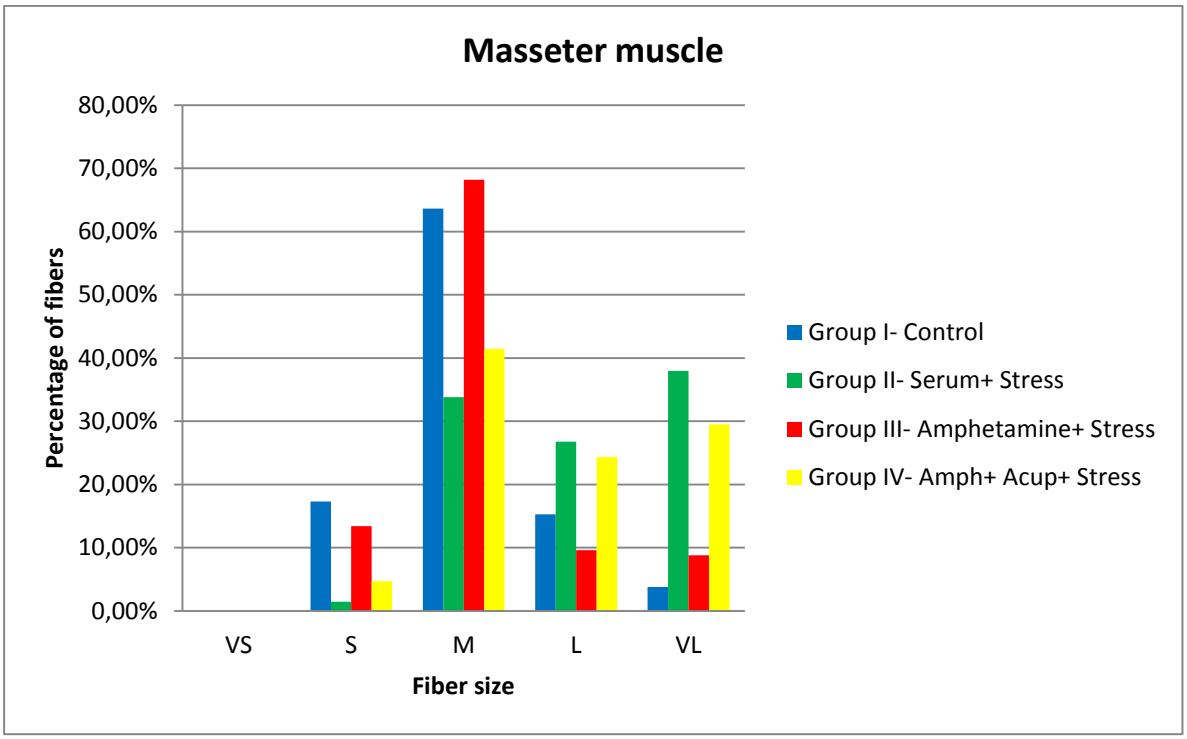

Figure.10- Masseter muscle morphometry graph.

Masseter	Group I	Group II	Group III	Group IV
Very Small	0,00%	0,00%	0,00%	0,00%
Small	17,29%	1,46%	13,39%	4,70%
Medium	63,66%	33,82%	68,20%	41,45%
Large	15,29%	26,76%	9,62	24,36%
Very Large	3,76%	37,96%	8,79	29,49%
Total	100%	100%	100%	100%

Figure.11- Table with the fibers dimensions percentages of the masseter muscle.

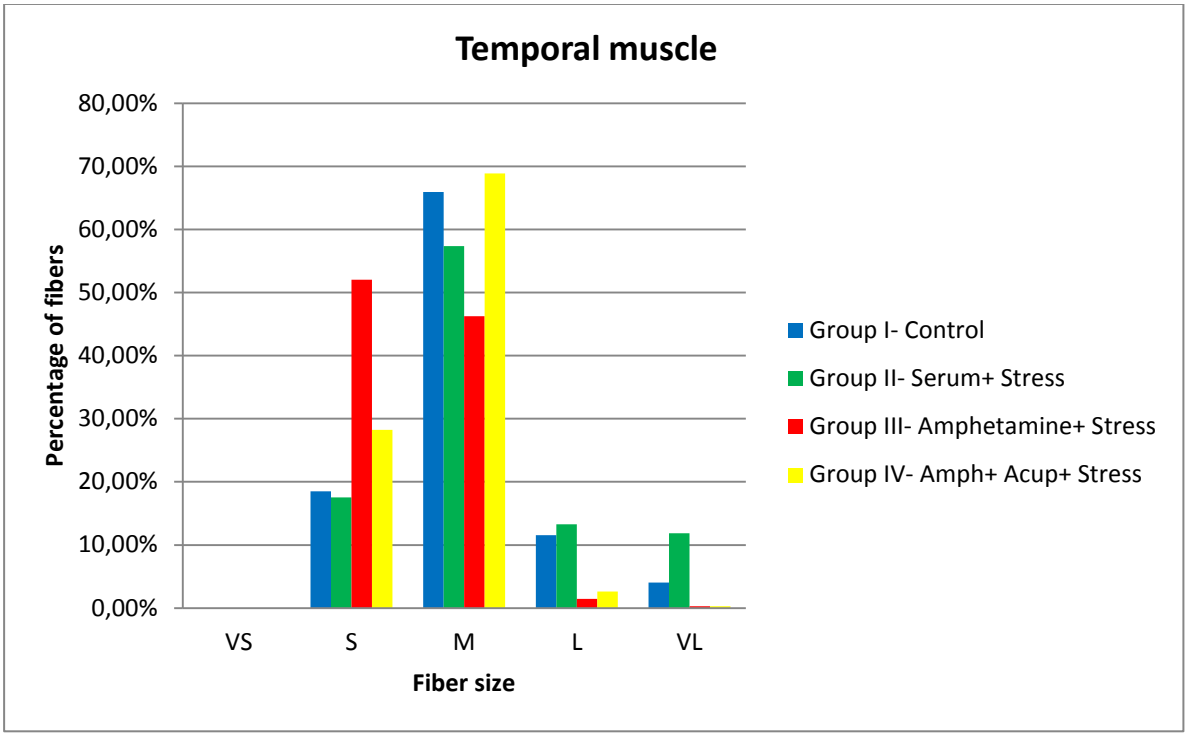

Figure.12- Temporal muscle morphometry graph.

Temporal	Group I	Group II	Group III	Group IV
Very Small	0,00%	0,00%	0,00%	0,00%
Small I	18,50%	17,55%	52,02%	28,24%
Medium	65,95%	57,35%	46,24%	68,88%
Large	11,53%	13,27%	1,45%	2,59%
Very Large	4,02%	11,84%	0,29%	0,29%
Total	100%	100%	100%	100%

Figure.13- Table with the fibers dimensions percentages of the temporal muscle.

5.5.1. Morphometric analysis of the masseter and temporal muscle

5.5.1.1Group I

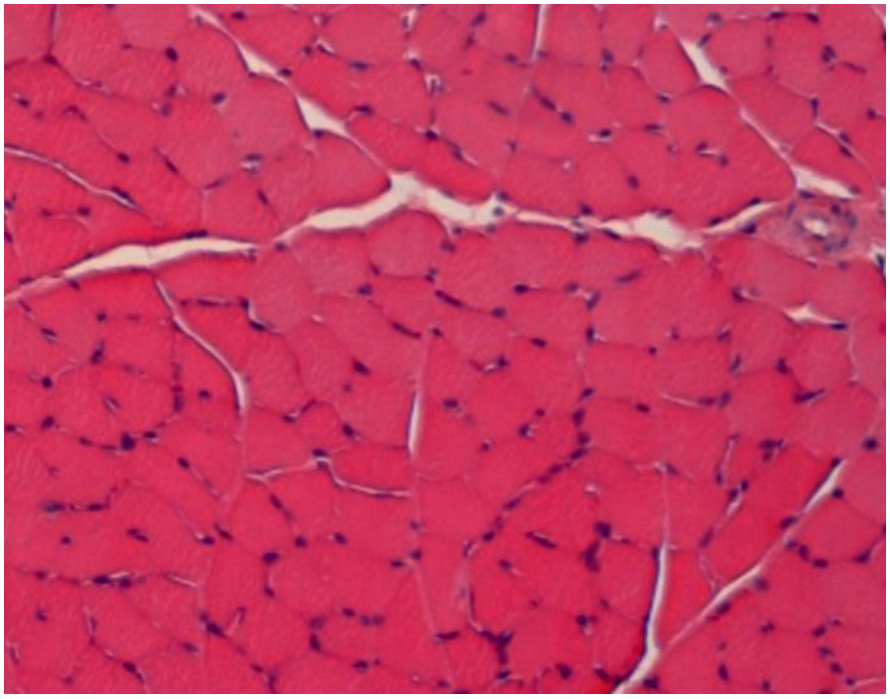

Figure.14- Photomicrograph of a section of the masseter muscle in the group I, HE, 100X the original.

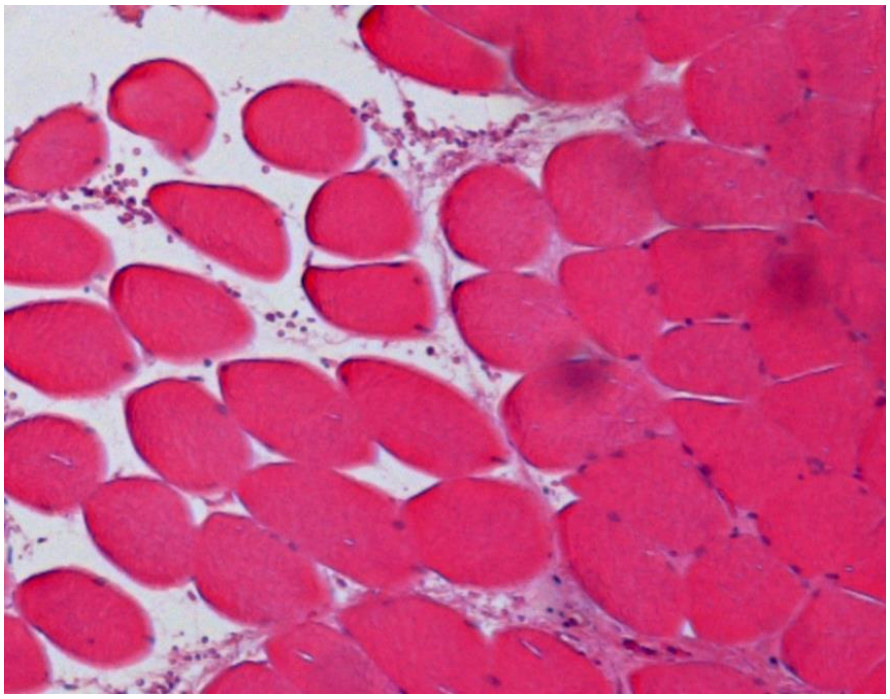

Figure 15- Photomicrograph of a section of the temporal muscle in the group I. HE, 100X the original.

The determination of the area occupied by each of the fibers of each muscle, in cross-section, showed for the masseter muscle that this area had a value of 1369,92 ± 518, 89 microns varying between 851,04 and 1888,81 microns for range of 95% confidence interval.

Considering the five classes mentioned above to describe the area occupied by each muscle fiber cross-section, there are no observed instances of very small fibers, 17,29% of the measured fibers are small; 63,66% of the measured fiber are medium, 15,29% of the measured fibers are large and 3,76% of the measured fibers are very large.

The determination of the area occupied by each temporal muscle fibers in cross-section, showed that this area had a value of 3356,34 ± 1324,72 microns varying between 2031,62 and 4681,07 microns for a range of 95% confidence interval.

Considering the five classes mentioned above to describe the area occupied by each muscle fiber cross-section, there are no observed instances of very small muscle fibers; 18,50% of the measured fibers are small, 65,95% of the measured fibers are medium; 11,53% of the measured fibers are large and 4,02% of the measured fibers are very large.

5.5.1.2. Group II

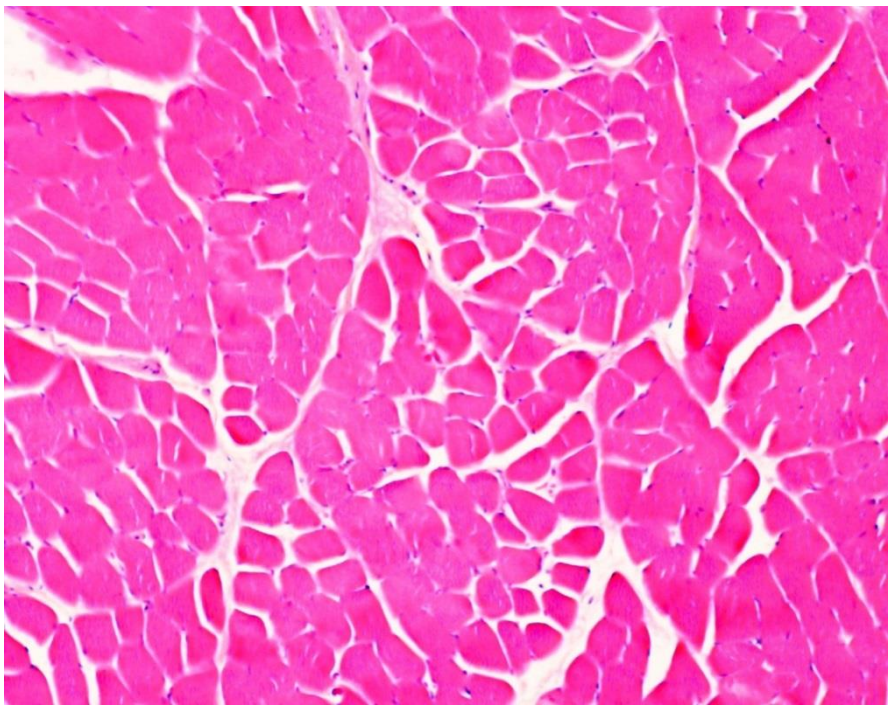

Figure 16- Photomicrograph of a section of the masseter muscle in the group II. HE, 100X the original.

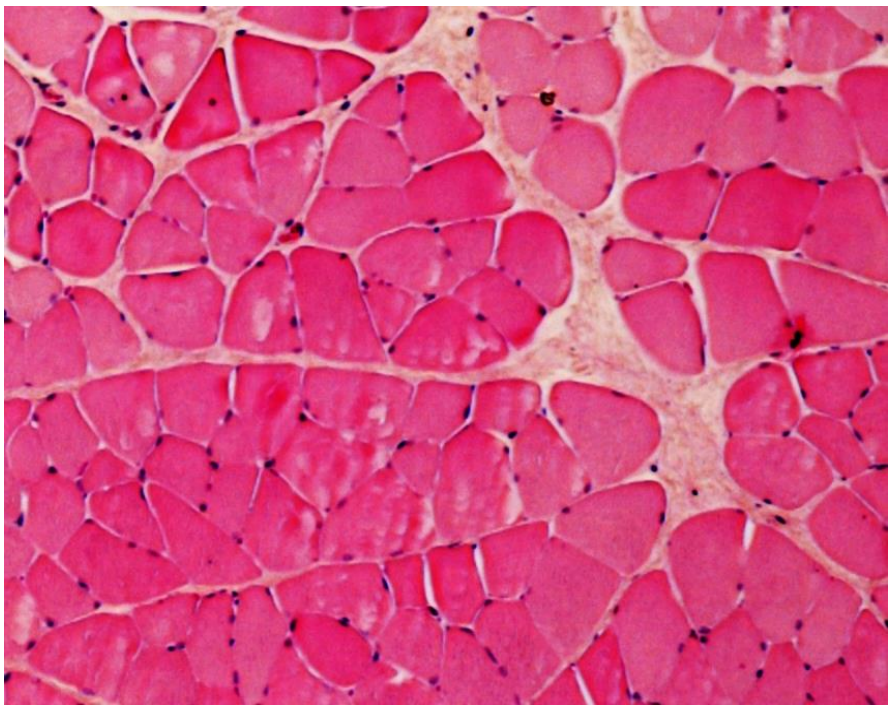

Figure 17- Photomicrograph of a section of the temporal muscle in the group II. HE, 100X the original.

The determination of the area occupied by each of the fibers of each muscle, in cross-section, showed for the masseter muscle that this area had a value of 2248,09 ± 822,83 microns varying between 1425,27 and 3070,92 microns for range of 95% confidence interval.

Considering the five classes mentioned above to describe the area occupied by each muscle fiber cross-section, there are no observed instances of very small fibers; 1,46% of the measured fibers are small; 33,82% of the measured fiber are medium; 26,76% of the measured fibers are large and 37,96% of the measured fibers are very large.

The determination of the area occupied by each temporal muscle fiber in cross-section, showed that this area had a value of 3283,32 ± 2358,62 microns varying between 924,70 and 5641,94 microns for a range of 95% confidence interval.

Considering the five classes mentioned above to describe the area occupied by each muscle fiber cross-section, there are no observed instances of very small muscle fibers; 17,55% of the measured fibers are small, 57,35% of the measured fibers are medium ; 13,27% of the measured fibers are large and 11,84% of the measured fibers are very large.

5.5.1.3. Group III

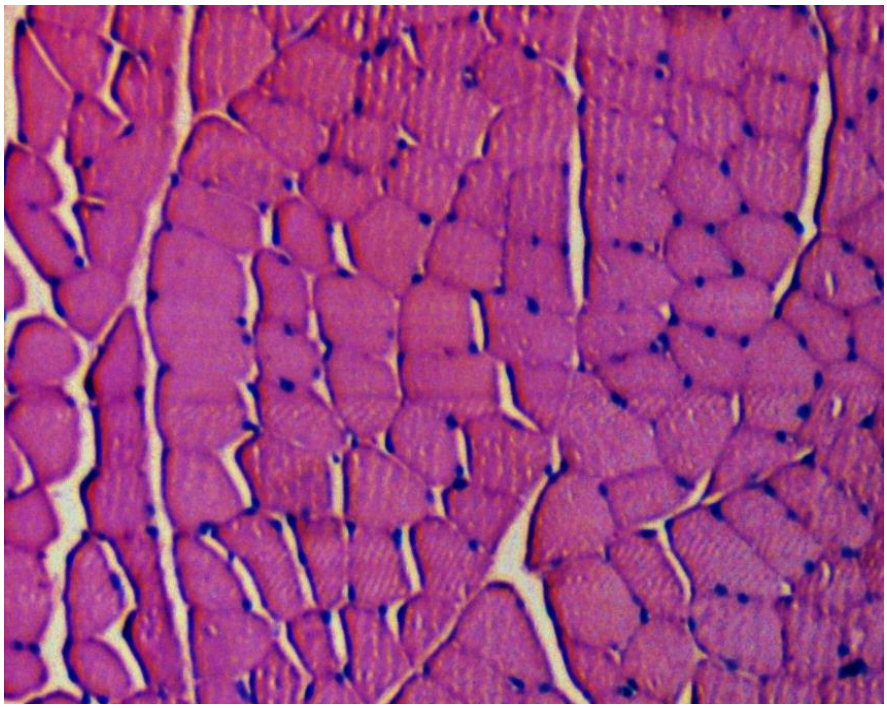

Figure 18- Photomicrograph of a section of the masseter muscle in the group III. HE, 100X the original.

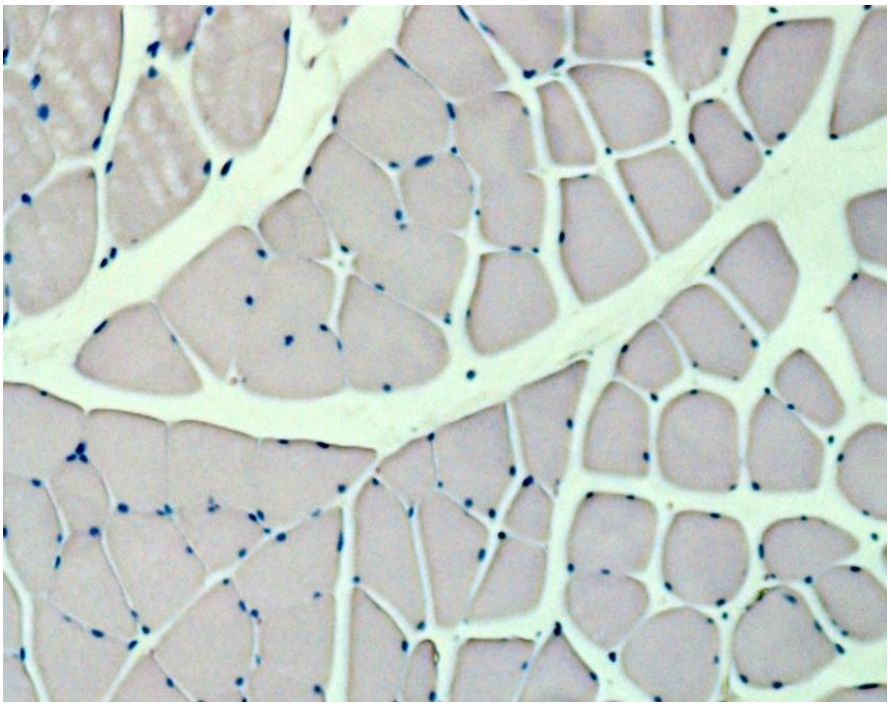

Figure 19- Photomicrograph of a section of the temporal muscle in the group III. HE, 100X the original.

The determination of the area occupied by each of the fibers of each muscle, in cross-section, showed for the masseter muscle that this area had a value of 1605,77 ± 856,80 microns varying between 748,97 and 2462,57 microns for range of 95% confidence interval.

Considering the five classes mentioned above to describe the area occupied by each muscle fiber cross-section, there are no observed instances of very small fibers, 13,39% of the measured fibers are small, 68,20% of the measured fiber are medium, 9,62% of the measured fibers are large and 8,79% of the measured fibers are very large.

The determination of the area occupied by each temporal muscle fiber, in cross-section, showed that this area had a value of 2429,05 ± 1173,23 microns varying between 1255,83 and 3602,28 microns for a range of 95% confidence interval.

Considering the five classes mentioned above to describe the area occupied by each muscle fiber cross-section, there are no observed instances of very small muscle fibers; 52,02% of the measured fibers are small, 46,24% of the measured fibers are medium, 1,45% of the measured fibers are large and 0,29% of the measured fibers are very large.

5.5.1.4. Group IV

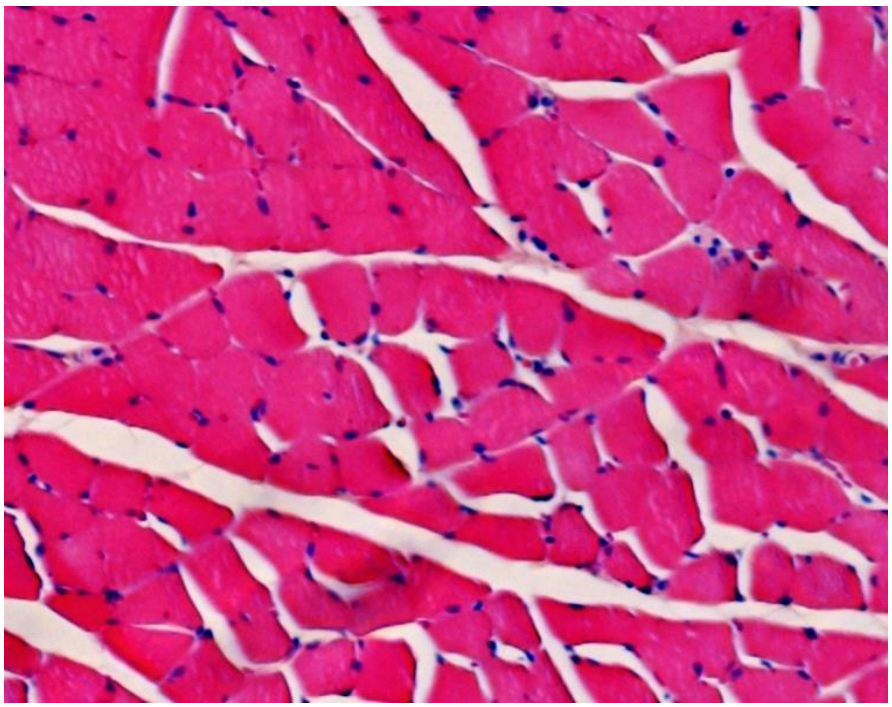

Figure 20- Photomicrograph of a section of the masseter muscle in the group IV. HE, 100X the original

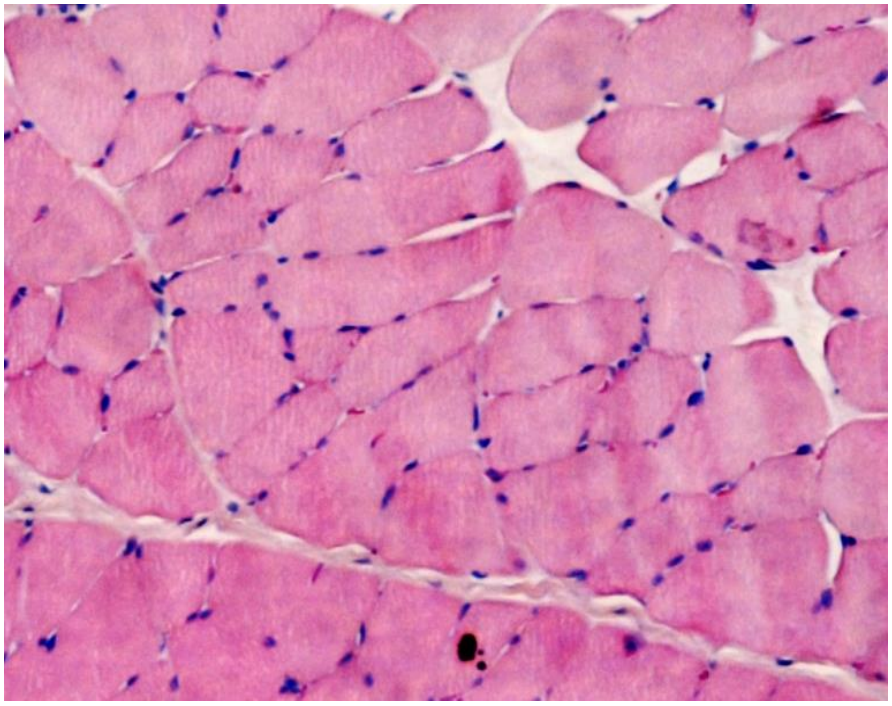

Figure 21- Photomicrograph of a section of the temporal muscle in the group IV. HE, 100X the original.

The determination of the area occupied by each of the fibers of each muscle, in cross-section, showed for the masseter muscle that this area had a value of 2030,19 ± 678,31 microns varying between 1351,89 and 2708,50 microns for range of 95% confidence interval.

Considering the five classes mentioned above to describe the area occupied by each muscle fiber cross-section, there are no observed instances of very small fibers, 4,70% of the measured fibers are small, 41,45% of the measured fiber are medium, 24,36% of the measured fibers are large and 29,49% of the measured fibers are very large.

The determination of the area occupied by each temporal muscle fibers, in cross section, showed that this area had a value of 2681,32 ± 918,71 microns varying between 1762,60 and 3600,03 microns for a range of 95% confidence interval.

Considering the five classes mentioned above to describe the area occupied by each muscle fiber cross-section, there are no observed instances of very small muscle fibers, 28,24% of the measured fibers are small, 68,88% of the measured fibers are medium, 2,59% of the measured fibers are large and 0,29% of the measured fibers are very large.

6. DISCUSSION

This experimental study is part of a broader work on non-functional masticatory movements, taking place at the Institute of Experimental Pathology Faculty of Medicine, University of Coimbra. Thus, this study used the animal model employed in this methodology.

6.1. Body Weight

At the start of the experiment, day 0, the different groups had different weight values. On this particular day only pairs GI-GII and GII-GIV were not different from each other ($p > 0.05$, LSD test). This data may correspond to an error in our study, because the mice should have at day 0, initial weights not statistically different. However, this weight difference is strange to us, because mice were randomly assigned to four groups and were requested to the supplier always with the same age. According to this data, our statistical analysis was based on the variation in body weight over the 14 days and not in weight being compared between groups. On the 2nd day of weighing, there was a decrease in body weight in all groups, being most notable in Group I. This decrease may be due to the environment in which the animals were inserted and when the experimental manipulation was made for the first time (taking of blood, administration of anesthetics and carrying out the incisal marks). According to Connell (1966), this weight loss could also be explained by an acute state of anxiety.[63]

After the second day to the last day of the trial, the animals of Group I and Group II individually and gradually recovered weight of the initial phase of the study (difference between day 0 and day 14). However, Group I (not subjected to stress) had a more marked increase (15.84 g). The difference between the final weight and the initial weight was lower in group II (7.85 g) and not statistically significant. It could be argued that stress and anxiety limited the mice of GII to increase weight. However, we cannot eliminate the possibility that they could gain weight if the experimental period were wider.

Groups III and IV showed a bigger weight loss after experimental manipulation. The weight change in the negative direction (weight loss), is significant for the Group III (-21.28 ± 16.43 g) where $t(9) = 4.094$, $p = 0.03$ (Kruskal Wallis) but even more significant (-49.37 ± 29.83 g) for Group IV $t(8) = 4.96$, $p = 0.001$ (Kruskal Wallis). This may be due to the fact that the manipulation of mice for the preparation of acupuncture protocol is itself a stressor agent, therefore leading to a greater loss of bodyweight.

The weight loss in Group III and Group IV seems to be potentiated by the

administration of d-amphetamine.

According to the study by Jones (1992),[64] which in turn is supported by other studies, chronic use of amphetamine suppresses food intake, reduces body weight due to increased energy expenditure and promotes an alteration of metabolic rate and metabolism fat in rats when administered during the day.[65-67]

As a consequence of dietary restrictions and the increase of tension, it can be seen rhabdomyolysis, that it is a rapid breakdown of skeletal muscle due to injury to muscle tissue and can be caused by physical, chemical or biological factors, which leads to muscle contraction (bruxism, clenching jaws and constant restless motion), and even muscle necrosis.[68, 69] There is also a functional replacement of muscle tissue by connective tissue, that leads to a loss total weight.[69] After morphometric analysis of the masseter and temporal muscles, different values in relation to the different dimensions of the muscle fibers were obtained. Those changes are discussed in a latter chapter of this thesis.

Shah HV *et al*, described a case of rhabdomyolysis involving the masseter muscle. It involved an episode of muscle lysis of the left and lower upper member and the left masseter, after ingesting an unknown quantity of alcohol, amphetamines and ecstasy with prolonged immobility for an unknown period after collapse. [70]

A study by Sund, demonstrates that post-dose amphetamine, there was a weight loss or no increase even during the first year. [71]In the study by Jones, it was concluded that amphetamine reduces body weight by altering metabolic rate and fat metabolism in mice when the drug is given during the day.[64]

However, the greater significance of weight loss in Group IV raises some questions regarding the anxiolytic effect of acupuncture in animal models, or at least, the effects of the protocol used in this study.

6.2. Attrition

The differences of the values of attrition between the four groups were statistically significant ($p < 0.009$, LSD test). However, contrary to what was expected, the attrition was higher in Group IV (7.84 ± 0.62 mm) subjected to acupuncture than in Group III (7.24 ± 0.41 mm). The average difference between Group I and Group II (0.443 mm) is greater than the difference between Group II and Group III (0.4225 mm) suggesting that stress can have an effect on isolated attrition greater than the actual amphetamine. However, the difference between Group III and Group IV (0.6067 mm) is greater than between any of those described above. As expected, the attrition proved higher in group II (6.82 ± 0.34 mm) subjected to

stress, than in the control group (6.37 ± 0.23 mm) not subjected to stress. The average daily attrition in the control group given the 14 day trial was 0.455 mm, and Group II was 0,487 mm. Although representing an average daily over these data are in agreement with those obtained by Gomez and Kiliaridis. [11, 72]The results of this study are relatively similar to those obtained by Gomez, with respect to the average value of attrition per day of experiment. Gomez obtained for the control group value of 0.45 mm / day while this experiment was obtained 0.455 mm / day. For groups subjected to stress, Gomez scores a attrition of 0.463 mm / day and the present study was 0.487 mm / day for Group II and higher for others. However, while this study found a statistically significant difference in the mean values of attrition between groups, Gomez, prompting him to say that stress in general, might not be a relevant factor in tooth wear. The fact that Gomez have failed to find this relationship statistically significant, does not mean it does not exist, but only that their study could not prove. This assumption is advocated by some authors, who claim that the stress protocol may not have been sufficiently effective to induce changes in the values of attrition. [11]The acupuncture protocol used in this study did not reduce attrition displayed by rats in Group IV.

There are no studies in the literature that relate the effect of acupuncture on the incisal attrition. However, considering that the induction of stress and amphetamine cause a significant increase in attrition, and that the manipulation of mice during the acupuncture protocol may be per se a stressor, the combination of both can further enhance and considerably dental attrition values.

6.3. Creatine-Kinase

Creatine-kinase is an biomarker composed by isoforms MM, MB and BB, often used to assess the damage in the muscle tissue.[73, 74]

After direct muscle tissue injuries such as produced by eccentric contractions, intramuscular injections, ischemia or excessive stress usually occurs an elevation of CK in serum.[73, 75, 76]

According to Howatson G *et al*, Exercise Induced Muscle Damage (EIMD) can be caused by novel or unaccustomed exercise and results in a temporary decrease in muscle force production, a rise in passive tension, increased muscle soreness and swelling, and an increase in intramuscular proteins in blood.[77] Since the MM is a predominant isoform in skeletal muscle, this assay may be indicative of an injured muscle type referred to above.[74]

The serum, in the absence of cardiac disease, only have the MM isoenzyme, which in non-pathological conditions represents 95% of the total CK.[78, 79]

Thus, making the determination of total CK is obtained, indirectly, the expression of

this isoform values, evaluating induced muscle damage.

It is possible to detect increased activity of the masseter muscle in animals with bruxism, as shown in studies by Landgren,[80] Weiner[81] and Sitthisomwong.[9, 82]

A common fact among these three authors is that they all study the pression of hypothalamically activated emotional behavior. For Sitthisomwong P *et al* the hypothalamically mediated emotional behavior is also accompanied by increased EMG activity in the jaw muscles.[82] These experiments were designed to examine the combined effects of administration of pentagastrin with activation of hypothalamically mediated emotional behavior upon jaw muscle EMG activity.[82]

For Weiner S *et al* electromyographic amplitudes resulting from stimulation at hypothalamic sites at which affective defense and quiet biting attack behaviors were elicited were compared with those recorded during stick biting that simulated mastication.[81] Finally their results suggest a relationship between hypothalamically elicited behaviors and increased levels of jaw muscle activity and, NFMM.[81]

This increased of muscle activity can be evaluated by the increase in serum CK. The models with rodents were considered good models of induction of muscle alterations, taking into account that physiology and adaptation are well studied

Hutchins M.O, *et al* in a study of Weakness in Mouse Masticatory Muscles by Repetitive Contractions with Forced Lengthening concluded that the masticatory muscles of humans and animals move the mandible by concentric and eccentric contractions and stabilize the mandible by isometric contractions.[76] Acute pain in masticatory muscles is usually brief and without injury. However, if pain is delayed or is maintained longer in response to masticatory muscle hyperactivity, the muscle fibers and tendons may be overstrained and structurally damaged [76] Skeletal muscle fibers are also susceptible to injury by eccentric contractions, particularly when contracting with a load and at high velocity.[76]

In the Analysis 1 there are no significant differences between groups ($p > 0.05$, Fisher's LSD test) because the values of CK are elevated only a few hours after muscle damage. [83] As blood collection has happened immediately after the induction of stress, it is likely that hasn´t passed time enough, to cause the effect of induction protocol.

Hutchins, in his study in Sprague-Dawley rats, assessed the relationship between the damage caused by muscle contraction and concentration of CK. Following the protocol of damage, only four hours later there was an increase in the concentration of CK in serum.[83]

Silva LA, *et al* in a study of 2013 in rats, concluded that muscle damage (creatine-kinase) and inflammation (myeloperoxidase) were not decreased with Trained Eccentric

Running (TER). They suggested that TER improves mitochondrial function, but does not reduce oxidative stress, muscle damage, or inflammation induced by eccentric contractions. [84]

Acupuncture does not seem to bring significant changes when comparing the results with the GI. A study of Lin noted that there were no statistically significant changes in CK activity after treatment with acupuncture, applied to the muscle pain induced by exercise. Thus, taking into account that amphetamine increases rhabdomyolysis, acupuncture may not have sufficient effect to counteract. Note that this does not happen only during the exercise, as described by this author.[85]

According to obtained in Matte study, in which rats were subjected to different types of stress, such as immobilization and isolation, the CK values were higher in the induction group compared to the control group. As stated by the author, and also through the present experimental study, the induction of stress may have resulted in an increase of CK activity.[85]

6.4. MORPHOMETRY

By analyzing the distribution percentages of the different types of muscle fibers from GI, we concluded that the medium fibers are predominant, followed in decreasing order by small, large and very large fibers. This relative distribution of the group I is verified in both the temporal and masseter muscles and will use it for comparison in the posterior analysis relatively to the different variables under study.

For group II - Masseter muscle, comparing to group I, the small and medium fibers decreased and large increased in number, but the increase is more pronounced for very large fibers. For group II - Temporal muscle the decrease in the small and medium fibers is less evident in the temporal than as seen in masseter of group II, as the increase in large and very large fibers is also smaller

. For group III - Masseter muscle, comparing to group I and group II, both large and very large fibers seem to decrease, with a predominant increase in the medium fibers. Hereafter, we could question ourselves whether the amphetamines could cause this decrease of large and very large fibers (as well as a decrease in weight) and its transformation into medium fibers? This is even more evident in the temporal muscle.

For group III - Temporal muscle, the decrease in large and very large fibers is more pronounced than in the masseter muscle, with essentially a marked increase in small fibers.

For group IV - Masseter muscle the small and medium fibers present a relatively similar distribution to group II. However large and very large fibers increase as compared to

those in group III. Is this an evidence that acupuncture could reduce the effect of amphetamines? More studies should be conducted in these issues. Although this has happened in muscle fibers, the same hasn't happened in weight and attrition. This suggests the question whether the attrition could be linked, or not, to the more marked increase of large and very large fibers in this group.

For group IV - Temporal muscle, there is essentially a marked increase in medium fibers.

Relative to the total weight of the animals, we observed that the group I gained weight between day 0 and day 14.The group II, which was subjected to stress, didn´t increased the weight. The group III, decreased weight, due to the effect of amphetamine and rhabdomyolysis. Harris SC *et al* concluded that the administration of amphetamine ("benzedrine") facilitates the reduction of weight. This effect of the drug was first reported in 1937, when patients receiving amphetamine for other purposes were observed to lose weight. [86] In group IV, subject to amphetamine and acupuncture, the weight also decreased.

Relatively to the morphometry of the masseter and temporal muscle, the group II, subjected to stress, presented less small and medium fibers, however, there is an increase of large and very large fibers. Although there are no studies that can clarify the evolution of fibers size relatively to the induction agents used, we can suggest that the stress can cause an increase of these fibers (evolution to the large and very large fibers). Although this difference is pronounced in masseter, it's less pronounced relatively to the temporal muscle. Could this increase of large and very large fibers justify the higher values of dental attrition in group II? Curiously, the same happens in group IV, however the data from group III, with a decrease in large and very large fibers, seem to be contradictories. However, there are no studies, that tell us that it is possible the transformation of small and very small fibers in large and very large fibers, in a trial period of 14 days.

In group III for masseter and temporal muscle, is possible to observe an decrease of large and very large fibers compared to the other groups. That could justify the increase of CK values, due to the, effect of rhabdomyolysis by the amphetamine. However the same explanation is not valid for group IV where an increase in large and very large can be seen. Tóth AR, *et al* concluded that the most frequent damage observed is rhabdomyolysis syndrome, which has been mainly described after cocaine or opium consumption. The laboratory tests showed renal and liver insufficiency; in addition the CK and CK-MB level increase suggested damage in striated muscles. [87] The weight loss can also be associated to the reduction of large and very large fibers; however, there are no morphometric studies that support this.

An interesting fact is that when amphetamine acts alone (G III), there is a decrease of large and very large fibers. When acupuncture is associated (GIV), these fibers increase in quantity, especially in the masseter. We can suggest that acupuncture reduces the effect of amphetamine. Although there are no morphometric studies in the literature that support this fact, Russell LC, *et al* in a pilot study of acupuncture for addicted patients with chronic histories of arrest, concluded that in methamphetamine-addicted patients, acupuncture improved program retention up to 30 days. These findings support addition of acupuncture to substance abuse treatment for criminal justice clients and indicate a need for acupuncture research focusing on withdrawal from methamphetamine. [88]

In group II, there was an increase of the CK values and so, we can conclude that there was an increase of muscle activity. Studies in rats undergoing stress, concluded that there is an increase of activity of CK compared to the mice control group, as indicated in the work of Woolf, Allen, Patterson, Ready and Watson. [89, 90]The present study shows that the stress increases the CK values, the amphetamine potentiated this increase in the CK values and acupuncture does not seem to bring any effect.

In small and medium fibers such data is not consistent, but it is in the large and very large fibers. There are no studies that support this increase of large and very large fibers, when the rats are subjected to stress. Chen YJ, et al, in a study of alterations in the ultrastructure and energy metabolism of masticatory muscles in rats (divided in control group, foot-shocked group and psychological stress induced group), concluded that in comparison to the control group, the psychological stress induced group showed evidence of swollen mitochondria with cristae loss and reduced matrix density in the masticatory muscles after three weeks of stimulation. After five weeks of stimulation, severe vacuolar changes to the mitochondria were observed. Increased vascular permeability of the masticatory muscle capillaries was found in the five-week in psychological stress induced group.[91] However, the effects of stress in the fibers dimensions are not studied in this work.

Regarding attrition, the group II, subjected to stress, shows an increase of attrition. By comparing the dimension of masseter and temporal muscle fibers, this increase is mainly due to the large and very large fibers increase. However, there are no morphometric studies, to tell us, why the increase of large and very large fibers, in stressful situations.

In group III, subject to amphetamine, the attrition also increased. However this fact is not consistent with what we saw in group II. In this group (III) we saw an increase of medium fibers in the masseter muscle and small fibers in the temporal muscle, which is comparable to the group I. In terms of morphometry, the fact why the attrition increases, without muscle changes, remains unknown.

In group IV the attrition increased, which is one surprising fact in this study. For group IV – the masseter fibers present a relatively similar distribution to GII. However large and very large fibers increase as compared to those in group III. Relatively to the temporal muscle there is essentially a marked increase in medium fibers. For this fact it remains unknown to us the relation between the fibers dimensions and dental attrition.

7. CONCLUSIONS

After the development of this experimental study we can conclude:

In rats, stress increases the attrition and the CK values and limits the weight gain.

Amphetamine potentiates the effects of stress in attrition and CK, and generates an increased weight loss.

Contrary to what was expected, the acupuncture protocol used did not reduce the incisal attrition, and led to a significant loss of total weight, causing no significant changes in CK.

After morphometric analysis of the masseter and temporal muscles, it was possible to verify that the percentage of muscle fibers varies among different experimental groups and muscles.

Large and Very Large Fibers increased with stress.

Acupuncture seems to attenuate the effect of amphetamine to reduce the large and very large fibers.

There are no studies in the literature to compare with this work. For that reason we cannot conclude and did not found an obvious relation between muscle fibers diameter and CK, attrition and weight loss.

Furthers studies should be conducted in this area in order to better understand the mechanisms of masseter hypertrophy, and masticatory muscle fibers diameter changes and stress or dental attrition.

8. FURTHER PERPECTIVES

This study, as well as any other scientific study has limitations. It should answer to some of the purposed questions, but also allow future investigations related to the topic.

After the results, it would be relevant to examine, according to the same experimental protocol, groups submitted separately to the variables amphetamine and acupuncture, in a longer time of experience.

Due to the scarcity of masticatory muscles morphometric studies in the literature, it would be interesting to conduct further studies in this area in order to better understand the mechanisms of masseter hypertrophy, masticatory muscle fibers diameter changes and stress or dental attrition.

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