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Changes in masseter muscle structure, membrane lipid peroxidation and Ca-ATPase activity as effects of different local anesthetics

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ABSTRACT

Local anesthetics (LA) can cause undesired effects such as sustained contraction of skeletal muscles as a result of structural and functional changes. Proper skeletal muscle function is controlled by intracellular Ca^{2+} concentration and efficient energy (ATP) production, which is closely related to cell ultrastructure. ***Aim:*** *The aim of this study was to identify the structural and functional changes caused by LAs.* ***Materials and Method:*** *Male Wistar rats weighing 200 to 250g were used (n:49). They were divided into seven groups. One group was not anesthetized or treated (Control). The other six groups underwent intramuscular (IM) anesthesia with xylazine 2% (0.05 ml) and ketamine 50 mg/ml (0.1 ml/100g rat weight), and one of the following was applied to the masseter muscle (MM): no further treatment (Anesthetic Control group, CA); 0.1ml physiological saline solution (group SF); Carrageenin (group Carr) 1% as positive control group; prilocaine (group Pri), mepivacaine (group Mepi); or articaine (group Arti) 0.3M, IM. The animals were euthanized by cervical dislocation one hour after treatment. The effects of the different anesthetics on the MM were evaluated histologically and by electronic microscopy (EM). Ca-ATPase and membrane lipid peroxidation (LPX) were evaluated in muscle homogenates under the same conditions as those used to prepare the histological sections.* ***Results:*** *In general, structural damage and increased muscle contraction were observed in tissues treated with anesthetics. The most extreme values of Ca-ATPase activity and LPX were observed in the positive control group (carrageenin). Results were analyzed by one-way ANOVA for multiple comparisons and Tukey's test ($p < 0.05$).* ***Conclusions:*** *The results suggest that in the short term, local anesthetics affect the muscle function and are associated to structural changes.*

Keywords: *local anesthetics - masseter muscle - electronic microscopy - Ca-ATPase - membrane lipid peroxidation - carrageenin.*

Cambios estructurales del músculo masetero, lipoperoxidación de membrana y actividad de la Ca-ATPasa por efecto de distintos anestésicos locales.

RESUMEN

Los anestésicos locales (AL), pueden causar efectos no deseados como la contracción sostenida de los músculos esqueléticos como consecuencia de cambios estructurales y funcionales. Como es sabido, la función adecuada del músculo esquelético está controlada por la concentración de Ca^{2+} intracelular y por la producción eficiente de energía (ATP), íntimamente relacionado con la ultraestructura celular. ***Objetivo:*** *El propósito de este trabajo fue relacionar los cambios estructurales y funcionales provocados por los AL.* ***Materiales y Método:*** *se utilizaron ratas Wistar macho de 200 a 250g de peso (n:49), bajo anestesia intramuscular (IM) de xilazina 2% (0,05 ml) y ketamina 50 mg/ml (0,1 ml/100g peso de rata) se les aplicó en el músculo masetero (MM): 0,1ml de solución fisiológica (grupo SF) o Carragenina (grupo Carr) 1% como grupo control positivo o Prilocaína (grupo Pri), Mepivacaína (grupo Mepi) y articaína (grupo Arti) 0,3M, IM. Un grupo no recibió tratamiento, grupo Control (C). Se realizó la eutanasia por dislocación cervical a la hora post tratamiento. Se evaluó histológicamente y mediante microscopía electrónica (ME) los efectos de los distintos anestésicos a la hora post-inyección en músculo masetero. Además, se evaluó la actividad Ca-ATPásica y la lipoperoxidación de membrana (LPX) en homogenatos de músculo con las mismas condiciones que el utilizado para realizar los cortes histológicos.* ***Resultados:*** *En general se observaron daños estructurales en los tejidos tratados con anestésicos y aumento de la contracción muscular. Los valores extremos de la actividad Ca-ATPásica y la LPX se observaron en el control positivo (carragenina). Los resultados obtenidos se analizaron por comparación múltiple ANOVA de 1 via y test de Tukey ($p < 0,05$).* ***Conclusiones:*** *los resultados sugieren que los AL, a corto plazo, afectarían la función muscular asociados a los cambios estructurales.*

Palabras Clave: *anestésicos locales - músculo masetero - microscopía electrónica - Ca-ATPasa - lipoperoxidación de membrana - carragenina.*

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INTRODUCTION

Local anesthetics (LA) have been used since 1905 to block pain temporarily and reversibly in specific parts of the body. LAs block the transmission of the nerve impulse in any part of the system, causing loss of sensitivity, which recovers completely when the effect wears off[1]. However, LAs may cause an undesired effect involving sustained contraction of skeletal muscles involved in mastication. This condition is called "trismus"[2, 3].

In skeletal muscle, the sarcoplasmic reticulum (SR) is the main regulator of calcium storage, release and reuptake. Glycolysis and mitochondria are responsible for cell production of the ATP required for cell metabolism[4,5].

Proper skeletal muscle function is controlled by intracellular Ca^{2+} concentration and efficient energy (ATP) production, which depend on: (a) the release and reuptake of Ca^{2+} by the sarcoplasmic reticulum (SR) during excitation-contraction (EC) coupling, which controls sarcomere contraction and relaxation; (b) Ca^{2+} uptake in the mitochondrial matrix, which stimulates aerobic ATP production; and (c) entry of Ca^{2+} from the extracellular space by activation of store-operated Ca^{2+} entry (SOCE), an important mechanism for preventing muscle fatigue[6-10].

Calcium uptake into the mitochondria increases ATP production by stimulating oxidative phosphorylation and mitochondrial ATP production, as well as production and/or detoxification of reactive oxygen and nitrogen species (ROS/RNS). Mitochondria in turn modulate the release and reuptake of calcium by the SR. This close spatial Ca^{2+}/ATP/ROS/RNS communication between the SR and mitochondria is facilitated by the structural attachment between mitochondria and the calcium release unit (CRU)[4,11]. While mitochondrial uptake of Ca^{2+} after release from the SR in the muscle can stimulate ATP production by excitation-metabolism coupling, Ca^{2+} signaling is also markedly influenced by mitochondrial function. Firstly, mitochondrial ATP production is used to drive both crossbridge cycling during muscle contraction and that mediated by SR Ca^{2+}-ATPase and elimination of myoplasmic Ca^{2+} during relaxation. Indeed, up to 80% of the ATP consumed during muscle contraction is used to drive elimination of Ca^{2+} by the SR during contractile relaxation[7]. The impact of mitochondrial ATP production is particularly important during activity when glycolytic reserves become depleted[8].

In skeletal muscle, the increase in intracellular Ca^{2+} returns to its physiological values due to the activity of SR Ca^{2+}-ATPase, which catalyzes the transport of two Ca^{2+} ions from the cytosol to the lumen of the SR per mole of hydrolyzed ATP, using the magnesium cation as cosubstrate[12-15]. Our group has published several studies on preparations of different rabbit masticatory muscles in which the composition of muscle fibers was determined and related to the distribution of different isoform, enzymatic activity and calcium transport[16-19]. These mechanisms are also altered by different injurious factors such as the action of LAs[20-25]. In addition, in striated muscle, LAs cause the appearance of ROS which may interact with lipids[7].

The current study analyzes at the effect of different LAs on changes in skeletal muscle fiber ultrastructure, SR Ca^{2+}-ATPase activity, and antioxidant response in rat masseter muscle.

MATERIALS AND METHOD

Animals

This study used 49 healthy male Wistar rats weighing 200 to 250g. They were given "ad libitum" access to balanced feed and water and were housed in 32x45x24cm galvanized wire cages containing not more than five animals each, at temperature 21 ºC to 24 ºC; humidity 52% to 56%; and 12/12 h light/dark cycles. The experimental protocol was approved by the Ethics Committee at the School of Dentistry (005/2016 CICUAL-ODONTO-FOUBA Buenos Aires, Argentina), and follows the National Health Institutes Guidelines for the care and use of laboratory animals.

The rats were divided into seven groups of seven animals. One group received no treatment and served as Control (C). The rest received intramuscular (IM) anesthesia in the back leg with xylazine 2% (0.05 ml) and ketamine 50 mg/ml (0.1 ml/100g rat weight) plus one of the following: no further treatment (Anesthesia Control group, CA); or masseter muscle (MM) injection with: 0.1ml physiological saline solution (group SF); carrageenin 1% (group Carr, positive control group)[26]; prilocaine (group Pri), mepivacaine (group Mepi), or articaine (group Arti) 0.3M, IM. The rats were euthanized by cervical dislocation one hour after treatment. Subsequently, the masseter muscle was dissected and processed for evaluation.

Reagents

All reagents were acquired from Sigma Chemicals (St. Luis, Mo., USA). All solvents were analytical grade.

Electronic microscopy

MM specimens were fixed in a solution of glutaraldehyde 2.5% in phosphate buffer 0.1M pH 7.4 for 4hs at 4ºC. Then they were washed twice with phosphate buffer 0.1M, first for 15 minutes and then overnight. The specimens were sent to the Lanais-Mie, Institute of Cell Biology and Neurosciences, run by CONICET, where the tissues continued to be processed. They were fixed for a second time with osmium tetroxide 1% in the same buffer for 60 minutes at 4ºC, rinsed twice for 15 minutes with bidistilled water, and then dehydrated in ascending alcohols (50º, 70º, 96º, 100º) with two 15-minute changes each followed by two 10-minute changes of acetone. Specimens were embedded in "Durcupan" epoxy resin, which was polymerized at 60ºC for 72 hours, and then cut into semi-fine 0.50 µm sections using an ultramicrotome (Reichert Jung Ultracut E) with a glass knife. Sections were mounted on slides and stained with toluidine blue for observation under optical microscope to determine the direction of the section of muscle fibers, and then observe them by electronic microscopy.

For observation by transmission electron microscopy (TEM), ultrafine 70-90 nm sections were cut using the same ultramicrotome. Sections were mounted on a copper grid, contrasted with uranyl acetate and lead citrate (Reynolds' method) and observed under a Zeiss 109 transmission electron microscope. Digital photographs were taken with a Gatan W10000 camera.

To provide information on contractile activity, the fibers on the sections were measured transversely, and the number and size of mitochondria were evaluated because, as mentioned above, they are closely related to contractile activity due to the structural attachment with the calcium release unit (CRU). Mitochondrial size was calculated by the point-counting stereological technique[27] on TEM micrographs taken at magnification 12,000X after superimposing an orthogonal matrix of points 0.50 µm apart on the electronic micrographs. The ratio between the number of points within the mitochondrial outlines and the total number of points covering the whole image was used to calculate the relative area of the fiber occupied by mitochondria. The same set of micrographs was used to determine number of mitochondria per field, and muscle contraction by measuring the distance between Z lines. Sections with any of the following structural alterations were classified as damaged: swollen mitochondria, interrupted Z line, vacuoles, disorganized CRU.

Determination of membrane lipid peroxidation

LAs in striated muscle may cause the appearance of ROS that can interact with lipids. Therefore, a homogenate of MM was prepared in which to determine membrane lipid peroxidation (LPX) by means of the reaction between thiobarbituric acid (TBA) and the aldehyde products derived from the rupture of polyunsaturated fatty acids (thiobarbituric acid reactive substances or TBARS) (µmol MDA/mg Prot) and assess the effects of ROS production[28].

Briefly, the MMs were homogenized in 320 ml of Tris buffer 0.1 N, pH 7.4. Twenty ml were separated to determine proteins, and the rest was centrifuged at 1500 g for 20 min at 4°C. Then, 250 ml of the supernatants were transferred to a tube containing 250 ml trichloroacetic acid. The mixture was centrifuged at 3000 g for 15 min at room temperature. The supernatants were mixed with 250 ml of TBA, boiled for 15 min, and cooled on ice for 5 min to stop the reaction. The resulting reactive TBA species-stained pink (TBARS) were determined in a spectrophotometer (Beckman DU 520) at 540 nm. The acid did not produce color when tested without addition of the sample, showing the absence of a direct reaction to the TBA. The calibration curve was prepared using malondialdehyde (MDA), and each point on the curve was subjected to the same treatment as the muscles. The TBARS were calculated as micromoles of MDA per milligram of protein[29].

Determination of Ca-ATPase activity

SR Ca-ATPase enzymatic activity was determined to corroborate that the pump was not inhibited, preventing muscle relaxation in groups C, CA, SF and Carr, since our team had not analyzed this in any previous study[30]. To do so, sealed rat MM SR membrane vesicles were obtained, with capacity to accumulate calcium, following Champeil et al.[31].

For all the procedures, protein concentration was determined using the technique described by Lowry et al.[32].

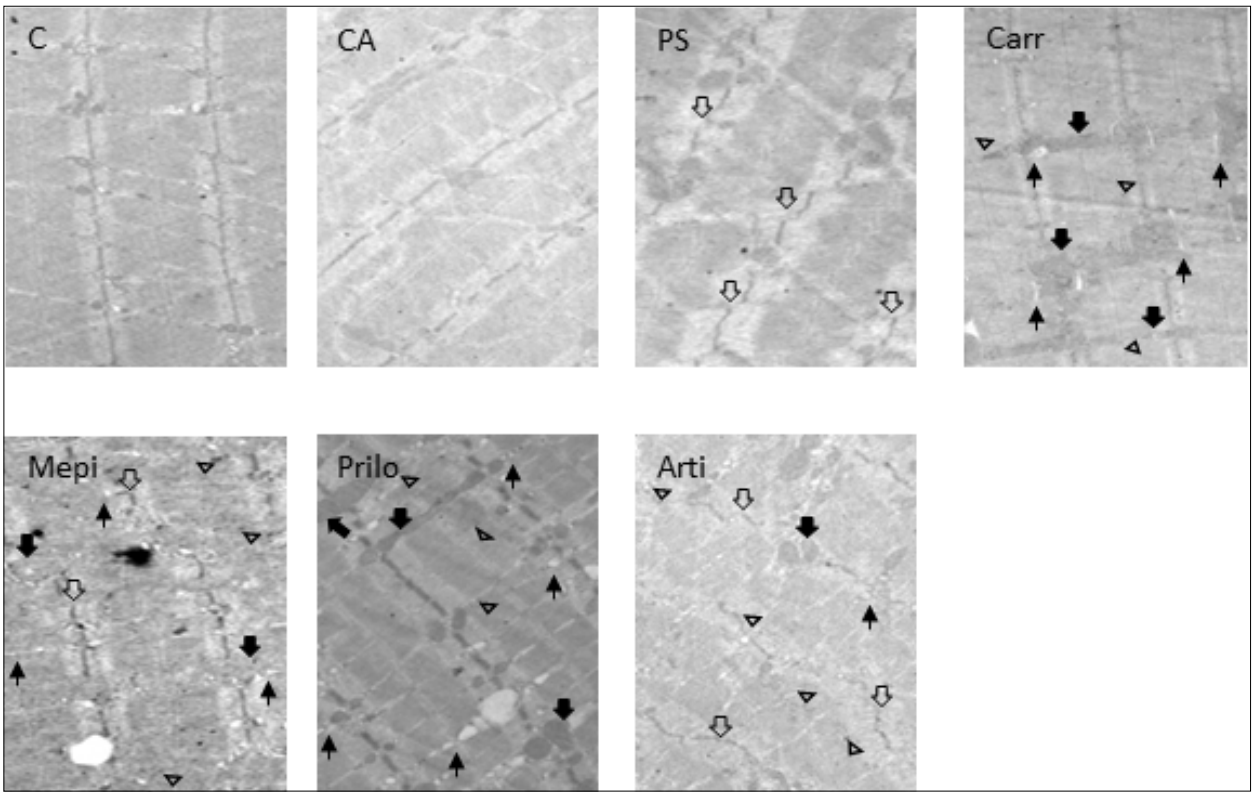

Fig. 1: Electron micrograph of masseter muscle (X12,000) C: Control. CA: Control Anesthetic SF: Physiological saline solution. These three longitudinal sections show that muscle structure is preserved. There is only a partial misalignment of microfibrils in muscles treated with PS (unfilled arrows). Carr: carrageenin. There are many calcium release units (CRU) (small arrows) near the mitochondria (filled arrows) showing clear signs of structural damage/swelling. There are also intermyofibrillar glycogen granules (unfilled arrowheads) near the longitudinal part of the sarcoplasmic reticulum. Mepi: Mepivacaine. Prilo: Prilocaine. Arti: Articaine. The three figures mainly show an increase in size and disorganization of structures, misalignment of myofibrils, and mitochondria with signs of damage/swelling.

The results were analyzed by one-way ANOVA multiple comparison and Tukey's multiple comparisons test, considering $p<0.05$.

RESULTS

Electronic microscopy images of MM showed that in groups C, CA, and SF, which were not treated with drugs, the structures were preserved. At ultrastructure level, partial misalignment of microfibrils (Z lines) was detected in groups SF, Mepi and Arti. In the groups treated with the different LAs, extensive areas with morphological anomalies (such as vacuoles) were observed, among which muscles in group Arti were more damaged than the others (Fig. 1). Muscle contraction values were significantly lower in all groups compared to control ($p<0.001$), with group Carr (positive control) having the greatest contraction (1.39 µm ± 0.03 µm) (Fig. 2).

Mitochondrial count was significantly lower in group Arti than the other groups, in which number of mitochondria was preserved (Fig. 3). Mitochondrial area represents mitochondrial volume and is related to structural damage. Group Prilo had the largest mitochondria (2.04 µm ± 0.42µm), compatible with mitochondrial inflammation, with significant difference ($p<0.001$) with respect to the other groups. In group Mepi, mitochondrial size was similar to that in the control group (C:1.10±0.33; Mepi:1.19±0.23), while the rest of the groups had smaller mitochondria (CA:0.61±0.30; SF:0.56±0.13; Arti:0.36±0.11; Carr:0.39±0.14) (Fig. 4).

Ca-ATPase activity (µmol Pi/ mg protein per hour) differed among groups, with much lower activity in the positive control group (Carr) (C:489.67±11.07; CA:443.47±18.35; PS:417.79±12.13; Carr:294.55±9.70) (Fig. 5).

LPX was significantly higher in the positive control group Carr ($9.85x10^{-3}$ ± $2.12x10^{-4}$) than in the other groups. LPX was also higher in group Prilo

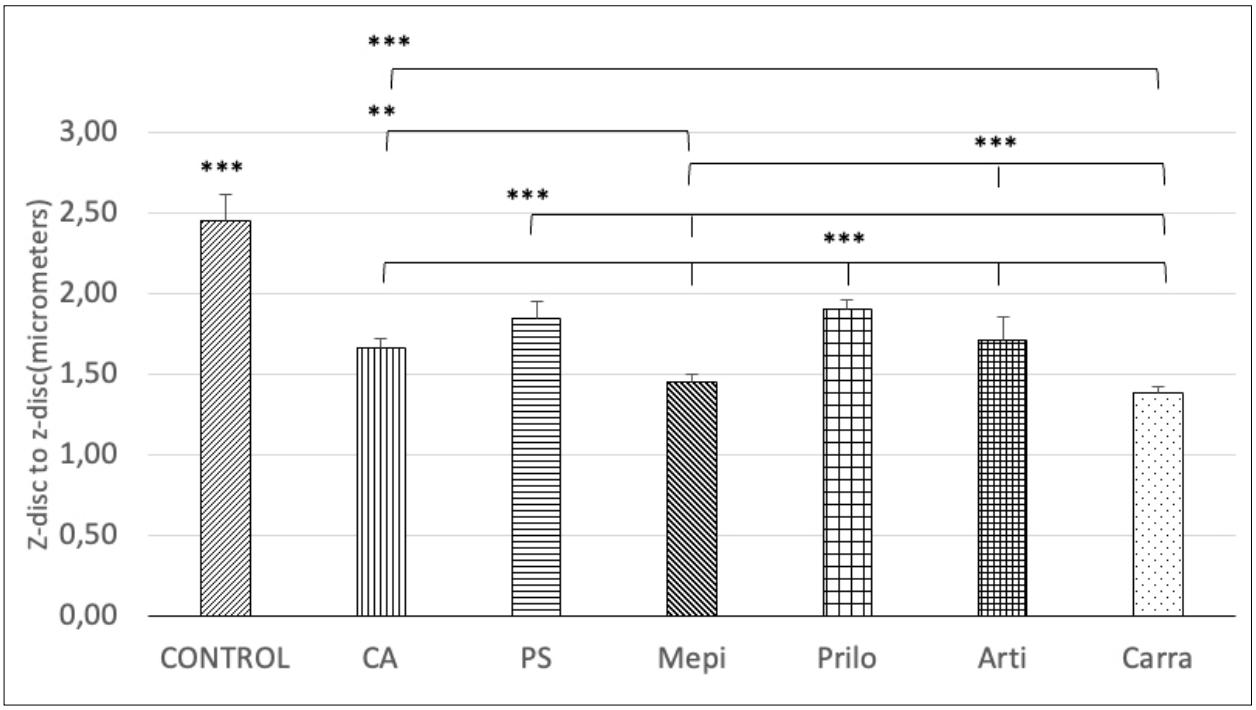

Fig. 2: ***Muscle contraction.*** *Expressed as distance in µm between Z lines. There is a significant reduction (p< 0.001) in all groups compared to control (2.45±0.16), with group Carr showing the greatest contraction (1.39 ± 0.03).*

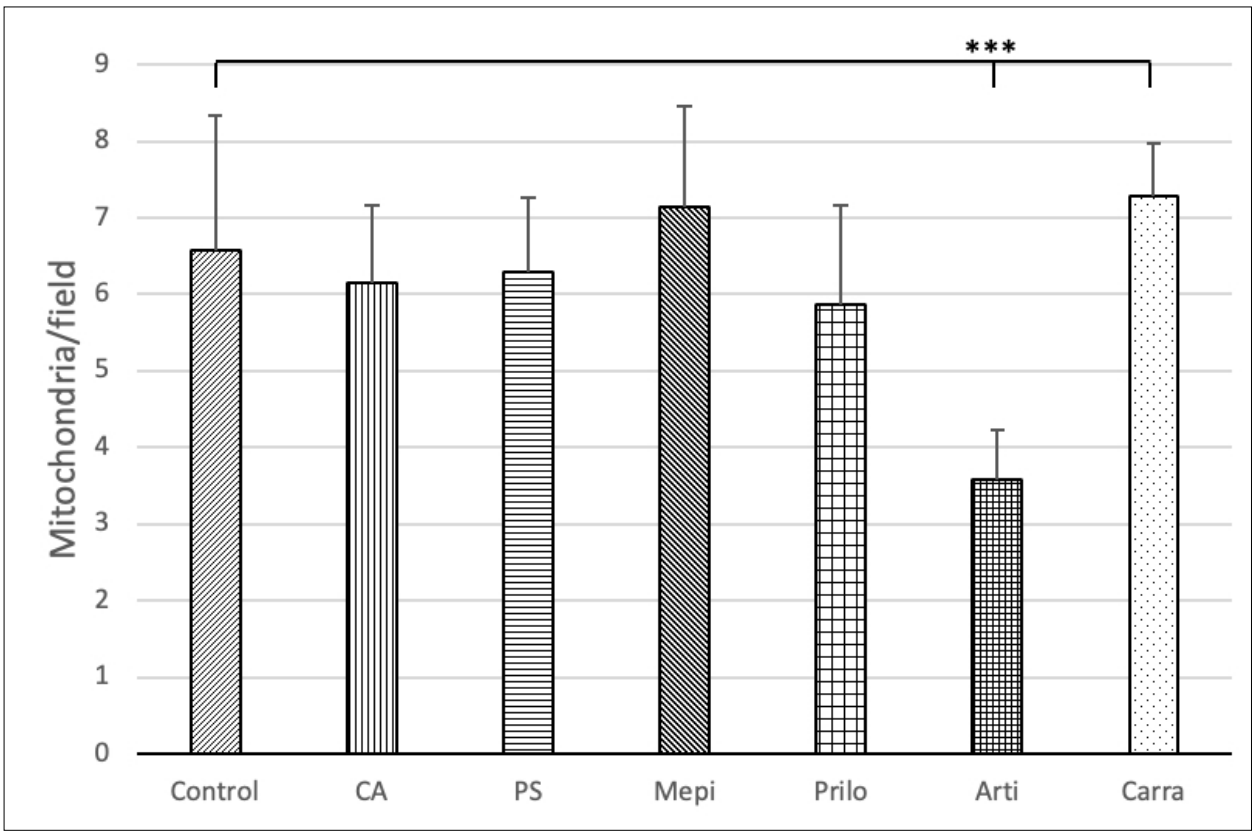

Fig. 3: ***Number of mitochondria.*** *The number of mitochondria per field is lower in group Arti (p=0.002) than in groups C, Carr y Mepi. No difference is observed with the other groups.*

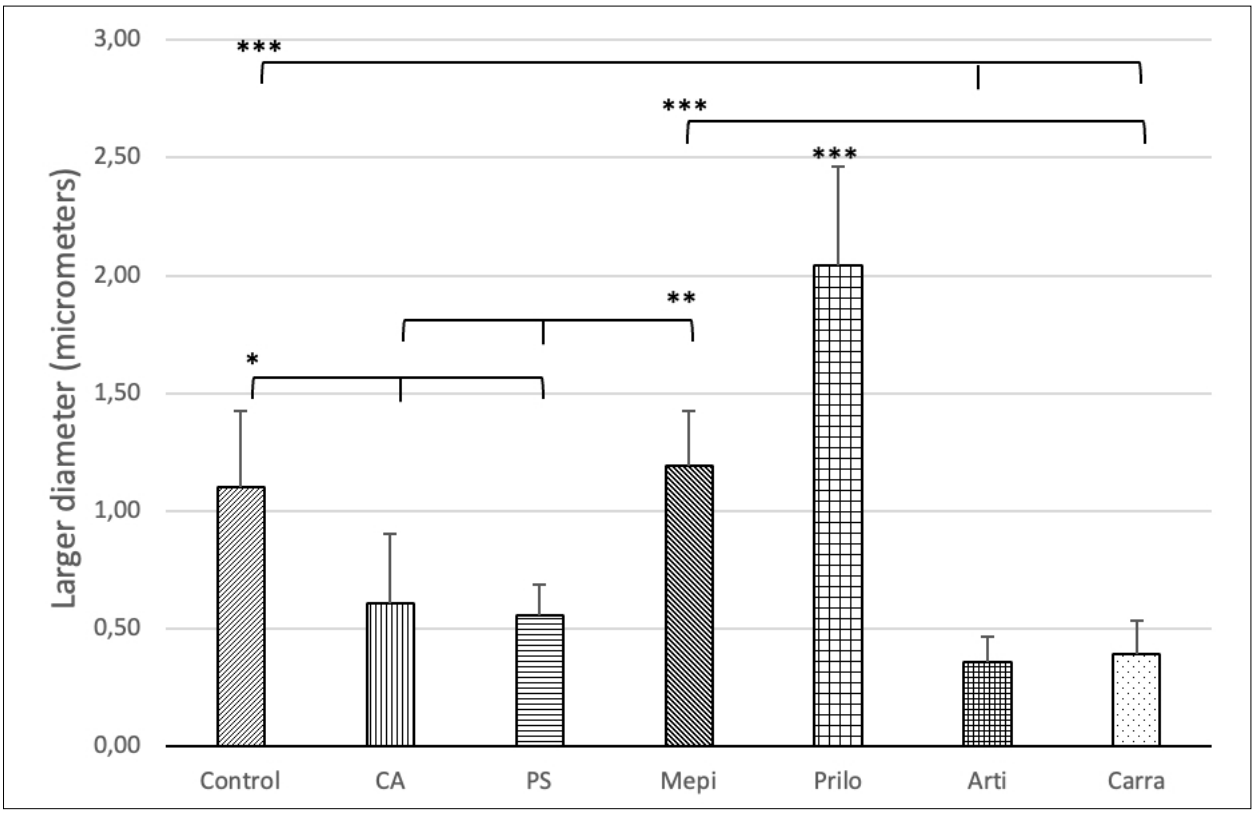

Fig. 4: ***Mitochondrial size.*** *Group Prilo has the largest mitochondria (2.04 µm ± 0.42µm) (p<0.001) compared to the other groups (C:1.10±0.33; CA:0.61±0.30; SF:0.56±0.13; Mepi:1.19±0.23; Arti:0.36±0.11; Carr:0.39±0.14).*

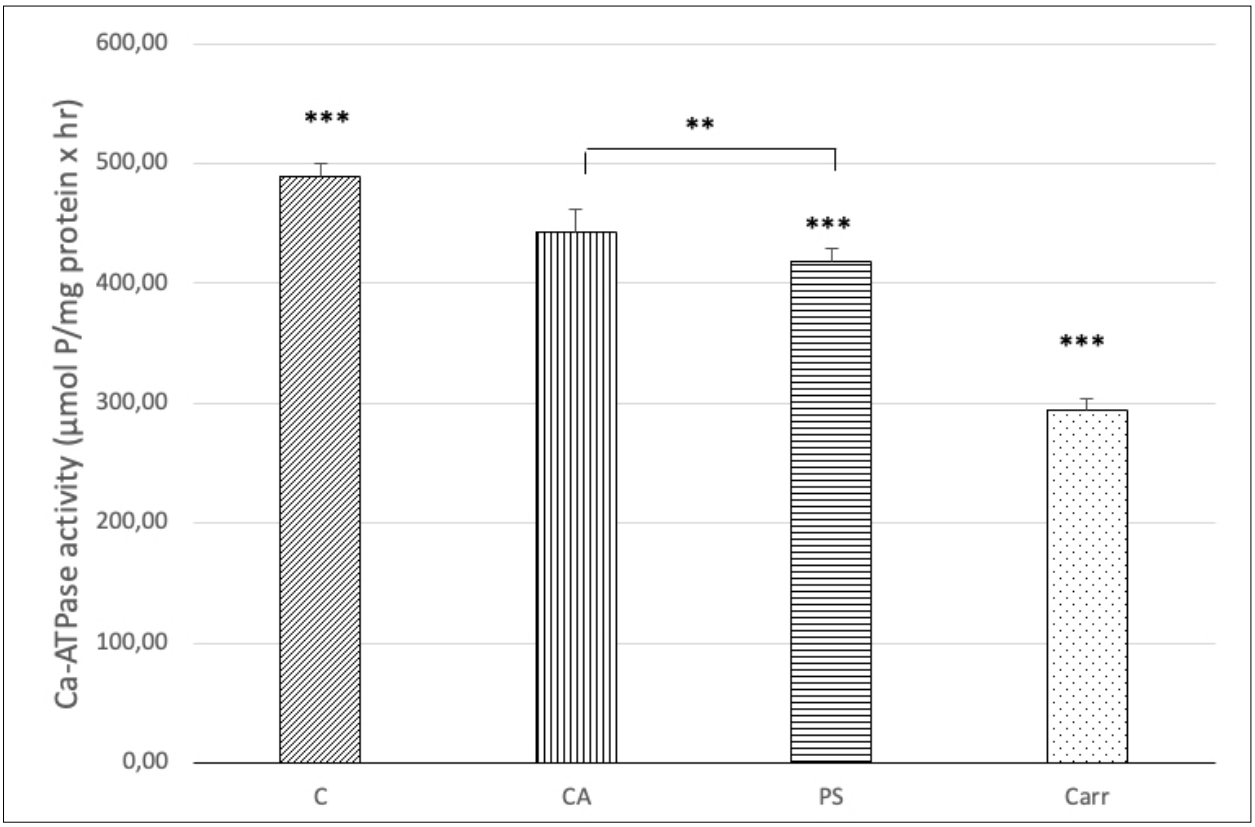

Fig. 5: ***Ca-ATPase activity.*** *There is less activity in all groups than in the control group, with group Carr (positive control) having the lowest enzymatic activity.*

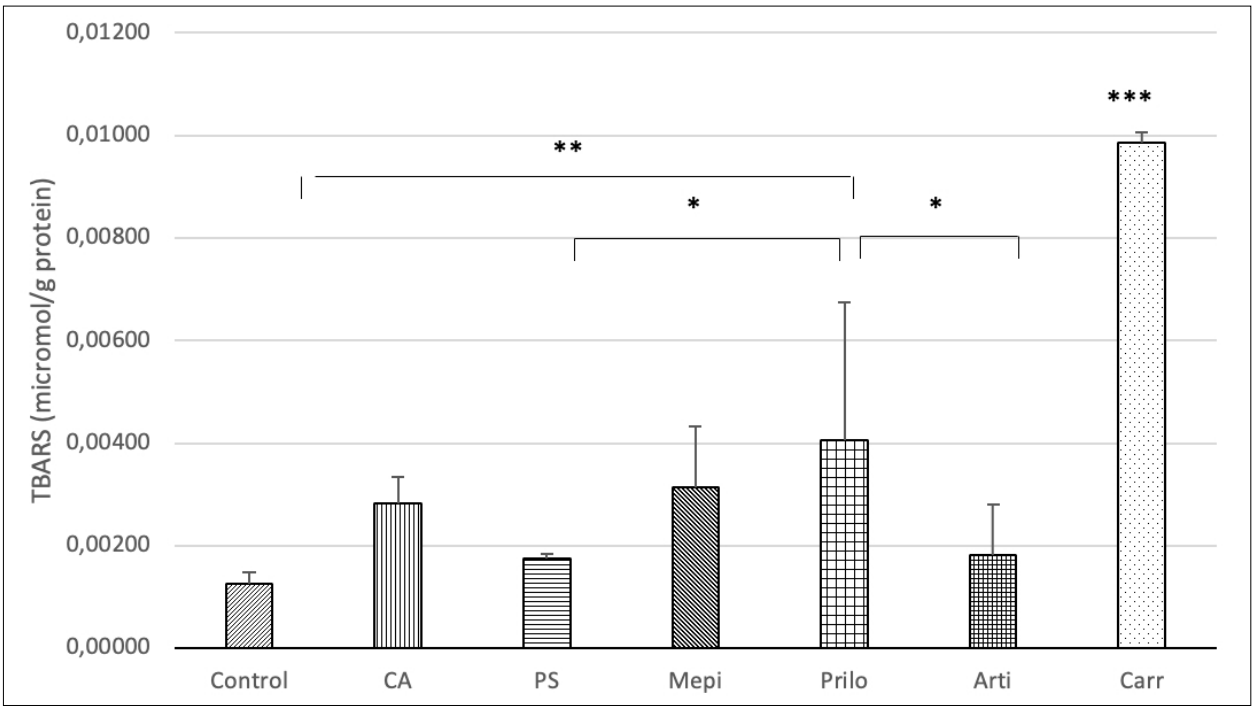

Fig. 6: ***Membrane lipid peroxidation.*** *Group Carr has a highly significant increase in membrane lipid peroxidation compared to the other groups. Group Prilo differs significantly from groups C, SF and Arti.*

($4.05x10^{-3} \pm 2.73x10^{-3}$) than in groups C, SF and Arti ($1.26x10^{-3} \pm 2.30x10^{-4}$; $1.75x10^{-3} \pm 7.78x10^{-5}$; $1.81x10^{-3} \pm 1.02x10^{-3}$) (Fig. 6).

DISCUSSION

All groups treated with LA presented morphological anomalies such as lack of continuity of the muscle tissue. Histological assessment suggests that all the treatments generate muscle contraction, but that in addition, the study LAs, as from the time they are applied, produce different structural changes in the MM, as reported by Gitman et al. (2019)[33]. Our results regarding mitochondrial size agree with several authors who suggest that the increase or reduction in size may be associated to mechanisms of mitochondrial fusion or fission[34,35] as a result of the stress produced in the environment, with the purpose of regulating mitochondrial morphology and function. Other authors attribute the increase in mitochondrial size to an inflammatory response[36].

The Ca-ATPase pump activity values in skeletal muscle found in groups C, CA, SF and Carr were consistent with the results published previously by our group in animals treated with LA, corroborating the interaction of these treatments in pump activity[22,25,37]. The increase in TBARS was used as an indicator of the appearance of ROS. Groups Mepi and Prilo have similar LPX values. It is worth highlighting that the micrograph of group Arti is similar to that of the control group, possibly as a result of destruction of muscle ultrastructures, which may prevent activation of the lipid peroxidation process. Similar effects have been described in salivary glands treated with ionizing radiation[29]. Qaisar et al. (2019) suggests that the increase in oxidative stress is closely related to the malfunction of the Ca-ATPase pump[38]. This is consistent with our results, where the increase in TBARS might be associated with reduction in pump activity.

It is important to consider that the differences observed among the study anesthetics could be owed to their chemical structure. They are all amide groups, with articaine having the greatest molar mass, in addition to containing sulfur and one additional ester group in its composition[39]. These structural features may be related to the extent of the damage recorded in the micrographs.

All of this suggests that in the short term, LAs cause structural changes that affect muscle function. Further medium- and long- term studies are needed on the evolution of muscle structure response such as recovery or increase in the muscle

destruction observed. With regard to the dental anesthetic cartridge, the absence of adrenaline in our determinations may modify the responses obtained in this study.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest concerning the publication of this article.

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