# AQUATIC TRAINING IN MENOPAUSE EXPERIMENTAL MODEL PROMOTE CHANGES IN THE SARCOMERES OF THE MYOTENDINOUS JUNCTIONS

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ABSTRACT

Myotendinous junction (MTJ) is the largest area of force transmission between skeletal muscle and bone tissue, which is directly associated with physical exercise that possible promotes morphological changes in muscle and tendon tissues. The aim of this study is to describe the ultrastructural characteristics of myotendinous junction and morphometric alterations in length sarcomeres of the anterior tibial muscle of ovariectomized aged female Wistar rats submitted to a swimming protocol. Twenty aged rats with 1 year and 8 months old randomly divided into four groups (n=5): Sedentary (S); Exercised (E); Menopause (M) and Menopause Exercised (ME). The exercising protocol consists at 40 sessions, one hour daily for a two months period and overload 5 % body weight of animals with adjustments weekly measured. Histological images were analyzed by transmission electron microscopy to demonstrate morphometric characteristics and ultrastructural elements of the cellular components. From the results obtained by transmission electron microscopy ultrastructural adaptations were observed in the MTJ region. The S and M groups demonstrated tissue disorganization in addition to lower density and length of sarcoplasmatic invaginations. The E and ME groups showed greater density, length and tissue organization, besides presenting sub-levels and communications between the sarcoplasmatic projections. Besides, they present adaptations in the plasticity of the MTJ evidenced by increase in the length of the distal sarcomeres. We concluded that the MTJ region presented adaptations in relation to the physical exercise during aging associated with ovariectomy, increasing sarcoplasmatic invaginations and changing length sarcomere distal, by improving the resistance and the transmission of force in the main injured area.

Keywords: myotendinous junction, aging, sarcomeres, menopause, Swinning training.

## INTRODUCTION

The muscular tissue is responsible for functions like mechanical protection and thermal insulation, besides generating force that is reversed in movements. This tissue has great capacity for anatomical and physiological adaptations such as increase in cross-sectional area, greater amount of Type I and II fibers due to training type, intensity and individual responses (MACDOUGALL, 2008).

One of the most important factors in the strength of muscle contraction is the length of the sarcomere - the morphofunctional unit of the skeletal striated muscle - which depends on the initial length of the sarcomere in addition to the tendinous stiffness that determines the shortening of the fibers. In addition, human skeletal muscle functions with a sarcomere length pattern that allows high levels of force production, however, in some cases the length of the fascicles changes perceptibly during maximal contractions (MACINTOSH, 2017).

The sarcomere is composed of two types of filaments, thick (nebulin / myosin) and fine (titin/actin). Muscle contraction requires a precise alignment between the actin and myosin filaments and this is characterized because there are accessory proteins such as α-actin, myomesin, titin, -H protein, desmin and myosin binding proteins (MyBP), -C and -H, that bind the different components and keep them aligned with each other (TAJSHARGHI, 2008).

At the distal end of the muscle tissue there is a complex and specialized region called myotendinous junction (CHARVET et al., 2012). The MTJ is the main area of predisposition to skeletal muscle injuries, especially in excessive effort and eccentric contraction (TIDBALL; SALEM; ZERNICKE, 1993).

The JMT presents a wide evolution and adaptations in the skeletal muscles, such as sintopy, topography, functions, disposition and histochemical composition of muscle fibers (CIENA et al., 2011). Furthermore, the main ultrastructural compromises the MTJ been demonstrated in the cervical muscles of rats with sarcopenia (CIENA et al., 2012).

There are many cellular and molecular mechanisms related to aging and sarcopenia, such as malnutrition, oxidative stress, inflammation, endocrine changes (decreased growth hormone secretion - GH) and inactivity (MENG; YU, 2010). Another possible cause of the significant decrease in muscle mass associated with the decline in skeletal muscle regenerative efficiency in elderly individuals is the decrease in the number and differentiation of satellite cells (VERDIJK et al., 2007).

Faced with an increasing life expectancy mainly in women, as a result of menopause, there is evidence of an increase in adipose tissue and a drastic decrease in muscle mass due to hormonal changes in sarcopenic women.

The aerobic swimming training for 8 weeks proved to be effective against abdominal fat deposition and furthermore to control blood pressure by lowering the concentration of angiotensin-II which is a vasoconstrictor (ENDLICH et al., 2013).

Muscular impairment in the elderly may be aggravated by the hormonal changes resulting from menopause which may lead to extensive morphological changes in the main area of the locomotor musculoskeletal system denominated myotendinous junction. In order to analyze the possible benefits that physical exercise can provide in this interface that has a direct influence on gait through the transmission of force, as well as to minimize the effects of aging potentiated in the experimental model of menopause in the ultrastructural anatomical complex of the myotendinous junction, aqua aerobic training was used.

Therefore, the aim of this study was to describe the ultrastructural and morpho-quantitative alterations of the myotendinous junction of the anterior tibial muscle of ovariectomized elderly Wistar rats submitted to a swimming protocol and the hypothesis that adaptations in the lengths of the sarcomeres occur in this region.

## MATERIALS AND METHODS

### Animals

Twenty Wistar rats, aged 1 year and 8 months, were randomly divided in four groups (n=5): **Sedentary (S)**: animals were not submitted to swimming training and ovariectomy; **Exercised (E)**: animals were only submitted to training-swimming; **Menopause (M):** animals were submitted only to ovariectomy; **Menopause/Exercised (ME):** animals submitted to ovariectomy and swimming training. The animals were kept in the Laboratory of Morphology and Physical Activity (LAMAF). They received standardized ration and water ad libitum, allocated 5 rats in each cage at controlled ambient temperature at 23 ± 2ºC and light/dark photo-period of 12h. All the procedures adopted in this study were approved by the Committee on Ethics and Research with Animals of the Institute of Biosciences of the Paulista State University – Protocol - nº 9376.

### Menopause Experimental Model

The M and ME groups were anesthetized with ketamine (50mg/kg) and xylazine (10mg) intraperitoneally, submitted to trichotomy and asepsis of the pelvic ventral region. The pelvic wall was sectioned longitudinally for exposure and removal of the ovaries followed by flat suture (nylon 6.0) (FERRETTI et al., 2014). The animals received in the first 7 days post-operative analgesic (Paracetamol-300mg/kg) via drinking fountain.

### Aquatic Training

In the 4th postoperative month the animals of the E and ME groups were submitted to aquatic training protocol in rectangular tanks individually separated by cylindrical tubes (24x50cm) to prevent agglomeration and dispersion immersed at 40cm with water heated to 31°C (PESTANA et al., 2012). To obtain the overloads during the training weights of lead fixed to the thorax with 5% of body mass were used which were checked and corrected weekly. Five weekly sessions lasting 60 minutes each were performed for a period of 8 weeks in a total of 40 sessions (CIABATTARI; DAL PAI; DAL PAI, 2005).

### Transmission Electron Microscopy

The animals from each experimental group (n=5) euthanized (overdose of anesthetic - ketamine 100mg / Kg, intraperitoneal route), the MTJ ($3mm^3$) samples of the anterior tibial muscle were dissected and immersed in modified Karnovsky solution for 48h at 4ºC. Subsequently the samples were post-fixed with 1% osmium tetroxide solution for 2 h at 4°C, washed with saline solution. The samples were then subjected to dehydration in an increasing series of alcohols. After dehydration the samples were included in resin (Low Viscosity Embedding Media Spurr's Kit Electron Microscopy Sciences, USA). The blocks were trimmed to make semi-thin sections of 10µm (Leica Ultracut UCT, Germany), and stained with the 1% toluidine blue method. After selecting the area of interest, ultra-thin sections of 60nm were obtained, which were collected in 200 mesh copper screens (Sigma-Aldrich, USA) and in contrast to the 4% uranyl acetate solution, for 3 minutes, washed with distilled water and then contrasted in aqueous solution of 0.4% lead citrate for 3 minutes (CIENA et al., 2012). The screens were examined through the Philips Transmission Electron Microscopy CM 100, Microscopy Laboratory of the Institute of Biosciences - UNESP.

### Morphometric Analysis

For morphometric analysis of sarcoplasmatic invaginations and evaginations were performed 30 measurements of length and 60 of thickness. Thirty measurements were made on the lengths of sarcomeres comprised between the pairs of Z lines of the last (distal sarcomere) and penultimate (proximal sarcomere) sarcomere present in the MTJ organization with the help of ImageJ® software. After defining the size of the bar in pixels with the "Straight Line" tool the scale of the image was determined in the "Analyze" option afterwards "Set Escale" and then the value in the "Know Distance" box was substituted for the value in the bar. Unit of scale in µm "Unit of Length". The mean value of the standard deviation was calculated by means of the ANOVA variance analysis, followed by BonFerroni post-hoc test. The $p<0.05$ was considered statically significant.

# RESULTS

## Ultrastructure

The ultrastructural aspects of MTJ of tibial anterior muscle from transmission electron microscopy revealed the cell as characteristics of the Groups: S, E, M and ME (Figure 1).

In the S Group the distal muscle cell, organization and parallel arrangement of the sarcomeres in series, adjacent to a large area of connective tissue were observed, in which it was possible to visualize the cellular parts of tenocytes (Figure 1A). At higher magnification the interaction of bundles of collagen fibers called sarcoplasmatic invaginations thin structures projecting towards muscle tissue and oblique sarcoplasmatic evaginations were noted at the muscle / tendon interface (Figure 1B).

In the E Group extensive tissue adaptation was demonstrated with the organization through the parallel arrangement with greater projection and volume of invaginations and sarcoplasmatic evagination (Figure 1C). In focus the tissue reorganization in relation to the positioning communications and sub levels of the sarcoplasmatic invaginations (Figure 1D).

In the M group they presented drastic reduction in the amount of collagen fibers as well as attenuation in the length and thickness of invaginations and sarcoplasmatic evagination (Figure 1E). At higher magnification, a conical shape of the muscle tissue was observed with tissue disorganization when sarcomere misalignment was revealed (Figure 1F).

In the ME group a higher tissue organization with a parallel arrangement was observed, evidenced by an increase in the length and thickness of the invaginations and sarcoplasmatic evagination as well as an increase in the deposition of collagen fibers in the adjacent region (Figure 1G). In greater magnification, it is possible to observe communications between the sarcoplasmatic invaginations, forming sub-levels (Figure 1H).

## Morphometric Analysis

### Length of Sarcoplasmatic Invaginations and Evaginations

Measurements of the lengths (Fig 2A) of sarcoplasmatic invaginations and evaginations were presented.

The lengths of the sarcoplasmatic invaginations in the E group presented an increase of 44.2% and 65.6% in the sarcoplasmatic evagination, in comparison with the S group, with statistical significance ($p<0.001$). In the M group, there was a reduction in the length, 14.1%, of sarcoplasmatic invaginations ($p>0.05$), and in sarcoplasmatic evaginations of 33.8% ($p<0.05$), compared to S group. Similar characteristics were observed in the M group, showing attenuation of sarcoplasmatic invaginations, 17.2%, and 21.8% in sarcoplasmatic evaginations in relation to the

ME group (p>0.05). In addition, ME group presented 0.6% greater sarcoplasmatic invaginations and sarcoplasmatic evagination 19.3% lower than S group (p>0.05).

**Thickness of Sarcoplasmatic Invaginations and Evaginations**

Measurements of the thicknesses (Fig 2B) of sarcoplasmatic invaginations and evaginations were presented.

The invagination and evagination thickness presented similar adaptations (p> 0.05).

In S group the thickness of sarcoplasmatic invaginations and evaginations were 30.8% (p<0.01) and 16.6% smaller in relation to E group (p>0.05), respectively. In the M group, they presented thicknesses of invaginations and evaginations 21.4% (p>0.05) and 26.1% smaller than S group. In the ME group, thicknesses of invaginations showed 34.3% greater (p<0.05) and sarcoplasmatic evaginations 37.6% smaller (p<0.001) than the M group. In addition, in the ME group, the thicknesses showed values 5.5% higher and 8.8% smaller in relation to S group (p>0.05).

**Morphometrics - Proximal and Distal Sarcomeres**

Measurements of the proximal and distal sarcomeres (Fig 2C) were presented.

The proximal sarcomere lengths were 25.8% lower in the S group than in the E group (p <0.05), as well as 50.5% shorter distal sarcomere lengths (p<0.001). The M group had proximal sarcomere lengths 41.2% (p<0.001), and distal 14.6% (p>0.05) higher than the S group. The ME group was 54.5% higher in the proximal sarcomere lengths, and, in the distal sarcomere, 47.8% higher in comparison to the S group (p <0.001). The ME group presented values 9.4% (p >0.05) and 28.8% (p<0.001) higher in the proximal and distal sarcomere lengths compared to the M group, respectively.

## DISCUSSION

The ultrastructural and morphometric aspects of the myotendinous junction of the anterior tibial muscle demonstrated extensive adaptive changes through the physiological process of aging and physical inactivity that were aggravated due to the hormonal deprivation- ovariectomy, and demonstrated before this the benefits of the association of physical training through swimming.

In group S, presented extensive tissue disorganization due to aging associated with physical inactivity revealing atrophic cellular characteristics and an increase of adjacent connective tissue corroborating with the findings of Ciena et al. (2012), which revealed the morphological characteristics of the muscle of the cervical region in aged animals. They also demonstrated the

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interaction between the muscular and tendinous tissues observed by the arrangement of the collagen fibers called sarcoplasmatic invaginations and the sarcoplasmatic evagination also called the finger-like process, arranged obliquely and parallel to the sarcomeres. According to Lang et al., (2009) there is a progressive decrease in muscle volume during aging, this change refers to a decrease in muscle strength and endurance and subsequently to functional loss and weakness with predisposition to falls, characteristics observed in sarcopenia.

In the MTJ region, studies have shown characteristics as structures adjacent to the projections, called sarcoplasmatic invaginations. These long and thin structures derived from the extracellular matrix are composed by connective tissue and increase the attachment surface between the tendon and muscle tissues (CIENA et al., 2010). Recent researches has shown that MTJ presents evolutions and / or adaptations in the skeletal muscles, such as sintopy, topography, functions, arrangement and histochemical composition of muscle fibers (CIENA et al., 2011).

According to Doral et al. (2010), tendinous cells, tenoblasts and tenocytes represent about 90-95% of tendon elements. According to Ippolito et al. (1980); Moore; de Beaux, (1987); Butler et al., (1990), tenocytes are responsible for the synthesis of proteins such as collagen in the extracellular matrix. The collagen fibers consists of an arrangement of primary and secondary parallel bundles diminished according to the surface of the tendon insertion.

According to Zhang et al., (2016) during aging there is predisposition to tendinopathies that lead to tendon ruptures. Furthermore, this physiological process induces increase in lipid deposition and vascular decrease, as well as attenuation of the tenocytes and their cellular activity in order to predispose a greater risk of injuries. And that aerobic exercise of moderate intensity as an injury prevention modality promotes rapid healing in the experimental model in the elderly.

According to Knudsen et al., (2015), MTJ is a dynamic structure that adapts to the stimuli that are imposed in various many ways, such as loading and unloading. In addition, they state that their results show similarities in relation to research with experimental models, such as protrusions of the extracellular matrix (sarcoplasmatic invaginations) and the "finger-like process" (sarcoplasmatic evagination).

In the present study, aquatic training (E), promoted greater tissue organization demonstrated by the parallel arrangement and significant increase in the length of invaginaition and sarcoplasmatic evagination, besides presenting communications and sub levels of sarcolemma projections, which demonstrates greater interface resistance due to greater projection and interaction between the extracellular matrix and the distal sarcomeres. These results corroborate to Tidball's (1991) statements about the ability of MTJ to transmit the generated force, which can be accentuated by the invaginations of the sarcoplasmatic membrane, in a way that decreases the share force due to the increase in the area of interaction between muscle and the tendon. According to

Ciena et al., (2012) MTJ, a region of muscle-tendon connection, can be identified macroscopic distal to the muscular belly, close to the mobile insertion point. In addition, its components have been described from the neonate phase to aging in the sternomastoid muscle (CIENA et al., 2012); in the adult stage the soleus muscle (ST PIEERE; TIDBALL 1994); its association with aerobic treadmill exercise in the anterior tibial and gastrocnemius muscles (KOJIMA et al., 2008); in a model of antigravity hypoactivity in the soleus muscle of primates (ROFFINO et al., 2006); related to exercise resistance to growth hormone (GH) in plantar muscle MTJ (CURZI et al., 2013); (MTJ) of the long extensor muscle of the fingers related to exercise resistance to IGF-1 expression factor (Curzi et al., 2016). In addition, Curzi et al. (2015) used aerobic exercise at different intensities, in moderate intensity the synthesis of structural proteins in the basal lamina and also in the cytoskeleton induce morphological alterations in the MTJ of the gastrocnemius muscle, increasing the area of connection between the tissues.

However, there are still some mechanisms not well understood in this region, mainly associated with training and its implications in different age groups and special groups, which advocates more MTJ morphological studies.

With hormonal deprivation (M), it was observed a decrease in collagen fibers as well as a conical shape of muscle tissue. In addition, they showed attenuation in the length of sarcoplasmatic invaginations - extracellular matrix projections, tissue disorganization without growth pattern, inferring tissue atrophy and sarcomere misalignment. According to Kadi et al. (2002), ovariectomy induces changes in the contractile properties of skeletal muscle and the association of exercise in ovariectomized animals with estrogen treatment maintains the pattern of myosin heavy chain expression (MyHC) in oxidative metabolism muscles ( soleus) and glycolytic (long extensor digitorum).

The results obtained in this study confirm the research of Fontinele et al., (2013) in which physical exercise has the ability to mimic the deleterious effects of ovariectomy and consequent interruption of hormonal levels evidenced by extensive adaptations in the anterior tibial myotendinous junction of rats the elderly.

In the experimental model menopause and submitted to the training of swimming, ME, presented benefits like greater deposition of collagen fibers and extensive organization of the sarcoplasmatic projections. In addition, these adaptations are similar to E, such as the formation of communications between the matrix projections, as well as the formation of sub-levels, inferring energetic tissue adaptation in order to reaffirm the potential of physical exercise in pathological environments.

With an increase in the contact surface between the tissues evidenced by an increase in the length of invaginations and sarcoplasmatic evaginations, it is possible to relate a better transmission of strength and consequently lower fragility in elderly individuals who also have physical stimulation. Our results revealed a wide adaptation due to the training associated to the hormonal reduction evidenced by the greater deposition of collagen fibers in the groups that received the physical stimulus, mainly in the ME.

According to Jakobsen et al., (2016), MTJ in human shows heterogeneity of collagen fibers, since the addition of collagen type XIV associated with four weeks of resistance training may increase the protection against injuries in this region. Takagi et al. (2016) have shown that the intense deposit of collagen in the superficial layer of skeletal muscle may indicate a more important role than other factors in the injured muscle region by means of subsequent eccentric stimuli.

According to Gordon et al. (1966) the amount of force that a muscle is capable of generating is directly related to the length of the sarcomeres. In addition, Plotnikov et al. (2008); Ralston et al. (2008) argue that the integrity of myofibril can be observed by sarcomere patterns as well as these may be useful in the diagnosis of muscular diseases.

The lengths of the proximal sarcomeres in M and ME groups increased according presented adaptive changes through aging associated with hormonal deprivation, possibly due to marked muscular atrophy. Such changes in the lengths of the proximal sarcomeres may be due to the adaptations of the adjacent sarcomeres, the distal sarcomeres.

The length of the sarcomere depends on several factors such as: total muscle length, quantity of sarcomeres, tendon length and stiffness (MACINTOSH, 2017). The distal sarcomere lengths were higher than the proximal sarcomeres and maintained their standard in the S and M groups. In the E and ME groups, there was an expressive increase in lengths caused by physical training that possibly promoted the synthesis of protein myofilaments at the MTJ, the site of addition of new sarcomeres.

In M, the lengths presented alteration in the distal sarcomeres, in addition, it was possible to conclude that the length of the sarcoplasmatic evaginations decreased and their thickness increased, probably due to the atrophy and reduction of the sarcomeres. It thus presents a correlation with the length and thickness of sarcoplasmatic invaginations also evidenced by tissue atrophy. In addition, the lengths of the proximal sarcomeres showed to be smaller than the S group and larger in relation to the ME group.

The length of the distal sarcomere and the length of the invaginations presented a pattern of similar alterations due to the adaptations through training and consequent traction of the sarcomeres in series associated with the extracellular matrix through the MTJ.

In the trained groups (E and EO), a similar increase in the length of the distal sarcomeres was observed, possibly associated to the traction of the bundles generated by the muscular contraction of the swimming in order to bring about adaptations in the sarcomere plasticity. In addition, it was observed that training in ME promoted adaptations that reestablish and even improve S patterns, demonstrated by greater lengths and thicknesses of sarcoplasmatic invaginations. Sarcoplasmatic evaginations, which are the precursors of new sarcomeres by addition, are adapted by increasing the length of sarcoplasmatic invaginations in proportion to the production and myofilament bundles due to training (E and ME). Our findings corroborate Moo et al.'s (2016) assertion that the lengths of the sarcomeres vary according to the muscle region; in the case of the anterior tibial muscle in dorsiflexion (state of shorter length) we observed an average length of 2.1 to 2.3 μm. In plantar flexion (longer length), the sarcomeres that are more elongated are those close to the distal sarcomere at the myotendinous junction and may stretch up to 25% more than in other regions of the TA.

We concluded that the association between ovariectomy and swimming training promoted alterations in the plasticity of the MTJ of the anterior tibial muscle, showing a greater resistance at the muscle / tendon interface due to the greater contact surface area, quantified by greater lengths of invagination and sarcoplasmatic evagination, in addition to longer lengths of the distal sarcomeres, possibly due to the traction of the fiber bundles and activation of the satellite cells fomenting better tissue regeneration, which requires further studies.

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Figures:

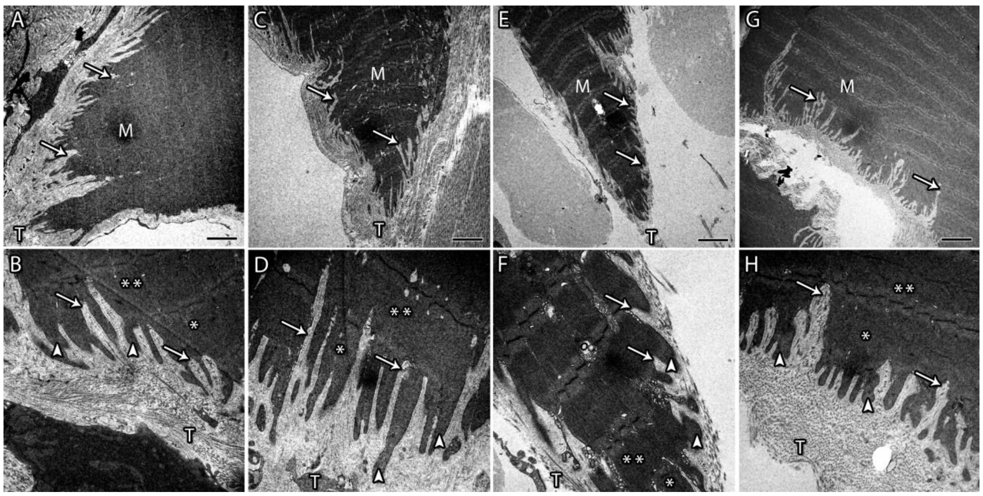

**Figure1 – Transmission electron microscopy micrography of the MTJ**: Sedentary (A;B), Exercised (C;D), Menopause (E;F), Menopause/Exercised (G;H). Distal Sarcomere (*); Proximal Sarcomere (**); Tendon Tissue (T); Muscle Fiber (M); Sarcoplasmatic invaginations (arrow) and Sarcoplasmatic evaginations (arrowhead). Magnifications: (A, C, E, G) 4.000x, (B, D, F, H) 15.000x.

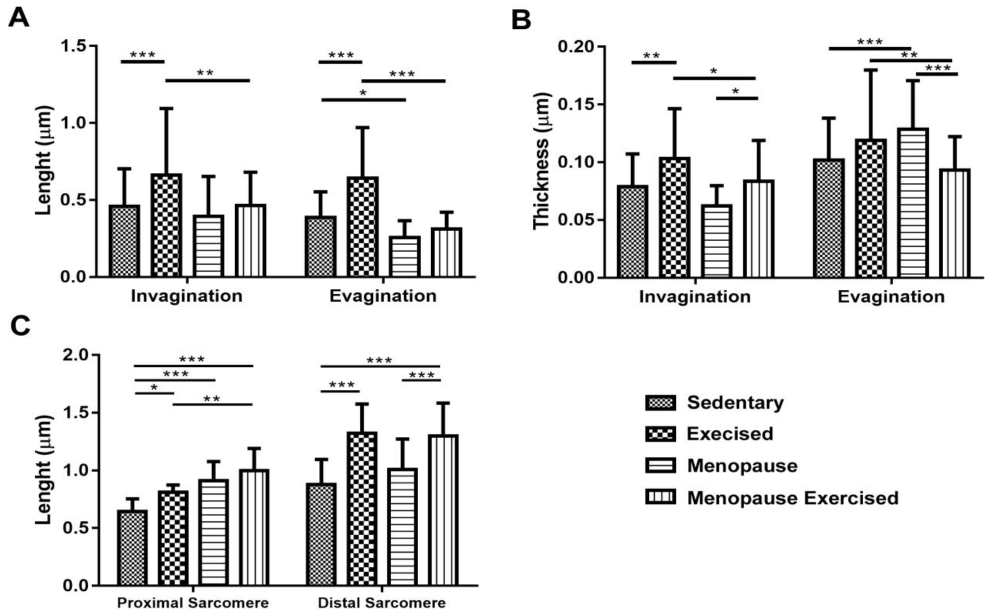


**Figure 2** – Morphometric data: Mean and standard deviation of the lenghts (A), thickness (B) of the invaginations and evaginations sarcoplasmatic and the lenghts of the proximal and distal sarcomeres (C). *p<0.05; **p<0.01; ***p<0.001.