



The effects of stretching on muscle morphometry of ovariectomized rats

Efeitos do alongamento na morfometria do músculo em ratas ovariectomizadas

Los efectos del ejercicio de estiramiento en la morfometria muscular de las ratas ovariectomizadas

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Abstract

Introduction: Ageing is responsible for structural alterations, declining of all physiological variables, including range of motion and skeletal muscle function, known as sarcopenia. **Objective:** The aim of the study was to evaluate the effects of stretching on muscle morphometry in ovariectomized rats. **Method:** 21 female Wistar rats (12 weeks, 218 ± 22 g) were divided into 4 groups: control (CONTROL, n = 3) intact; ovariectomized and hysterectomized (OH, n = 6); Stretching (STRET, n = 6); ovariectomized and hysterectomized and stretching (OHS, n = 6). The rats were subjected to ovariectomy and hysterectomy. The stretching protocol of the soleus muscle lasted 10 repetitions of 1 minute with 45s interval between each repetition performed 3 times a week for 3 weeks. After 3 weeks, the rats were weighed and the muscles of both hind limbs were removed weighed and analyzed at muscle length; serial sarcomere

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number; sarcomere length; muscle fiber cross-sectional area (MFCSA) and percentage of connective tissue. **Results:** The final body weight increased in all groups. The serial sarcomere number of STRET was greater than the OH. The muscle fibers' cross-sectional area of OHS was higher than CONTROL. **Conclusion:** It can be concluded that ovariectomy and hysterectomy prevented sarcomerogenesis even when stretching was applied. However, the stretching protocol enhanced muscle trophism of ovariectomized and hysterectomized rats. It might be suggested that longitudinal growth (serial sarcomeres) and radial (ASTFM) are differently regulated by stretching in intact and/or estrogen depleted (ovariectomy and hysterectomy) skeletal muscle.

Keywords: Ovariectomy. Hysterectomy. Skeletal Muscle. Muscle Stretching Exercise.

Resumo

Introdução: O envelhecimento é responsável por alterações estruturais, com declínio de todas as variáveis fisiológicas, incluindo amplitude de movimento e função muscular esquelética. **Objetivo:** O objetivo do estudo foi avaliar os efeitos do alongamento na morfometria muscular de ratas ovariectomizadas. **Método:** Assim, 21 ratas Wistar (12 semanas, 218 ± 22 g) foram divididas em 4 grupos: Controle (CONTROL, $n = 3$) intacto; Ovariectomizadas e hysterectomizadas (OH, $n = 6$); Alongamento (ALONG, $n = 6$); Ovariectomizadas e hysterectomizadas e Alongamento (OHA, $n = 6$). As ratas foram submetidas a ovariectomia e hysterectomia. O alongamento do músculo sóleo foi composto de 10 repetições de 1 minuto com intervalo de 45s entre cada repetição, realizado 3 vezes por semana, durante 3 semanas. Após 3 semanas, as ratas foram pesadas e os músculos sóleos de ambas as patas foram retirados, pesados e analisados: comprimento do músculo; número de sarcômeros em série; comprimento do sarcômero; área da seção transversal das fibras musculares (ASTFM) e porcentagem de tecido conjuntivo. **Resultados:** O peso corporal final aumentou em todos os grupos. O número de sarcômeros em série do ALONG foi maior que o OH. A área de seção transversa das fibras musculares do OHA foi superior ao CONTROL. **Conclusão:** Conclui-se que a ovariectomia e hysterectomia impediram a sarcomerogênese mesmo quando realizado alongamento. Porém, o protocolo de alongamento incrementou o trofismo muscular em ratas ovariectomizadas e hysterectomizadas. Sugere-se que o crescimento longitudinal (número sarcômeros em série) e radial (ASTFM) respondem diferentemente ao alongamento em músculo esquelético intacto e/ou com depleção estrogênio (ovariectomia e hysterectomia).

Palavras-chave: Ovariectomia. Hysterectomia. Músculo Esquelético. Exercício de Alongamento Muscular.

Resumen

Introducción: El envejecimiento es responsable de cambios estructurales del organismo, con disminución de todas las variables fisiológicas, incluyendo la amplitud de movimiento y función muscular esquelética, conocida como sarcopenia. **Objetivo:** El objetivo del estudio fue evaluar los efectos de los ejercicios de estiramiento en la morfometria muscular de ratas ovariectomizadas. **Método:** Fueron seleccionadas 21 ratas Wistar (12 semanas, 218 ± 22 g) que fueron divididas en 4 grupos: control (CONTROL, $n = 3$) intacto; ovariectomizadas y hysterectomizadas (OH, $n = 6$); estiramiento (ALONG, $n = 6$); ovariectomizadas y hysterectomizadas y estiramiento (OHA, $n = 6$). Las ratas han sido sometidas a ovariectomía y hysterectomía. El protocolo de estiramiento del músculo sóleo fue realizado en 10 repeticiones de 1 minuto con intervalos de 45 segundos entre cada repetición, realizada 3 veces por semana, durante 3 semanas. Después de 3 semanas, las ratas fueron pesadas y los músculos de ambas patas fueron retirados para analizar el peso muscular; la longitud del músculo; número de sarcómera en serie; longitud de sarcómera; área de la sección transversal de las fibras musculares (ASTFM) y porcentaje del tejido conjuntivo. **Resultado:** El peso corporal final aumentó en todos los grupos. El número de sarcómera en serie de ALONG fue mayor que el OH. El área de la sección transversal de las fibras musculares del OHA fue superior al de CONTROL. **Conclusión:**

En conclusión, ovariectomía y histerectomía impidieron la sarcomerogénesis mismo cuando se realizó el estiramiento. Sin embargo, el estiramiento aumentó el trofismo muscular de las ratas ovariectomizadas y histerectomizadas. Sugiere que el crecimiento longitudinal (número sarcómeros) y radial (área de sección transversal) responden diferentemente al estiramiento en músculo esquelético intacto y/o con depleción estrógeno (ovariectomía y histerectomía).

Palabras Clave: Ovariectomía. Histerectomía. Músculo Esquelético. Ejercicios de Estiramiento Muscular.

Introduction

An age-related skeletal muscle function decline, clinically referred as sarcopenia, results from complex interactions among innervation disturbances, hormone deficiency, inflammatory cytokines and restriction in caloric-protein ingestion [1, 2]. The effect of sarcopenia is more pronounced on women because the menopausal transition is associated to a decline in female hormones, especially estrogen, which induces changes on skeletal muscle and/or lifestyle modifications [3]. In addition, loss of muscle mass and strength diminish muscle function that can result in significant morbidity and mortality for the elderly [4]. Therefore, musculoskeletal alterations can induce decrease on range of motion (ROM) limiting the older adult's ability to perform daily activities [5].

Many treatments have been developed with the aim of preventing sarcopenia, including estrogen replacement, bisphosphonate compounds and physical activity programs [4, 6, 7].

Physical rehabilitation has used many techniques as resistance exercise, stretching, to minimize the physiological changes induced by increasing age [8, 9]. It has been reported that stretching programs can ameliorate the loss of muscle mass, performance, flexibility, and improve well-being and independence of ageing people, especially women [10-13].

It was documented that 1 min of static stretching of the hamstring muscles resulted in gains of ROM in elderly individuals [9]. However, the cellular mechanisms of skeletal muscle adaptation induced by stretching exercise in ageing people or rats were not shown.

Thus, it is important to investigate the underlying effects of stretching to improve the appropriate

exercise programs to gain ROM. The experimental studies have contributed to clarify the understanding about the histological, cellular and molecular mechanisms induced by stretching in young male rats [14-17]. Some adaptations include increase in the serial sarcomere number, cross-sectional area and also in gene expression of hypertrophic factors [14-17].

However, there is no description in the literature of the chronic (more than one week) effects of stretching on skeletal muscle morphometry of ageing rats. Based on this, this study was undertaken to determine the effects of stretching exercise on skeletal muscle fiber morphology of ovariectomized rats to mimic the ageing process.

Materials and Methods

Animal care and groups

This study was conducted in accordance to the Guide for Care and Use of Laboratory Animals and approved by the Research Ethics Committee of Faculdade Evangélica Paraná (number 2166/05). The animals were kept in plastic cages with free access to water and food pellets.

The female rats ($n = 21$; aged 12 weeks, 218 ± 22 g) were divided into 4 groups: CONTROL ($n = 3$), rats kept freely moving in their cage for 7 weeks; OH ($n = 6$), rats were submitted to ovariectomy, hysterectomy and then kept in their cages for 7 weeks (18); STRET ($n = 6$), rats stayed for 4 weeks freely on their cages and on the 5th week the stretching protocol initiated on the left soleus muscle, 3 times per week, for 3 weeks; OHS ($n = 6$), rats were submitted to ovariectomy, hysterectomy, waited 4 weeks and then the stretching protocol for 3 weeks [18].

The animals of all groups were anesthetized by anesthetic overdose of xylazine (12 mg/kg) and ketamine (95 mg/kg) for dissection of left and right soleus muscles. Afterwards, the animals, aged 19 weeks, were euthanized by anesthetic overdose.

Surgical procedure of ovariectomy and hysterectomy

For the surgery, the female rats were anesthetized with an intraperitoneal injection with a mixture of xylazine (12 mg/kg) and ketamine (95 mg/kg). Later on, a small incision was made on the right side over the lower back. After, the uterine cervix was sewn with absorbable 3-0 and their horns and the ovaries were sectioned from, and then were removed. Afterwards, the muscle and skin were sewn separately with absorbable 3-0 (ETHICON) and non-absorbable 6-0 silk (ETHICON), respectively [18].

Stretching Procedure

To stretch the left soleus muscle, the animals were previously anesthetized by intraperitoneal injection of xylazine (12 mg/kg) and ketamine (95 mg/kg). After that, they were weighted and later on, the soleus muscle stretching was performed holding the left ankle in full dorsal flexion and maintaining manually in that position. The stretching protocol consisted of a bout of 10 repetitions of 1 minute each stretching with 45 seconds of rest between each repetition, controlled through a chronometer (*Technos YP2151/8P*, Technos) [19]. The stretching procedure was performed 3 times a week (Monday, Wednesday and Friday), for 3 consecutive weeks. Thus, at the end of the experiment 9 stretching sessions were completed.

Morphology and sarcomere measurement

The soleus muscles were dissected free from surrounding tissue. After the dissection each muscle was weighed, subsequently, the tendons of the soleus muscle were clamped with the muscle at resting position, which was defined as initial length (L_0), as previously reported by Ansved [20] and muscle length was determined. Each soleus muscle was then divided longitudinally into

two similar parts: the medial one was used for histology, while the lateral portion was used for the sarcomere measurements.

The number and length of the sarcomeres along a single muscle fiber were determined as previously described by Williams and Goldspink [21]. The muscle was fixed at resting position (L_0 length) in 2.5% glutaraldehyde for 3 h and then removed, placed in 30% HNO₃ for two days, and stored in 50% glycerol. Next, five individual fibers of the whole muscle were teased from tendon-to-tendon from each soleus muscle and mounted and their length was measured using a caliper rule. The number and length of the sarcomere along a 300- μ m portion were quantified at different points in the middle region of each single fiber using a projection microscope (Nikon, Eclipse E 200 model). The total number of sarcomeres in each muscle fiber was identified by the correlation between the number of sarcomeres along the 300- μ m portion and the total fiber length [21]. In view of the conflicting reports on the literature about the sarcomere length along the muscle fibers, particularly at the ends of the stretched fibers, in this study we assumed that sarcomere length is homogeneous along the entire length of the muscle fiber [22].

Muscle fiber area

For histology, serial cross-sections (10 μ m) were then obtained from the middle part of the soleus muscle and stained with haematoxylin and eosin (H&E) for the morphometry measurement of the muscle fiber cross-sectional area (CSA). Other serial slices were stained with Mallory's trichrome to analyze the percentage of connective tissue.

The cross-sectional area of 100 muscle fibers randomly chosen from the central region of one cross-section of each soleus muscle was measured using a projection microscope (Axyophot, Carl Zeiss, Oberkochen, Germany) and a video-image system (Applied Spectral Imaging, MigdalHa'emek, Israel) through the software Case Data Manager Expo (Applied Spectral Imaging, MigdalHa'emek, Israel, version 4.0) in Laboratory at Biology Cell Department from the Federal University of Parana, Curitiba, Brazil. In each cross-section we measured the CSA and the percentage of connective tissues through the free software UTHSCSA Image Tool 3.0 (developed

at the University of Texas Health Science Center at San Antonio, Texas, and available from: <http://ddsdx.uthscsa.edu/dig/itdesc.html>.

Connective tissue area of one cross-section of each soleus muscle was measured first using the whole slice area (100%) of the Mallory's trichromeslide (10x objective). All muscle fiber cross-sectional areas were marked and then excluded, remaining only the connective tissue, i.e., perimisium and endomisium. Afterwards, the connective tissue area was expressed as the percentage of the whole slice area [23].

Statistical analysis

The data were inspected for normality using Shapiro Wilk's test and to verify the homoscedasticity or heteroscedasticity with Levene's test. Thus, as the body weight data were normal distributed and presented homoscedasticity, the paired Student's *t*-test was used to compare the final body weight within each group. Comparisons among the groups were evaluated by ANOVA or Kruskal Wallis for non-parametric data. Significant level accepted was 5% ($p \leq 0.05$).

Results

Body weight

The data showed normal and homogeneous distribution ($p = 0.1$, $p = 0.72$, respectively). Increase in the final body weight compared to initial was observed in all groups: CONT ($p = 0.007$); OH ($p = 3.98E-05$); STRET ($p = 0.003$); OHS ($p = 0.0005$).

The final body weight of the OH was higher than STRET (294 ± 9 g vs 238 ± 9 g, $p = 0.001$). Still, the CONT showed final body weight higher than the STRET (293 ± 7 g vs 238 ± 9 g, $p = 0.02$). The results are shown in Table 1.

Muscle weight

The results showed normality ($p = 0.38$) and homogeneous distribution ($p = 0.13$). There was no statistically significant difference in muscle weight (Figure 1A).

Table 1 – Effects of stretching in the body weight of ovariectomized rats

Groups	Initial (g)	Final (g)	Relative Diference (%)	p Student's t test
CONT*	234±10	293±8	20	0.007
STRET	227±13	238±10	4.5	0.003
OH+	235±5	294±9	20	3.98E-05
OHS	197±19	259±13	24	0.0005

Note: Data are mean \pm standard deviation. CONT ($n = 3$), intact-control group; OH ($n = 6$) ovariectomy and hysterectomy group; STRET ($n = 6$) stretching group, left soleus muscle was stretched 3 times a week for 3 weeks; OHS ($n = 6$) ovariectomy, hysterectomy and stretching group, after 30 days of the surgery the left soleus muscle was stretched 3 times a week for 3 weeks. +compared to STRET ($p = 0.001$); *compared to STRET ($p = 0.02$) (Kruskal Wallis).

Muscle length

The data showed normal distribution ($p = 0.88$) and homogeneity ($p = 0.39$). There were no statistically significant results in muscle length (Figure 1B).

Serial Sarcomere Number

The results showed normal ($p = 0.98$) and homogeneous distribution ($p = 0.17$). The STRET increased serial sarcomere number compared to the OH (7762 ± 531 vs 6209 ± 566 , $p = 0.01$, Figure 1C).

Sarcomere length

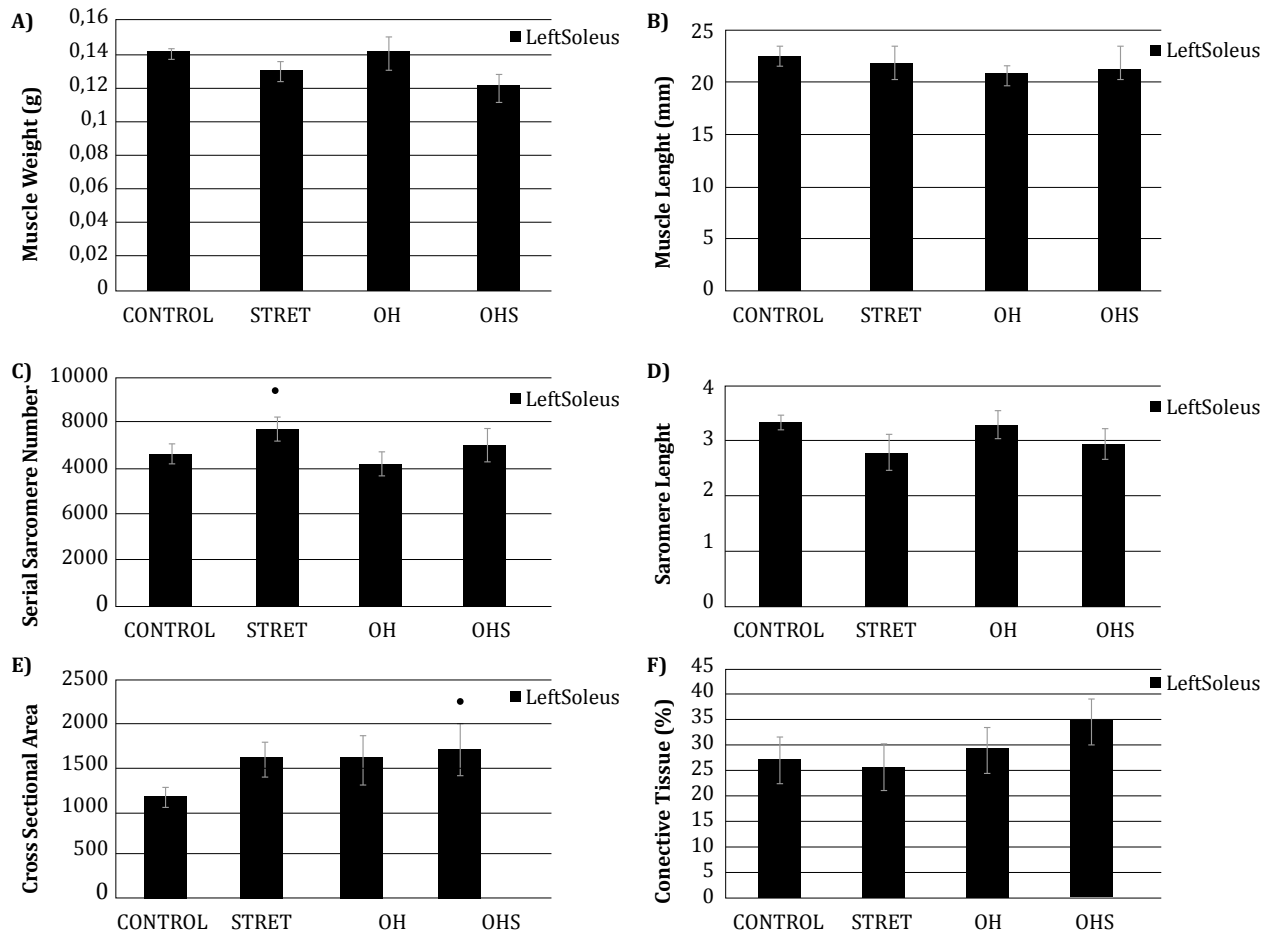
The data showed normality ($p = 0.78$) and homogeneous distribution ($p = 0.71$). There were no statistically significant results in sarcomere length.

Cross Sectional Area (CSA)

The data presented normality ($p = 0.75$) and homogeneity ($p = 0.11$). The OHS showed an increase in the muscle fiber cross-sectional area compared to CONT ($1682 \pm 292 \mu\text{m}^2$ vs $1139 \pm 98 \mu\text{m}^2$, $p = 0.02$, Figure 1E).

Percentage of connective tissue

No change was found in the percentage of connective tissue (Figure 1F).



Note: CONT (n = 3), intact-control group; OH (n = 6) ovariectomy and hysterectomy group; STRET (n = 6) stretching group, left soleus muscle was stretched 3 times a week for 3 weeks; OHS (n = 6) ovariectomy, hysterectomy and stretching group, after 30 days of the surgery the left soleus muscle was stretched 3 times a week for 3 weeks. Data are mean \pm standard deviation. **A)** Muscle weight: It was not found statistical difference. **B)** Muscle length: It was not found statistical difference. **C)** Serial sarcomere number: • $p \leq 0.05$ compared to OH (ANOVA). **D)** Sarcomere length: It was not found statistical difference. **E)** Cross-sectional area: • $p \leq 0.05$ compared to CONT (ANOVA). **F)** It was not found statistical difference in the percentage of connective tissue.

Figure 1 – Effects of stretching in the soleus muscle morphometry of ovariectomized rats.

Discussion

The results of this study showed that ovariectomy and hysterectomy (OH) impaired sarcomerogenesis even when soleus muscle was stretched. However, rats subjected to ovariectomy, hysterectomy and stretching had greater muscle fiber cross sectional area (CSA) than intact rats and showed no loss of sarcomeres in series. These results were very interesting because demonstrated that stretching exercise on muscle under estrogen depletion condition was enough to prevent sarcopenia.

All the experimental groups showed an increase in the final body weight compared to initial ones similar to the normal growth of animals [15]. In contrast, the

animals submitted to OH, showed a slight increase in the final body weight when compared to STRET. This outcome suggests that ovariectomy and hysterectomy promote increase in body weight while some mechanisms induced by stretching could prevent body weight gain. It could be supposed that the association of stretching under anesthetic status and the estrogen depletion can induce some stressing condition to the animal that does not permit the normal weight gain [16]. Instead it has not been found in the body weight gain in male rats submitted to passive stretching, 3 times a week, even under anesthetic for 40 min, maybe the animal sex could have interfered in this response [8, 15].

In this study the association of estrogen depletion and stretching was determinant to induce an increase

in CSA which can suggest some inflammation process such as damage of muscle structural proteins and/or muscle fiber swelling and/or hypertrophy [24, 25]. As stated previously, estrogen has generally been shown to inhibit inflammation related to leucocyte infiltration into skeletal muscle and accentuate factors related to muscle repair after unaccustomed exercise [25, 26].

An increase has been reported in CSA when ovariectomized and hysterectomized rats underwent 10 minutes of stretching, twice a week, during 6 weeks [24]. Therefore, it might be supposed that the augment in CSA of the OHS found in the current study might not be explained for muscle damage or swelling but it can indicate hypertrophy as previously reported [24]. However, the cellular and molecular mechanisms by which stretching exercise exerts on skeletal muscle estrogen-depleted must be well investigated. Furthermore, whether its influence extends to differential rates of muscle repair, recovery and/or hypertrophy also requires more investigation.

It is known that stretching can increase serial sarcomere number in skeletal muscle of young male rat [25, 26]. In the current study we found sarcomerogenesis in the STRET group as confirmed by an increase in the serial sarcomere number. This result was remarkable because it confirms that the soleus muscle of female rats responds similarly to male when submitted to passive stretching 3 times per week [15]. Instead, the present stretching protocol was applied only for 10 min (10 repetitions of 1 min) different from Coutinho et al. 2004 [15] that maintained during 40 min. It has been also reported that 10 min stretching on rat soleus muscle is able to increase gene expression of factors associated with muscle growth (myo-D) [17].

Even so, muscle stretching regulates serial sarcomere number and the cross-sectional area of muscle fibers by different mechanisms [14]. Previous studies have demonstrated that the pathway for addition of serial sarcomeres number is different from that regulating the parallel sarcomere number. The nitric oxide (NO) derived from the neuronal isoform of NO synthase (nNOS) is a positive modulator of serial sarcomere addition [27], while Ca²⁺/calmodulin-dependent protein phosphatase calcineurin and insulin-like growth factor 1 (IGF-1), by activating phosphatidyl- inositol 3-kinase (PI3k)-Akt (a serinethreonine kinase) pathways, appear to regulate skeletal muscle hypertrophy [28, 29]. In contrast, the ubiquitin-proteasome pathway regulates

the binding and degradation of ubiquitinated proteins in the atrophy process [30].

No difference was found in the percentage of connective tissue as well as observed by other authors in male rats submitted to stretching [16, 31]. In addition, it was reported that the matrix metalloproteinases (MMP-2) activity, responsible for remodeling process of muscle fibers and extracellular matrix, in the rat soleus muscle submitted to a similar stretching protocol, was not altered probably because the stretching protocol applied did not cause collagen reorganization [19].

These outcomes can contribute to highlight the effect of stretching on muscle estrogen-depleted which may provide a better understanding for its prescription for older people or women in or post the menopause period.

Conclusions

Ovariectomy and hysterectomy prevented sarcomerogenesis even when soleus muscle was stretched. However, the stretching protocol increased muscle trophism in ovariectomized and hysterectomized rats. The outcomes are indicating longitudinal muscle growth (sarcomere number) and radial (cross-sectional area) remodeling by stretching through different pathways in intact and/or hormone-depleted skeletal muscle.

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